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Review

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ABSTRACT

Ensuring consistent beef eating quality is paramount for meeting consumer demands and sustaining the meat industry. Electrical stimulation (ES) is a post-slaughter intervention used to accelerate post-mortem glycolysis, to avoid cold shortening, to control the tenderization rate of meat through sophisticated physical, chemical and biochemical mechanisms including proteolysis, to improve beef tenderness and to achieve normal pHu that might lead to positive impact on color. This review comprehensively examines the multifaceted effects of ES on beef quality, encompassing factors and settings influencing its efficacy and the underlying biochemical mechanisms revealed using traditional biochemistry methods. It then delves into the molecular pathways modulated by ES, as unveiled by muscle proteomics, aiming to provide a second look and an unprecedented understanding of the underlying biochemical mechanisms through an integrative proteomics analysis of low-voltage ES (LVES) proteomics studies. The proteins changing as a result of ES were gathered in a compendium of 67 proteins, from which 14 were commonly identified across studies. In-depth bioinformatics of this compendium allowed a comprehensive overview of the molecular signatures and interacting biochemical pathways behind electrically stimulated beef muscles. The proteins belong to interconnected molecular pathways including the ATP metabolic process and glycolysis, muscle structure and contraction, heat shock proteins, oxidative stress, proteolysis and apoptosis. Understanding the intricate interplay of molecular pathways behind ES could improve the efficiency of beef production, ensuring consistent meat quality and meeting consumer expectations. The integrative analysis approach performed in this study holds promise for the meat industry's sustainability and competitiveness.

1. Introduction

The meat industry must strive to consistently provide sustainable and high-quality meat in order to meet the expectations of discerning consumers in terms of both intrinsic and extrinsic qualities. The supply of meat that is sustainable, safe, wholesome, nutritious, and of high quality ensures continued consumption of meat and meat products (Henchion, McCarthy, Resconi, & Troy, 2014; Troy & Kerry, 2010). After cooking, the eating quality of meat is largely determined by the attributes of tenderness, flavor and juiciness (O'Quinn, Legako, Brooks, & Miller, 2018; Santos et al., 2021), which have also been identified as the most important determinants of beef palatability by consumers (McIlveen & Buchanan, 2001). Some other studies have focused on beef tenderness, considered as the primary determinant of satisfaction for consumers (Gagaoua, Monteils, Couvreur, & Picard, 2019; Miller, Carr, Ramsey, Crockett, & Hoover, 2001; Watson, Gee, Polkinghorne, & Porter, 2008). Moreover, several consumer research studies confirmed the market demands quality by indicating the willingness to pay a premium for

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guaranteed tender meat (Polkinghorne & Thompson, 2010; Reicks et al., 2011; Santos et al., 2021). Therefore, providing consistently tender beef to consumers, while guaranteeing other quality attributes such as flavor or color, should be of utmost importance to the meat industry.

The variability in beef tenderness has many origins and stems from intrinsic and extrinsic factors ranging from farm-to-fork including the effects of end-point cooking temperatures and country origin of the consumers (Gagaoua et al., 2016; Gagaoua, Picard, & Monteils, 2019; Gagaoua, Terlouw, Richardson, Hocquette, & Picard, 2019; Holman & Hopkins, 2021; O'Quinn et al., 2018; Santos et al., 2021) as a result of intricate and highly interconnected molecular pathways (Gagaoua et al., 2021; Purslow, Gagaoua, & Warner, 2021). However, it is widely recognized that post-mortem factors play a crucial role in determining beef tenderness, outside of extreme situations like much older animals and high preslaughter stress levels. Post-mortem factors encompass the biochemical dynamics that unfold in the muscle from the initial minutes after animal bleeding (Ouali et al., 2013), persisting actively throughout the early post-mortem period and ageing processes (Gagaoua, Troy, & Mullen, 2021). For example, the interactions between pH, temperature and time during the early post-mortem period have significant effects on the extent of rigor and rate of meat tenderization (Ouali et al., 2013; White, O'Sullivan, Troy, & O'Neill, 2006a). Such biochemical dynamics are of paramount importance for the final meat quality determination. Therefore, several post-mortem tenderization interventions, being traditional or emerging methods, have been applied to make significant improvements in tenderness outcomes (for review: Bhat, Morton, Mason, and Bekhit (2018)). While many technological advancements have been made in the processing chain to improve beef tenderness, electrical stimulation (ES) is one of the methods widely applied in commercial processing plants in many countries to improve meat quality (Devine, Hopkins, & Strydom, 2024).

Electrical stimulation, a technology first implemented on an industrial scale in New Zealand (Davey, Gilbert, & Carse, 1976), had as its main objective to prevent the phenomenon of cold shortening during the rapid chilling of carcasses (Hwang, Devine, & Hopkins, 2003). This technology soon gave rise to other interests. The first was its use to enable the development in the best possible conditions of hot-boning (removal of muscle from the carcass before chilling, mainly within 90 min post-slaughter). In fact, hot-boned muscles are much more susceptible to cold shortening, due to the reduced mass to be cooled and the elimination of existing tensions on the carcass. The second is the apparent acceleration of the tenderizing process, which reduces storage time. In this context, ES has sometimes been used, erroneously, as a commercial argument of guaranteed tenderness, which, as we shall explore, is not always the case and depends on multiple factors. Overall, the effect of ES has been evidenced in three main ways: i) to prevent cold shortening (first major reason) through the acceleration of the rate of post-mortem glycolysis and, thus, decrease pH decline to below 6.0 within 3 to 4 h post-slaughter to allow rapid refrigeration; ii) to improve tenderness in the absence of cold shortening, which has been mainly suggested through the acceleration of proteolysis by activating certain endogenous muscle proteases and enhancement of the concentration of free calcium ions; iii) to initiate the physical disruption and damage of muscle myofibrils, which can be evidenced by the presence of contracture nodes and changes in the sarcomeres at the internodal zones that experienced stretching or fracturing (Hwang et al., 2003). In the literature, there is a general consensus among meat scientists that a combination of these interrelated effects can be beneficial in terms of quality, although a lot of variation in improving tenderness has also been observed.

In order to broaden and update our knowledge of the applications of ES in beef research and related variability, this review explores the various factors and settings contributing to the effectiveness of ES on tenderness and other quality traits. It further covers the diverse and emerging ES systems currently in use and their characteristics. It then delves into the biochemical mechanisms unveiled by traditional methods at the muscle level, by discussing certain of the biochemical indicators used to investigate the rate of proteolysis. Finally, and for the first time, this report offers a second look and an unprecedented understanding of the underlying biochemical mechanisms through an integrated analysis of proteomics studies. Such an approach allowed exploring the proteins so far identified to change as a result of ES along with providing, thanks to bioinformatics, novel insights and a comprehensive overview of the molecular signatures and interacting biochemical pathways behind electrically stimulated beef muscles.

2. Electrical stimulation as a way to improve meat quality

Electrical stimulation (ES) involves passing an electric current through the carcass of a freshly slaughtered animal using contact electrodes (Devine et al., 2024). This post-mortem processing technique causes extensive muscle contraction in the carcasses (Fig. 1), which accelerates glycolysis and the rate of pH drop (Chrystall & Hagyard, 1976; Hope-Jones, Strydom, Frylinck, & Webb, 2010), ensuring earlier rigor onset and less rigor contraction (Devine et al., 2024). An earlier study by Ducastaing, Valin, Schollmeyer, and Cross (1985) demonstrated that muscle pH can drop by 0.5 units during 60 s of ES in beef carcasses, a process which can take three or more hours in unstimulated muscle representing a 180-fold increase in the rate of muscle glycolysis. In addition, there may also be a temperature-dependent acceleration in the subsequent rate of glycolysis following ES, above that observed in unstimulated muscle (Chrystall & Devine, 1978) although this tends to be more associated with carcasses that have been subjected to high-voltage electrical stimulation (HVES) and is not necessarily triggered by low-voltage electrical stimulation (LVES) (Kastner et al., 1993).

As a consequence, *rigor mortis* occurs at higher temperatures in stimulated carcasses (Hwang et al., 2003), hence, preventing cold shortening in light carcasses (Devine et al., 2024) and reducing the time to onset of *rigor mortis*, occurring both during and after stimulation (Matarneh, England, Scheffler, & Gerrard, 2017). Additionally, ES produces contracture nodes and supercontracture of the muscles that results in microtears of the myofibrils and connective tissue (Hwang & Thompson, 2002). ES also causes early activation of proteolytic enzymes (Lee, Polidori, Kauffman, & Kim, 2000), hence, accelerating proteolysis (Hwang et al., 2003). All of these mechanisms result in ES contributing to the tenderization of meat, which has been widely reported in the literature (Supplementary Table S1).

On the other hand, the rapid pH drop in stimulated carcasses affects other meat quality attributes such as color. As pH decreases, muscle fiber diameter decreases and extracellular space increases, resulting in more light being scattered and, thereby, the meat appears lighter (Hughes, McPhail, Watkins, Stark, & Warner, 2023). In addition, at lower pH the activity of mitochondrial enzymes is reduced (Gagaoua et al., 2021), which leads to a reduced oxygen consumption rate and better oxygenation of the meat surface; hence, increasing the redness of meat. Apart from the pH decline, the extensive muscle contraction resulting from ES also contributes to improvements in meat color. In this context, the contraction nodes observed in stimulated muscle fibers lead to these fibers bending, contributing to the increase of lightness in the muscle (Hughes et al., 2023). Additionally, ES damages the muscle fibers, enabling oxygen to penetrate deeper into the tissue (Bakker, Underwood, Grubbs, & Blair, 2021), as well as the enzymes involved in oxygen consumption, lowering oxygen consumption rate (Bekhit, Suwandy, Carne, Ahmed, & Wang, 2017). These structural changes additionally contribute to a bright red color of the meat. The improvement in meat color by ES has been well described in the scientific literature (Supplementary Table S1).

Under the conditions established right after ES, *i.e.,* low pH and high temperature conditions, a high amount of protein denaturation is expected, which leads to a lower water-holding capacity (Bekhit et al., 2017). Nevertheless, there is minimal evidence showing that ES increases drip significantly (Rosenvold et al., 2008). According to Devine

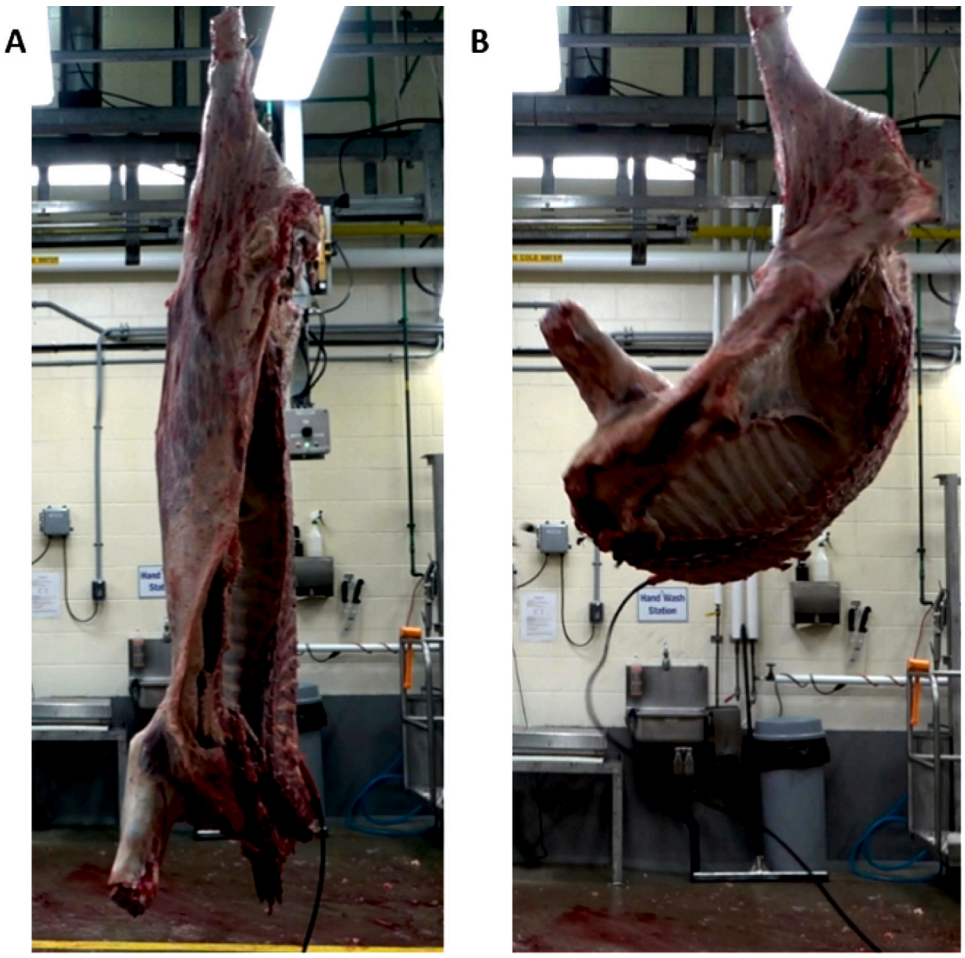


Fig. 1. Constant current electrical stimulation (CCES), a new generation of ES systems. Carcass before A) and after B) CCES. (Reproduced with permission from Leighton et al. (2023)).

et al. (2024), the early appearance of drip in stimulated carcasses is a direct consequence of rapid and extensive muscle tenderization at high temperatures, but there is no more drip for equivalent tenderization when compared to unstimulated carcasses.

Despite the fact that most of the studies have evaluated the effect of ES on the above-mentioned meat quality characteristics, recent findings also suggest an effect of ES on meat eating quality. In this context, ES contributed to the increase of bloody/serumy flavor and typical beef texture, and the reduction of texture attributes such as rubbery and spongy ((Leighton et al., 2023); Supplementary Table S1). It is important to note that, despite bloody/serumy traditionally being considered a negative flavor (Campbell, Hunt, Levis, & Chambers IV, 2001), some consumers have recently considered bloody/serumy flavor to be a positive attribute (Maughan, Tansawat, Cornforth, Ward, & Martini, 2012; Miller, 2020). These positive effects of ES could be due to the supercontraction of muscles during stimulation that leads to physical disruption of myofibers and release of both iron (Alahakoon, Oey, Bremer, & Silcock, 2019) and lysosomal enzymes that hydrolyze myofibrillar and connective tissue proteins (Aalhus, Jones, Best, Robertson, & Lutz, 1994; Mikołajczak et al., 2019). Additionally, ES may further improve flavor by altering the concentrations of flavor precursors and enhancers, such as nucleotides, in the muscles (Calkins, Dutson, Smith, & Carpenter, 1982; Yang, Dashdorj, & Hwang, 2019). As such, Mikami, Nagao, Sekikawa, Miura, and Hongo (1994) demonstrated that the ES of beef leads to an accumulation of peptides and glutamic acid that are thought to be associated with flavor enhancement.

Overall, ES has potential to favor beef tenderization, thereby, stimulated meat is ready for the customer earlier. Consequently, this translates into a reduction of refrigerated storage capacity required for longer ageing, which leads to an important reduction in costs and, hence contributes to the sustainability of the meat industry. The improvements in color and palatability are also benefits of ES that must not be overlooked. In fact, improvement in color is an important issue in the willingness to buy and also associated with normal pHu, which has an impact on shelf life. It is worth mentioning that most of the studies we discussed above are from *Bos taurus* cattle, and our current knowledge is limited in the context of *Bos indicus* cattle, which warrants future investigations.

3. Factors and settings impacting the efficiency of electrical stimulation in relation to meat quality

For many years, HVES has been applied in beef. Nevertheless, a new generation of ES systems such as constant-current electrical stimulation (CCES), medium-voltage electrical stimulation (MVES), and LVES have been developed to mitigate the safety risks and high costs associated with the HVES. A summary of these ES systems applied in beef and the different electrical specifications are shown in Table 1. However, it is not yet clear how MVES and CCES systems can be well distinguished, due to the lack of studies on their use. Also, the CCES system doesn't seem to be yet commercially applied.

The effect of ES on the acceleration of the glycolytic rate and pH decline, favorably impacts meat tenderness (evaluated as shear force

Table 1
Summary of characteristics of electrical stimulation (ES) methods applied to beef carcasses based on the large literature[1], which may not reflect the current conditions used at the industry scale.

Stimulation system [2]	Time	Voltage	Frequency	Amperage	Duration [3]	Pulse length	Pulse width
HVES	During exsanguination−60 min post-mortem	400–3600 V	2–60 Hz 0.33–15 pulses/s	0.5–7.9 A	10 s − 2 min	0.68–3 s on 0.32–3 s off	5–10 ms
CCES	30–45 min post-mortem	300–552 V	15–50 Hz	0.55–2.04 A	20–60 s	2 s on, 2 s off	1–2 ms
MVES	Immediately after stunning−Up to 50 min post-mortem	200–400 V	15–60 Hz	0.3–1.0 A	30–120 s	1–5 s on 1–2 s off	0.5–2 ms
LVES	During exsanguination−30 min post-mortem	21–150 V	13.3–60 Hz 14.3–40 pulses/s	0.2–9 A	15 s − 4 min	0.1–4 s on 0.012–2 s off	2–40 ms

[1] The characteristics in this table may not fully reflect the conditions currently used at the industrial scale, but they are representative of the variation in the conditions reported in the experimental designs of the studies some of which are cited in the manuscript and Table 2.
[2] HVES: high voltage electrical stimulation; CCES: constant current electrical stimulation; MVES: medium voltage electrical stimulation; LVES: low voltage electrical stimulation.
[3] There is one MVES study whose duration is much longer (30 min) and amperage higher (1.94 A) than the rest (Tang & Henrickson, 1980). These unusual durations and amperages have not been considered in the ranges shown in this table. Overall, the duration of ES should not exceed 2 min, since after this time the muscle no longer responds, or responds much less effectively to stimulation.

and/or sensory tenderness scores), and improvement of meat color has been widely studied and documented in the literature (Supplementary Table S1). Nevertheless, according to the recommendations of Devine (2009), comparisons among studies are difficult due to factors that are not accounted for in the studies, such as different animal types, populations or different efficiency of chilling along with possible disparity in muscle typing (Houlier, Valin, Monin, & Salé, 1984). There are, however, some studies comparing the effect of several ES settings impacting the efficiency of ES within the same body of work (Table 2). Among those settings, application time, type and duration of ES, electrical settings (current, voltage, and pulse width), as well as chilling efficiency and carcass size have been reported as factors affecting the ES efficiency concerning meat quality along with frequency as evidenced for sheep meat (Pearce et al., 2009).

In this context, Aalhus et al. (1994) evaluated **several ES systems** in the same study, applying HVES at 40 min post-mortem, LVES for 20 s in the bleeding area, and a combination of both HVES and LVES on beef carcasses. In that study, the three ES systems reduced pH at 3 and 24 h and shear force and overall led to improved color, without affecting drip and cooking losses, when compared to unstimulated carcasses (Supplementary Table S1). Nevertheless, these authors found that the pH at 3 h post-mortem was reduced more in HVES and combined HVES + LVES systems than LVES, and LVES was less tender and had lower a^* and b^* values than the HVES meat at 6 d post-mortem. However, there were no differences in the reduction of pH at 24 h or the effect on shear force, drip loss, juiciness and L^*, a^* and b^* at 24 h and L^* at 6 d post-mortem among stimulation systems (Table 2). It is worth mentioning that HVES is more expensive and has greater safety implications than LVES, however, it has been suggested that HVES results in a greater rate of pH decline and one that is less variable than LVES (Hwang et al., 2003). To avoid intrinsic variations among studies using animals from different backgrounds, Leighton et al. (2024) have recently evaluated the efficacy of CCES and HVES when applied to carcass sides from the same animal and observed that CCES decreased pH early post-mortem (*i.e.*, at 3 h) and purge and drip losses, and improved meat color and some flavor profile attributes compared to HVES (Table 2).

When compared to unstimulated, Hwang and Thompson (2001) observed that LVES applied at 40 min post-mortem reduced the pH at 70, 120 and 180 min post-mortem, and shear force, increased tenderness, and tended to increase juiciness (Supplementary Table S1). In addition, these authors performed further experiments to evaluate if the **time of application of ES** is a setting that can affect the efficiency of the ES (Table 2). In this context, Hwang and Thompson (2001) compared the performance of HVES and LVES at 3 and 40 min post-mortem and reported that pH collected at 70 and 120 min tended to be lower and shear force values were higher when stimulated at 3 compared to 40 min post-mortem in both HVES and LVES. However, meat from HVES applied at 3 min post-mortem was less tender than meat from the other treatments, which were similar to each other. The authors further evidenced shorter sarcomeres due to rigor shortening after ES at 3 min compared with ES at 40 min post-mortem. Nevertheless, no effect of timing or **type of ES** was found on juiciness. Hwang and Thompson (2001) also studied the effect of time of stimulation in the application of HVES (40 *vs.* 60 min post-mortem). These authors observed that early pH measured at 70- and 120-min post-mortem was lower when stimulated at 40 compared to 60 min. Additionally, they reported an interaction only between the timing of stimulation and ageing for shear force, as the HVES applied at 60 min post-mortem initially had a higher shear force value (5.54 kg for HVES 60 min *vs.* 4.50 kg for HVES 40 min at 1-day post-mortem) but had a faster rate of tenderization. Furthermore, the timing of HVES (40 or 60 min) did not affect the sensory tenderness and juiciness of meat. For LVES, it is known that its efficiency is higher if applied within a few minutes post-slaughter (Valin, 1986). As depicted in Fig. 2, the decrease in the rate of pH drop is reduced when LVES (100 V, 2 min) is applied 15 min post-mortem compared to the effect of similar stimulation applied 2 min post-mortem. After this latter time (2 min post-mortem), the rate of the time course in pH decline is almost comparable to the one obtained using HVES (750 V, 2 min) applied 30 min post-mortem.

Regarding the **duration of the stimulation**, Strydom and Frylinck (2014) evaluated the effect of applying LVES for 15, 45 and 90 s. All stimulation treatments increased the rate of pH decline, reduced shear force, increased purge losses, and overall led to improved color (Supplementary Table S1). Nevertheless, the pH decline was more rapid and the L^* was higher in meat stimulated for 45 s and 90 s than for 15 s. Meat stimulated for 15 s, however, had the lowest purge losses measured at 2 d post-mortem. Regarding shear force, applying LVES for 15 s and 45 s reduced the shear force at 2 d post-mortem more than stimulating for 90 s, but only LVES applied for 15 s improved shear force at 14 d post-mortem (Table 2).

Apart from the duration of the stimulation, electrical settings of the stimulation can also impact the efficiency of the ES. In this context, Zhang et al. (2019) applied CCES and evaluated the **current, pulse width, and duration** by using three treatments (1 A/1.55 ms/27 s *vs.* 0.55 A/1 ms/34 s *vs.* 0.55 A/2 ms/20 s). Overall, all these treatments reduced pH, shear force and improved color (although with some differences in color values), without significant effect on cooking losses compared to unstimulated carcasses (Supplementary Table S1). Nevertheless, these authors found that the 1 A/1.55 ms/27 s treatment accelerated pH decline and increased L^* and h° in retail display the most,

Table 2
Summary of the effects of different factors on the efficiency of electrical stimulation based on a non-exhaustive list of studies.

Stimulation system and settings	Factor and/or settings	Reduce pH	Reduce shear force	Increase purge loss (PL), drip loss (DL), cooking loss (CL)	Increase L^*, a^*, b^*, chroma (C^*) & hue angle (h°)	Increase sensory tenderness (T) and juiciness (J)	References
HVES and LVES HVES, 470 V, 60 Hz, 1.5 A for 1 min at 40 min post-mortem. LVES 21 V, 60 Hz, 0.25 A for 20 s immediately after exsanguination.	**Stimulation system**						Aalhus et al. (1994)
	HVES	++ 3 h + 24 h	+	↔ DL, CL	+ L^*, a^*, b^* 24 h, a^*, b^* 6 d ↔ L^* 6 d	+ T ↔ J	
	LVES	+ 3 h + 24 h	+	↔ DL, CL	+ L^*, a^*, b^* 24 h ↔ L^*, a^*, b^* 6 d	↔ T ↔ J	
	Combined HVES and LVES	++ 3 h + 24 h	+	↔ DL, CL	+ L^*, a^*, b^* 24 h ↔ L^*, a^*, b^* 6 d	+ T ↔ J	
CCES *vs.* HVES CCES, 50 Hz, 2.04 A_{RMS}, 380–554 V_{RMS} (2 s on, 2 s off for 60 s) at 45 min post-mortem. HVES, 60 Hz, 477 V_{RMS} (2 s on, 2 s off for 60 s) at 45 min post-mortem.	**Stimulation system**						Leighton et al. (2024)
	CCES *vs.* HVES	++ 3 h CCES	–	– CL ++PL, DL in HVES	+ L^*, b^* 3, 6, 10 d CCES + a^* 3 d CCES – C^*, h°	– T, J	
HVES and LVES 1. LVES, 70 V, 1.4 A, 7 ms width, 14.3 pulses per s applied for 40 s at 3 min post-mortem. 2. HVES, 800 V, 7.9 A, 10 ms width 14.3 pulses/s for 40 s at 3 min post-mortem. 3. LVES, 70 V, 0.6 A, 7 ms width, 14.3 pulses per s applied for 40 s at 40 min post-mortem. 4. HVES, 800 V, 6.5 A, 10 ms width 14.3 pulses/s, for 55 s at 40 min post-mortem.	**System and time of stimulation**						Hwang and Thompson (2001)
	HVES, 3 min	++	+	/	/	+ T ↔ J	
	HVES, 40 min	+	++	/	/	++ T ↔ J	
	LVES, 3 min	++	+	/	/	++ T ↔ J	
	LVES, 40 min	+	++	/	/	++ T ↔ J	
1. HVES, 800 V, 6.5 A, 10 ms width 14.3 pulses/s for 55 s at 40 min post-mortem. 2. HVES, 800 V, 6.0 A, 10 ms width 14.3 pulses/s for 55 s at 60 min post-mortem.	**Time of stimulation**						
	HVES, 40 min	++	↔	/	/	↔ T & J	
	HVES, 60 min	+	↔	/	/	↔ T & J	
LVES 150 V, maximum amplitude = 17 Hz, 5 ms pulse width, RMS voltage below 50 V applied immediately after exsanguination.	**Stimulation duration**						Strydom and Frylinck (2014)
	15 s	+	++ 2 and 14 d	+ PL	↔ L^* + C^*	/	
	45 s	++	++ 2 d ↔ 14 d	++ PL	++ L^* and C^*	/	
	90 s	++	+ 2 d ↔ 14 d	+++ PL	++ L^* and C^*	/	
CCES 300 V, 15 Hz before entry into the chiller.	**Amperage, pulse width, duration**						Zhang et al. (2019)
	1 A, 1.55 ms, 27 s	++	+	↔ CL	++ L^* 1, 8 and 15 d + b^* 1 and 15 d, C^* 1 d, and h° 1, 8 and 15 d ↔ a^* 1, 8 and 15 d, b^* 8 d and C^* 8 and 15 d	/	
	0.55 A, 1 ms, 34 s	+	+	↔ CL	++ C^*1d + a^*, b^*, C^* 8 d and h° 15 d ↔ L^* 1, 8 and 15 d, a^*, b^*, C^* 15 d and h° 1 and 8 d	/	
	0.55 A, 2 ms, 20s	+	+	↔ CL	++ C^* 1d + L^* 1, 8 and 15 d a^*, b^*, C^* 8 d, and h° 15 d	/	

(*continued on next page*)

Table 2 (*continued*)

Stimulation system and settings	Factor and/or settings	Reduce pH	Reduce shear force	Increase purge loss (PL), drip loss (DL), cooking loss (CL)	Increase L^*, a^*, b^*, chroma (C^*) & hue angle (h°)	Increase sensory tenderness (T) and juiciness (J)	References
					↔ a^*, b^*, C^* 15 d and h° 1 and 8 d		
	Voltage						de Alvarenga et al. (2024)
MVES 15 Hz, 0.3–0.5 A, 5 s on, 2 s off for 25–30 s after carcass splitting.	200 V	+ 0, 6 and 24 h	+ 3, 7 and 10 d ++ 14 d	↔ CL	+ b^* 7 d ↔ L^* and a^* 3, 7, 10 and 14 d ↔ b^* 3, 10 and 14 d	/	
	300 V	+ 0 and 6 h ↔ 24 h	+ 3, 7, 10 and 14 d	↔ CL	Decrease b^* 10 d ↔ L^* and a^* 3, 7, 10 and 14 d ↔ b^* 3, 7 and 14 d	/	
	400 V	+ 0 and 6 h ↔ 24 h	+ 3, 7 and 10 d ++ 14 d	↔ CL	↔ L^*, a^* and b^* 3, 7, 10 and 14 d	/	
LVES 4 s pulse with 2 s between pulses applied for 60 s at 45 min post-mortem	**Voltage**						(Bakker et al., 2021)
	80 V	++	+	↔ CL	+ L^*, a^*, b^*	/	
	40 V	+	+	↔ CL	+ L^*, a^*, b^*	/	
	Voltage and # of pulses						(Roeber et al., 2000)
MVES and LVES 60 Hz, impulse cycles 1 s on, 1 s off at 50 min post-mortem	11 impulse cycles of 100 V	/	+	↔ PL (inside round) & CL	+ L^*, a^*, b^*	/	
	16 impulse cycles of 100 V	/	+	↔ PL (inside round) & CL	+ L^*, a^*, b^*	/	
	6 impulse cycles of 100 V plus 5 impulse cycles of 300 V	/	+	↔ PL (inside round) & CL	+ L^*, a^*, b^*	/	
	6 impulses of 100 V plus 10 impulse cycles of 300 V	/	+	↔ PL (inside round) & CL	+ L^*, a^*, b^*	/	
	Rapid chilling (−12 ° C for 2 h)						Li et al. (2006)
LVES 24 V, 50 Hz for 30 s immediately after exsanguination	ES	++	+	++ CL ↔ PL	/	/	
	ES + rapid chilling	+	↔	+ CL Decrease	/	/	
	Carcass size						
HVES 470 V, 60 Hz, 1.5 A for 1 min at 40 min post-mortem	Heavy	/	+ 2 d ↔ 6, 13, 21 and 27 d	/	/	/	Juárez et al. (2016)
	Light	/	+ 2, 6, 13, 21 and 27 d	/	/	/	
HVES	Heavy	/	/	/	↔ L^*, a^* and b^* of *Psoas major* (PM)	/	Djimsa et al. (2022)
	Light	/	/	/	+ L^* of *PM* ↔ $a^*$and $b^*$of *PM*	/	
	Conditioning						(Mikołajczak et al., 2019)
MVES 350 V, 17 Hz, 20 min post-mortem	ES	+ 45 min ↔ 48 h, 3d	+	/	/	/	
	ES + conditioning (18 h at 12–15 °C immediately after slaughter)	+ 45 min ↔ 48 h, 3d	+	/	/	/	

ES: Electrical stimulation.
Effects compared to unstimulated: medium (+), strong (++), very strong (+++); no effect compared to unstimulated carcasses (↔); quality traits not measured in the study (/).
[γ]: Electrical stimulation treatments were applied to carcass sides from the same animal and compared to each other; no differences between treatments (−).
Results are from *longissimus* muscle unless otherwise specified.

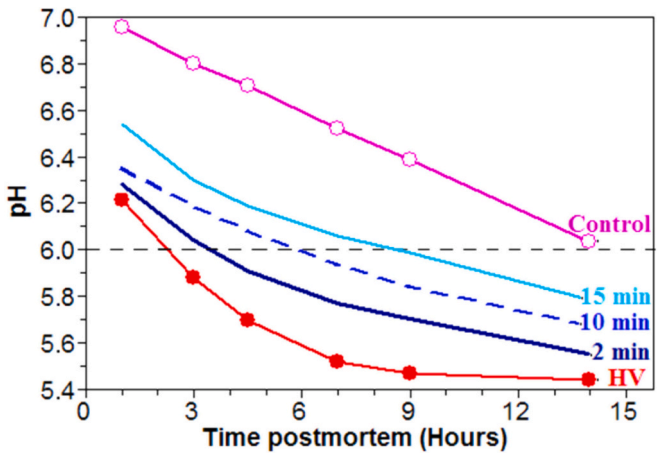


Fig. 2. pH decline curves of beef *Longissimus* muscle following different electrical stimulation conditions. **Control:** the control animals (unstimulated carcasses), **HV:** HVES, **Curves 2, 10 and 15**: LVES at 2-, 10- and 15-min post-mortem. Sampling times for LVES are the same than for the control and the HVES. Each point is the mean for three animals (5–6-years-old Friesian cull cows). The horizontal dashed line corresponds to the pH limit for prevention of cold shortening, *i.e.,* pH 6.0, The current voltage was 750 and 100 V for HVES and LVES stimulation, respectively. (Adapted with permission from Valin (1986)).

but decreased a^* and C^* compared to the other two treatments (Table 2). The positive effect on shear force values was not affected by the different currents, pulse widths, or durations applied. Other settings such as different **impulse cycles** in MVES and LVES seem to have no differential impact on color and shear force (Roeber, Cannell, Belk, Tatum, & Smith, 2000).

When applying ES, the **voltage** of the stimulation has also been examined. de Alvarenga et al. (2024) applied HVES and MVES using 200, 300 and 400 V and found that all treatments reduced pH at 0 and 6 h and shear force at different ageing times, without affecting cooking losses and most of L^*, a^*, b^* values (Supplementary Table S1). However, 200 V increased b^* at 7 d and 300 V decreased b^* at 10 d post-mortem compared to unstimulated meat. Additionally, the 200 V treatment also decreased pH at 24 h compared to no stimulation, whereas no reduction was observed for the other two treatments. The decrease in shear force after 3, 7 and 10 d of ageing was similar among the 200, 300 and 400 V treatments, however, the reduction in shear force after 14 d was numerically higher for the 200 V compared to 300 and 400 V (Table 2). Such results warrants further investigation to decipher the underlying mechanisms. Bakker et al. (2021) applied LVES using 40 and 80 V and found that both treatments reduced pH at 1 h post-mortem and shear force and increased L^*, a^*, b^* at 3 d compared to unstimulated meat, although no differences were found for cooking losses (Supplementary Table S1). However, the pH at 1 h post-mortem was reduced more when applying 80 than 40 V, although no voltage effect was observed for shear force or color (Table 2).

Rapid chilling can be another factor affecting the efficiency of ES. Li et al. (2006) applied LVES after exsanguination and observed a reduction in the pH at 1 h post-mortem and shear force and an increase in cooking losses compared to unstimulated meat (Supplementary Table S1). However, when they applied LVES after exsanguination followed by rapid chilling (−12 °C for 2 h), the rapid chilling decreased the rate of pH decline and increased shear force (to the extent that no longer were differences observed compared to unstimulated meat), but there was reduced purge and cooking losses compared to stimulated meat without rapid chilling (Table 2). In contrast, **conditioning treatment** (carcasses stored at 12–15 °C for 18 h starting immediately after slaughter) combined with MVES did not provide significant differences in the decrease of pH or shear force compared to the application of MVES alone (Mikołajczak et al., 2019).

The beneficial effect of ES is also well known to be closely dependent on the **muscle type**, and more specifically on its anatomical location (Devine, Ellery, & Averill, 1984; Valin, Touraille, Ouali, & Lacourt, 1981). These authors show that during the first 24 h, ES accelerates the maturation process in the most superficial muscles, such as the *Longissimus* and the *Triceps brachii*, both of which can contract at low temperature. On the other hand, under the same conditions, the electrically stimulated *Semimembranosus* muscle slows down this process. Unlike the two preceding muscles, the position of the *Semimembranosus* muscle protects it from any risk of cold shortening, even when subjected to fast chilling. Indeed, whatever the chilling conditions, the *Semimembranosus* muscle shows a slow decrease in temperature and a rapid drop in pH (Tarrant & Mothersill, 1977). Additionally, **carcass size** has been found to affect the impact of HVES. In this context, Juárez et al. (2016) and Djimsa, Nair, Hess, Belk, and Woerner (2022) observed a decrease in HVES efficacy in heavier carcasses to reduce shear force values and increase L^* in meat compared to unstimulated carcasses, respectively (Table 2). Although it depends on the carcass weight and fatness of the carcasses, the application of CCES seems, however, to solve the issues of HVES, as a significant reduction of shear force up to 12 d post-mortem and an increase of L^* were observed in meat from stimulated heavier carcasses compared to unstimulated ((Leighton et al., 2023); Supplementary Table S1). This could be due to the customized voltage for each carcass provided by the CCES as, in this new generation of ES systems first developed in Australia, the voltage varies based on carcass impedance, thereby enabling the electrical input to match the requirements of a particular carcass.

Considering the points mentioned above, several aspects merit emphasis regarding the enhancement of meat quality through ES, including the reliability of data and the comparison of results within the same experiment. In this context, three key areas require attention:

i) **Utilization of objective and consumer thresholds:** while studies have compared results between unstimulated and stimulated meat using mean values of quality traits, none have taken into account objective or consumer thresholds (Holman & Hopkins, 2021). Incorporating these thresholds, using individual data, would provide valuable insights into the frequency of high and low-quality meat, enhancing the interpretability of the results along with decision-making for carcass categorization. This could be further substantiated by incorporating the end-point cooking temperatures of beef and the country of origin of the panelists, as previously demonstrated with unstimulated beef (Gagaoua et al., 2016).

ii) **Sample size and representation:** many studies have utilized a limited number of carcasses, failing to accurately reflect the production rate and type of animals slaughtered by the current beef industry. This limitation hinders the development of prediction models for meat quality. Increasing the sample size and ensuring representation across different production contexts and systems would enable more robust predictions and generalization of ES treatments. Such an aspect can be addressed first using a meta-analysis of electrically stimulated bovine carcasses, as performed on small ruminants (Abhijith et al., 2020).

iii) **Holistic evaluation of beef quality traits:** Existing studies have tended to evaluate quality traits individually, overlooking the potential benefits of holistic approaches. It is essential to adopt methodologies that consider the interplay between various quality traits. For instance, employing clustering and decision tree approaches (Gagaoua, Monteils, & Picard, 2019; Gagaoua, Picard, Soulat, & Monteils, 2018; Listrat et al., 2020) can facilitate a more comprehensive assessment of meat quality and enable meaningful comparisons within treatments.

Table 3
Summary of reports measuring the effect of electrical stimulation on different indicators of proteolysis in beef muscles.[1]

Indicator(s) of proteolysis	Type of stimulation/conditions	Main outcomes	References
Degradation of desmin and troponin-T	Medium voltage electrical stimulation (ES; ~300 V, constant current 550 mA peak, 1 ms pulse width, 15 Hz, 34 s; at 45 min post-mortem. Used Chinese crossbred yellow cattle (18–24 mo old).	Both ES and prior ageing significantly reduced the initial toughness evaluated by an objective assessment (Warner-Bratzler shear force). The combination of ES and prior ageing shortened the storage time needed to reach an acceptable tenderness of superchilled beef. Desmin was the only proteolytic indicator that gradually degraded into small fragments as storage time extended, and each treatment resulted in different degrees of degradation. Troponin-T was changing as a consequence of ageing only.	Ji et al. (2021)
Myofibril fragment length (MFL), calpain-1	110 V, 17 Hz, 5 m/s pulse. ES duration was 30 s or 60 s, ES time was late stimulation (45 min post-mortem) or early stimulation (7 min post-mortem). Breed of cattle unspecified.	Early stimulation coincided with marginally longer MFL at 3- and 14-d, while late stimulation produced the shortest MFL at 14 d in (**LL**). Higher decline in calpain-1 concentration (mean 36.2 %) was recorded in the early stimulated carcasses compared with the late stimulated carcasses (mean 29.7 %) from 1 to 24 h PM, while calpastatin concentration decreased at a similar rate (mean 24 %)	Webb and Agbeniga (2020)
Myofibril fragmentation index (MFI) and muscle ultrastructure	ES was applied using a low-voltage Jarvis Es-4 stimulator (output: 21 V, 50 w) after bleeding for 30 s. The stimulation time was 72 s, 90 s, or 108 s. Used yak bulls.	ES severely damaged muscle fibril structure of yak (**LL**), and the myofibril damage was aggravated with prolonged stimulation, ES 90 s group showed a unique contracture zone. Higher MFI for all stimulated samples compared with non-stimulated ones from day 0 to 9.	Chen et al. (2020)
Degradation of titin and desmin and changes in heat shock proteins - HSP20, HSP27 and αβ-crystallin	The LL from either the right or left side of the carcass was randomly assigned for immediate treatment with low voltage electrical stimulation 30 s after boning (frequency = 13.3 Hz, pulse width = 5.4 ms, peak voltage = 104 V), with beef samples aged up to 28 days.	The concentrations of αβ-crystallin and HSP20 were higher in stimulated muscle at 1- and 2-d post-mortem. Stimulation did not increase the rate of degradation of titin or desmin, but the 30 kDa degradation product of troponin-T was detected earlier in stimulated (**LL**).	Contreras-Castillo et al. (2016)
Activity of Cathepsin B and L, and μ- and m-Calpains	Direct current, 80 V for 35 s at 30 min after stunning. The LL was removed immediately after stimulation and placed at approximately 10 °C for 12 h, then transferred to 4 °C. Used Swedish Red Cattle.	ES promoted early μ-calpain activation and increased free lysosomal cathepsin B and L activities of (**LL**).	Li et al. (2012)
Myofibril filament length (MFL)	Carcasses were split and the left sides were electrically stimulated for 30 s (400 V peak, 5 ms pulses at 15 pulses per second) within 30 min of death.	Stimulation did not decrease MFL when measured in muscle (**LL**) aged for either 3- or 14-d.	Hope-Jones et al. (2010)
Degradation products of troponin-T	Stimulated hot boned excised muscles from 8 carcasses within 90 min of death – 90 V at 14 Hz. The stimulated and non-stimulated muscles were halved and stored at 2 °C for 48 h or 10 °C for 10 h followed by 2 °C until 48 h. All muscles were vacuum packaged at 48 h post-mortem and stored at 2 °C for up to 14 days post-mortem.	The 30-kDa subunit appeared in stimulated (**LL**) after 14 days of ageing and non-stimulated (**LL**) after 7 days of ageing.	White et al. (2006a)
Degradation of titin and nebulin	50 V, 60 Hz for 20s within 3 min of death. Used Hanwoo bulls.	Degradation rate increased with stimulation and conditioning at higher temperatures (**LL**).	Rhee, Ryu, Imm, and Kim (2000)
Myofibrillar protein solubility	85 V, 14 Hz for 15 s straight after death. Used Friesian/Holstein cattle.	There was no effect of simulation on protein solubility in either the (**LL**) or (**SM**) muscle.	den Hertog-Meischke et al. (1997)
Degradation of titin, nebulin, desmin and troponin-T	200 V, 20 Hz for 15 or 20 s, 3 times with 30 s breaks between within 1 h of death. Used Brahman x Simmental cattle.	Stimulation did not increase the degradation rate of the proteins (**LL**).	Ho et al. (1997)
Degradation of titin, nebulin, desmin and troponin-T	200 V, 20 Hz for 15 s 3 times with 30 s breaks between within 1 h of death. Used Angus x Jersey cattle.	Titin, nebulin and troponin-T were degraded faster in **some** stimulated muscle (**LL**).	Ho et al. (1996)
Degradation of troponin-T and myofibrillar fragmentation index (MFI) changes	85 V, 14 Hz for either 8 or 64 s within 5 min of death. Breed of cattle unspecified.	Stimulation for 64 s increased the appearance rate of the 30-kDa fragment in (**LL**), but by 21 days there was no difference in the amount of the fragment. Day 1 MFI's were higher in stimulated muscle (64 s), but there was no difference to stimulated muscle (8 s) or control at 7, 14 or 21 days.	Geesink et al. (1994)
Degradation of troponin-T and myofibrillar fragmentation index (MFI) changes	85 V, 14 Hz for 60 s within 30 min of death. Used Friesian/Holstein veal calves.	Stimulation had no effect on the appearance rate of the 30-kDa fragment in 3 different muscles (**LL; SM; PM**). MFI was higher in stimulated muscle (**LL**), but had no effect on MFI in the **SM**.	Geesink et al. (1993)

(*continued on next page*)

Table 3 (*continued*)

Indicator(s) of proteolysis	Type of stimulation/conditions	Main outcomes	References
Degradation of titin, nebulin, filamin and troponin-T.	600 V, 50 Hz for 120 s, within 1 h of death. Used Belgian white-red cattle.	After 1-day post-mortem less troponin-T was present in stimulated muscle (**LL**) and by 6 days there was less titin	Uytterhaegen et al. (1992)
Degradation troponin-T and myofibrillar fragmentation index (MFI) changes	45 V, for 40 2 s pulses, 2 s apart giving a total of 40 s, straight after death. Used Holstein steers.	No effect of stimulation on the rate of degradation of troponin-T, but a product of degradation did appear earlier in stimulated muscle (**LL**). MFI was higher for stimulated muscle with slow chilling procedure (placed for 4 h at room temperature) than for control muscle at 24 h.	Pommier (1992)
Myofibrillar protein solubility	115 V, 60 Hz for 5 12 s pulses, 3 s apart giving a total of 60 s, within stimulation 1 h post-mortem. Used Charolais crossbred cattle.	No effect of stimulation on protein solubility (**ST**) when measured at 7 days.	Bruce et al. (1990)
Degradation troponin-T	45 V, for 40, 2 s pulses, 2 s apart giving a total of 40 s, straight after death. Used Holstein veal calves.	Stimulation decreased the rate of degradation of the protein (**LL**)	Pommier et al. (1987)
Myofibrillar protein solubility and degradation products of troponin-T	550 V, 60 Hz for 80, 1 s pulses, 0.5 s apart for a total of 2 min, within 30 min of death. Breed of cattle unspecified.	Increased protein solubility (**LL**) when measured at 3 days post-mortem. Reported that 30 kDa and 32 kDa subunits appeared earlier in stimulated muscle (4-h post-mortem).	Ducastaing et al. (1985)
Degradation of ∝ − actinin and troponin-T	500 V, 60 Hz for 20, 2 s pulses, 1 s apart giving a total of 40 s, within 45 m of death. Breed of cattle unspecified.	Less α-actinin and troponin-T was found in stimulated muscle (**LL**), but there was an interaction with chilling temperature.	Salm et al. (1983)
Degradation troponin-T and myofibrillar fragmentation index (MFI) changes	300 V, 60 Hz for 80, 1.5 s pulses, 0.5 s apart giving a total of 120 s, within 30 min of death. Breed of cattle unspecified.	MFI was increased by stimulation (**LL, SM, TB**), but not significantly and there was no effect on the degradation rate of troponin-T.	Sonaiya et al. (1982)

[1] Indicates the muscle used where **LL** = *longissimus*, **ST** = *semitendinosus*, **PM** = *psoas major*, **SM** = *semimembranosus*; **TB** = *triceps brachii*.

In summary, addressing these aspects could contribute to a more nuanced understanding of the impact of ES on meat quality, thereby promoting evidence-informed decision-making processes within the beef industry and advancing research in this domain.

4. Biochemical mechanisms behind electrical stimulation revealed using traditional biochemistry methods

Although it is well established that ES increases the rate of post-mortem glycolysis, other biochemical and biophysical effects on tenderness have been involved with the use of this technology (Hwang et al., 2003). For example, Hwang and Thompson (2002), reported that ES resulted in stretched and disrupted sarcomeres, whereas Luo, Zhu, and Zhou (2008) found that ES, applied immediately after slaughter, produced no evidence of super contraction in ES samples without ageing, however, in the study of Zhang et al. (2019), partial physical disruption was evidenced.

As reported, a rapid drop of pH at higher temperatures promotes early release of free calcium (Ca^{2+}) from the sarcoplasmic reticulum, enhancing the activity of endogenous proteases such as calpain (Whipple, Koohmaraie, Dikeman, & Crouse, 1990) and triggering apoptosis onset (Ouali et al., 2013). Here we focus on the biochemical effects arising from the enhancement of the rate of proteolysis stimulated by the release of free calcium, which is considered the main factor driving these biochemical changes.

The degradation of myofibrillar proteins during the post-mortem period in muscles subjected to ES has been studied using several techniques, including fragmentation of myofibrils (Geesink et al., 1993; Smith, Bush, van de Ven, & Hopkins, 2016), measurement of protein solubility (Ducastaing et al. (1985) and gel electrophoresis, to analyze the changes in the intensity of certain protein bands or appearance/disappearance of specific protein fragments, as a consequence of proteolysis (Ho, Stromer, & Robson, 1996).

Although gel electrophoresis using one-dimensional SDS-PAGE gels can highlight the impacts of ES and differences among similar experiments, it has some potential limitations, which can be exemplified by comparing the results of Ho et al. (1996) and Ho, Stromer, Rouse, and Robson (1997). In fact, very similar electrical inputs were used in both experiments, with different cattle breeds (*Bos taurus vs. Bos indicus*), but, otherwise, the designs were similar, yet Ho et al. (1996) in one set of results found that stimulation caused a faster degradation of proteins such as titin and troponin-T in some muscles, whereas, Ho et al. (1997) reported no such effect. It should be noted that a large tenderness advantage of *Bos taurus* cattle over *Bos indicus* has been reported when ES was not used, but HVES (1130 V peak, 14.28 pulses/s) mostly eliminated this difference (Gursansky, O'Halloran, Egan, & Devine, 2010). In both studies of Ho and co-workers, the interpretation of the gels was based on visual assessment, whereas in the work reported by Uytterhaegen, Claeys, and Demeyer (1992) and Hopkins and Thompson (2001a), techniques to quantify the change in protein bands were used. In this latter study, an unidentified protein fragment was in greater quantity in stimulated muscle pre-rigor, and the rate of decline was greater up to 2 d post-mortem. Even this approach based on quantification, however, can produce contrary results as seen by comparing the reports of Pommier, Poste, and Butler (1987) and Pommier (1992). Related to this, Contreras-Castillo, Lomiwes, Wu, Frost, and Farouk (2016) and Ho et al. (1997), who both used *Bos indicus* cattle, did not report that ES promoted the degradation of cytoskeletal proteins, but the former study was conducted with hot-boned muscle, which was maintained at 15 °C similar to the study of Martin, Hopkins, Gardner, and Thompson (2006). This latter study was designed to eliminate the impact of the rate of pH fall on the degradation of the muscle structure by holding the excised muscle at 15 °C. Indeed, this did remove all differences in the extent of the myofibrillar degradation (Table 3). This is a revealing study, as it suggests that changes in the myofibrillar proteins are driven by changes in the pH-temperature relationship which is

altered by stimulation and that, there is no intrinsic effect of stimulation apart from this on myofibrillar protein degradation. By contrast, the study of White, O'Sullivan, Troy, and O'Neill (2006b) produced very unusual results that are hard to reconcile with the earlier appearance of degradation products of troponin-T in stimulated hot-boned compared to non-stimulated hot-boned muscles (Table 3).

As mentioned earlier, the effect of ES on endogenous enzymes may be related to the stimulation time. Geesink, van Laack, Barnier, and Smulders (1994) showed that a brief application of ES (8 s) did not affect the activity of μ-calpain and calpastatin at 1.5 h post-mortem, whereas prolonged ES time (64 s) resulted in a significant decrease in the activities. Recently, Chen et al. (2020) found increased myofibrillar fragmentation index (MFI) values in yak meat when the stimulation duration was extended from 72 s to 108 s. The impact on protein solubility has, however, varied from no effect (Bruce, Jones, & Ball, 1990; den Hertog-Meischke, Smulders, Van Logtestijn, & van Knapen, 1997) to an effect in the *Longissimus* muscle after 3 d of ageing in beef (Ducastaing et al., 1985), and with different electrical settings used between experiments it does make it difficult to unequivocally claim that ES always results in specific myofibrillar changes. It is noteworthy that Hopkins and Thompson (2001b) reported an interaction between stimulation and ageing such that between sampling at pH 6.0 and 2-d post-mortem, stimulated muscle exhibited greater solubility. This, along with data from gel electrophoresis, suggests that ES favors an acceleration of myofibrillar degradation early post-mortem.

The ease with which myofibrils can be broken or fractured (MFI) has been a useful simple measurement for following myofibrillar degradation. Early work found that ES had no impact on this trait (Sonaiya, Stouffer, & Beerman, 1982) and other more recent studies reported that stimulation did not decrease myofibril filament length (MFL) when measured in *Longissimus* muscle aged for either 3 or 14 d (Hope-Jones et al., 2010). However, Geesink et al. (1993) reported an effect of stimulation on MFI of beef *Longissimus* muscle only for samples aged for 7 d. Hopkins and Thompson (2001b) reported that there was a significant interaction between stimulation and ageing for MFI, such that as stimulated *Longissimus* muscle from lamb aged, the rate of change of MFI was greater.

Therefore, the process by which ES promotes muscle tenderization through free calcium release may exhibit different effects depending on the type of muscle fibers. Studies have shown that type II muscle fibers respond more strongly to ES than type I muscle fibers (den Hertog-Meischke et al., 1997). There are differences in the composition of muscle fiber types in different parts of muscles (Picard & Gagaoua, 2020b), which may be one reason for the different effects of ES on different parts of muscles (Houlier et al., 1984). For example, the density of L-type calcium channels, an important parameter affecting free calcium release, is associated with the fast oxido-glycolytic type of muscle fibers, but not with the slow oxidative or fast glycolytic fiber types (Mänttäri, Pyörnilä, Harjula, & Järvilehto, 2001; Picard & Gagaoua, 2020b). In addition, earlier studies also pointed out that red muscles are relatively susceptible to cold shortening (Cornforth, Pearson, & Merkel, 1980), but are rarely affected by ES, while white muscles are rarely affected by conditions that cause cold shortening (Pearson, Dutson, Pearson, & Dutson, 1985). Further, Dutson, Smith, and Carpenter (1980) reported that lysosomal membranes were disrupted with decreasing pH, hence, leading to the release of cathepsins and enhanced proteolysis. Li et al. (2012) also found that ES promoted early μ-calpain activation and increased free lysosomal cathepsin B and L activities of *Longissimus* muscle.

The techniques described above are specific for looking at targeted proteins or protease activity only, using one-dimensional electrophoresis, but they are not powerful enough for in-depth characterization of all the changing proteins and their release or disappearance as fragments or in looking at the multiple proteins. However, the analysis of post-mortem muscle proteomes allows, thanks to advanced mass spectrometry (MS) and bioinformatics, for a more global examination of the dynamic processes unleashed by ES early or during the post-mortem period. Recent developments in proteomics offer the scope to further understand these changes in the form of both individual proteins and biochemical pathways at interplay.

5. New insights into the biochemical mechanisms behind electrical stimulation revealed by proteomics

With the advances in high-throughput foodomics technologies and the multiple applications of MeatOmics in meat research (Gagaoua et al., 2024), proteomics has emerged as a method of choice compared to the traditional biochemistry methods to analyze the series of physical and biochemical reactions in post-mortem muscle, namely those related to protein changes and their interrelated effects with several meat quality traits (Purslow et al., 2021). Recent advances in proteomics technologies have enabled simultaneous measurements of biological systems at different biochemical levels (Gagaoua et al., 2024). In the meat research field, proteomics has been demonstrated as a powerful tool that helps to decipher several pathways that are impacted by the post-mortem interventions, such as cooking (Gagaoua, Terlouw, Richardson, et al., 2019; Paredi et al., 2013; Salim et al., 2020; Tian et al., 2016; Yu, Morton, Clerens, & Dyer, 2017), alternating electric field-assisted freezing–thawing and ageing (Wu et al., 2022) or early post-mortem carcass interventions such as chilling (Zhang et al., 2018), hanging (Cai et al., 2023) or electrical stimulation (see studies listed in Table 4). Given the potential effects of ES on muscle structure, metabolism and glycolysis, proteolysis, and increased free calcium release and enzyme activities, proteomics represents an appropriate strategy for a deeper understanding of the biochemical mechanisms thanks to the in-depth quantitative and bioinformatics analyses it allows. In the following sections, we discuss, the current knowledge and main proteins reported to change in electrically stimulated bovine carcasses, based on an integrative analysis approach of published proteomics studies.

5.1. Integration of electrical stimulation muscle proteome studies is key for in-depth understanding of the underlying biochemical mechanisms

Through a datamining integrative analysis approach, in the frame of integromics, as recently described (Gagaoua, Suman, Purslow, & Lebret, 2023), we identified for the purpose of this work, six proteomics studies that all applied LVES, to investigate the protein changes triggered by ES in post-mortem bovine muscle (Bjarnadóttir, Hollung, Høy, & Veiseth-Kent, 2011; Contreras-Castillo et al., 2016; Kim et al., 2013; Li et al., 2012; Li et al., 2015; Shen, Li, Mao, Zhang, & Luo, 2012). These studies, published between 2011 and 2016, are described in Table 4. Although the number of ES muscle proteomics studies is low, a valuable strategy to expand our knowledge on the underlying mechanisms behind ES, which to the best of our knowledge has not been attempted so far, is their integration to create a compendium of 67 putative protein biomarkers (Fig. 3). Such a repertoire, in the frame of cross-study analyses, provides a more holistic understanding of the ES processes and their interactions.

The compendium has been analyzed for the degree of overlap in the changing proteins, the type of abundances in response to ES and/or to reveal the major molecular signatures at interplay. Actually, the integration of the changing proteins across ES studies in one repertoire constitutes a rich source of information, especially for performing bioinformatics analyses, which none of the 6 studies performed. We created a database of 67 unique proteins (including their proteoforms) that were changing in bovine *Longissimus* muscle as a result of LVES (Fig. 3). The studies applied different proteomic methodologies, but all were gel-based approaches coupled with MS (Table 4), but none have yet performed label-free quantitation of proteins, also known as shotgun proteomics. In these studies, researchers have reported that LVES can influence meat quality traits by promoting rapid pH decline (Bjarnadóttir et al., 2011; Kim et al., 2013), resulting in more tender meat in some cases, as demonstrated by two studies (Bjarnadóttir et al., 2011; Li et al., 2012). However, other studies have only focused on the

Table 4
Summary of studies investigating the effects of low-voltage electrical stimulation (LVES) on skeletal muscle proteomes (*Longissimus*) and possible effects on meat quality traits.

Study ID and reference	ES protocol	Breed / animal type	Number animals	Sampling at post-mortem for protein extraction	Meat quality traits/ conditions	Impacts ES on meat quality traits	Proteomic approach
ID1_ Bjarnadóttir et al. (2011)	0–95 V for 8 s; 95 V for 32 s	Norwegian Red/ bulls	10	1 h and 1-d post-mortem	N/A	Lower muscle pH during the first 10 h and lower WBSF	2DE + MALDI-TOF/TOF
ID2_ Li et al. (2012)	80 V for 35 s	Swedish Red Cattle/ bulls and steers	9	3 h post-mortem	pH + temperature (0, 3, 10, 24, 72, and 168 h post-mortem) and WBSF (24, 72, and 168 h post-mortem)	Accelerated meat tenderization; More tender meat at 168 h	2DE + MALDI-TOF and enzyme activity assays
ID3_ Shen et al. (2012)	42 V for 40 s	Chinese yellow crossbred/ bulls	10	3-d post-mortem	N/A	Changes in protein abundance.	2DE + MALDI-TOF/TOF
ID4_ Kim et al. (2013)	100 V for 30 s	N/A. Steers	8	1-day and 9-days post-mortem	pH (1.5, 3.5, 5.5, 8.5, 24 h post-mortem); WBSF + Sensory traits (tenderness, chewiness and juiciness) 24 h and 9 d post-mortem;	More rapid pH decline during the first 24 h of post-mortem chilling; No impacts on meat tenderness (sensory or WBSF)	SDS-PAGE + Western blotting; 2D-DIGE + ESI/MS
ID5_ Li et al. (2015)	80 V for 35 s	Swedish Red Cattle/ bulls and steers	9	0, 3, and 10 h post-mortem	N/A	Changes in protein abundance. Increase in the total phosphorylation level.	SDS-PAGE + LC-MS/MS + Phosphoproteins
ID6_ Contreras-Castillo et al. (2016)	104 V for 30 s; Frequency = 13.3 Hz; pulse width = 5.4 ms	Angus crossbred/ bulls	15	1- and 2-days post-mortem	pH (0.5 h, 3 h, 6 h, 18 h, 24 h and 48 h post-mortem) and WBSF (24 h, 2, 7, 14- and 28-days post-mortem)	No effect on the rate of pH decline. No differences on WBSF. Changes in protein abundance.	SDS-PAGE + Western blotting and enzyme activity assays.

2DE: Two dimensional-electrophoresis; MALDI-TOF: matrix assisted laser desorption ionization - Time of flight. SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. 2D-DIGE: Two-dimensional difference gel electrophoresis. LC-MS/MS: liquid chromatography tandem mass spectrometry; post-mortem: post-mortem.

changes in protein abundances (Contreras-Castillo et al., 2016; Kim et al., 2013; Li et al., 2015; Shen et al., 2012), without details or impacts on meat quality traits (details in Table 4).

Briefly, 2D-PAGE coupled with MALDI-TOF/TOF MS was applied in four studies (Bjarnadóttir et al., 2011; Kim et al., 2013; Li et al., 2012; Shen et al., 2012). For example, Bjarnadóttir et al. (2011) explored the insoluble protein fraction (myofibrillar proteome) collected at 1 and 24 h post-mortem and identified 23 proteoforms (12 unique proteins) changing due to ES intervention (Fig. 3). When investigating the changes in the sarcoplasmic proteome linked with meat quality and endogenous enzyme activity, Li et al. (2012) reported accelerated meat tenderization from Swedish Red cattle by inducing a faster pH decline, earlier activation of μ-calpain along with a release of lysosomal enzymes. The 2D-PAGE analysis revealed a decrease in the phosphorylation of energy metabolic enzymes as a consequence of LVES and reported 7 changing proteins. Shen et al. (2012) found that LVES accelerated proteolysis by reducing the abundance of seven protein spots that were identified to belong to 5 unique proteins (Fig. 3). Using 2D-DIGE, a variant of 2D-PAGE, in combination with LC-ESI/MS, Kim et al. (2013) analyzed the influence of LVES on both the sarcoplasmic and myofibrillar proteomes after 1- and 9-d post-mortem. The authors also targeted titin degradation by SDS-PAGE and μ-calpain autolysis and Troponin-T degradation using Western-Blotting to monitor proteolysis in relation to meat quality. Although the data suggested that LVES did not influence proteolysis and tenderness after 9 d of ageing, the proteomics analysis on early post-mortem samples revealed some insights regarding the underlying mechanisms and detected 12 changing proteins. As a consequence of LVES treatment, a greater abundance of myofibrillar proteins was found in the insoluble fraction, whereas, a greater content of several metabolic enzymes and cell death proteins were detected in the soluble (sarcoplasmic) fraction in accordance with their regulatory role in post-mortem glycolysis, Ca^{2+} release/uptake cycle, and activation of calcium-dependent proteases (μ-calpain). The fifth study by Li et al. (2015) applied a NuPAGE proteomics-based approach to unveil both sarcoplasmic and myofibrillar proteome changes after 0, 3, and 10 h post-ES. The gel-based phosphoproteomics analysis revealed a significant effect of LVES ($n = 50$ unique proteins) on the phosphorylation level of several metabolic enzymes, indicating a higher metabolic flux (pivotal role in regulating glycogen breakdown to produce ATP) in ES muscles. Finally, Contreras-Castillo et al. (2016) used SDS-PAGE and Western blotting to examine the muscle proteome differences of bulls in relation to pH decline and shear force. Although those authors found that LVES had no effect on the tenderization rate and myofibrillar protein degradation (titin, desmin, troponin-T), greater abundances of stress proteins were detected in LVES muscles (Fig. 3).

5.2. In-depth bioinformatics analyses on the compendium of beef muscle proteins triggered by electrical stimulation reveal major interconnected biochemical pathways at interplay

The 67 proteins identified to change in the *Longissimus* muscle of bovine carcasses following ES were in different directions or fold-changes and indicated as down (blue), up (red) or both directions (orange) in Fig. 3. The proteins were manually annotated using the gene ontology of UniProt Knowledgebase (https://www.uniprot.org/uniprotkb) and categorized into seven biological pathways, these being in their order of importance: Metabolic pathways and ATP process ($n = 21$); Structural and contractile proteins ($n = 15$); Response to stress & folding ($n = 8$); Binding and transport ($n = 7$); Oxidative stress ($n = 6$); Cell deaths: apoptosis & autophagy (n = 6) and Proteolysis & related regulating pathways ($n = 4$). The proteins and their corresponding pathways were significantly interrelated with a high degree of interconnectedness as evidenced in the protein-protein interaction network built from the STRING database (Fig. 4).

Out of the 67 proteins, 14 were identified across studies, *i.e.,* ≥ 2 studies (Fig. 5A), indicating around 21 % overlap. This score is for

Bovine Uniport ID	Gene name	Full protein names retrieved from Uniprot database	Biological Pathways	Study 1 (ID1)	Study 2 (ID2)	Study 3 (ID3)	Study 4 (ID4)	Study 5 (ID5)	Study 6 (ID6)	Σ Identifications	Number of proteoforms	≥ 2 times
Q9XSC6	CKM	Creatine kinase M-type	Metabolic pathways and ATP process	2	2	2	1	4		5	11	
B3IVN4	**PKM**	Pyruvate kinase			1		1	1		3	3	
A6QLL8	**ALDOA**	Fructose-bisphosphate aldolase A			2		3	3		3	8	
Q3ZC09	**ENO3**	Beta-enolase			3			2		2	5	
Q5E956	**TPI1**	Triosephosphate isomerase				2		3		2	5	
P10096	**GAPDH**	Glyceraldehyde-3-phosphate dehydrogenase					12	3		2	15	
Q3T0P6	**PGK1**	Phosphoglycerate kinase 1					1	1		2	2	
Q9XSJ4	**ENO1**	Alpha-enolase		1						1	1	
P00829	ATP5F1B	ATP synthase subunit beta, mitochondrial			1					1	1	
Q5EA88	**GPD1**	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic					3			1	3	
P11966	**PDHB**	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial					1			1	1	
F1MHT1	AGL	Glycogen debranching enzyme						1		1	1	
P79334	**PYGM**	Glycogen phosphorylase, muscle form						1		1	1	
P33097	GOT1	Aspartate aminotransferase, cytoplasmic						1		1	1	
P19858	**LDHA**	L-lactate dehydrogenase A chain						1		1	1	
Q32KV0	**PGAM2**	Phosphoglycerate mutase 2						1		1	1	
P00570	AK1	Adenylate kinase isoenzyme 1						1		1	1	
P20004	ACO2	Aconitate hydratase, mitochondrial						1		1	1	
Q08DP0	**PGM1**	Phosphoglucomutase-1						1		1	1	
Q0IIG5	**PFKM**	ATP-dependent 6-phosphofructokinase, muscle type						1		1	1	
Q3T0Z7	QDPR	Dihydropteridine reductase						1		1	1	
P68138	ACTA1	Actin, alpha 1, skeletal muscle	Structural and contractile proteins	3				1		2	4	
Q3SZE5	MYL2	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform					1	1		2	2	
Q0P571	MYLPF	Myosin regulatory light chain 2, skeletal muscle isoform					1	1		2	2	
Q8MKH9	TNNT3	Troponin T, fast skeletal muscle type		2						1	2	
O62654	DES	Desmin		1						1	1	
Q148F1	CFL2	Cofilin-2			1					1	1	
Q0VBZ1	MYBPH	Myosin binding protein H				1				1	1	
Q5KR48	TPM2	Tropomyosin beta chain					1			1	1	
Q5KR49	TPM1	Tropomyosin alpha chain					1			1	1	
E1BF23	MYOM2	Myomesin 2						2		1	2	
P80473	MYOM1	Myomesin-1						1		1	1	
Q148H2	MYL6B	Myosin light chain 6B						1		1	1	
F1MRC2	MYH2	Myosin heavy chain 2						2		1	2	
Q9BE39	MYH7	Myosin-7						1		1	1	
Q148C2	TNNC2	Troponin C type 2 (Fast)						1		1	1	
P02510	CRYAB	Alpha-crystallin B chain	Response to stress & folding	1				1	1	3	3	
Q3T149	HSPB1	Heat shock protein beta-1		7				2	1	3	10	
Q148F8	HSPB6	Heat shock protein beta-6		2				1		2	3	
P19120	HSPA8	Heat shock cognate 71 kDa protein		1						1	1	
Q27965	HSPA1B	Heat shock 70 kDa protein 1B		1						1	1	
A0A8J8YBH4	HSPB2	Heat shock 27kDa protein 2						1		1	1	
G5E531	TCP1	T-complex protein 1 subunit alpha						1		1	1	
P15246	PCMT1	Protein-L-isoaspartate(D-aspartate) O-methyltransferase				1				1	1	
Q5E9F8	H3-3B	Histone H3.3	Binding and transport			1				1	1	
P02769	ALB	Albumin						2		1	2	
P02192	MB	Myoglobin						3		1	3	
P62261	YWHAE	14-3-3 protein epsilon						1		1	1	
Q3SYU2	EEF2	Elongation factor 2						1		1	1	
Q3SYR3	APOBEC2	Probable C->U-editing enzyme APOBEC-2						1		1	1	
Q3SZX4	CA3	Carbonic anhydrase 3						1		1	1	
Q5E947	PRDX1	Peroxiredoxin-1	Oxidative stress					1		1	1	
Q9BGI3	PRDX2	Peroxiredoxin-2						1		1	1	
O77834	PRDX6	Peroxiredoxin-6								1	1	
P28801	GSTP1	Glutathione S-transferase P						1		1	1	
Q5E946	PARK7	Parkinson disease protein 7 homolog						1		1	1	
P41976	SOD2	Superoxide dismutase [Mn], mitochondrial						1		1	1	
P31800	UQCRC1	Cytochrome b-c1 complex subunit 1, mitochondrial	Cell deaths	1						1	1	
Q3ZBT1	VCP	Transitional endoplasmic reticulum ATPase						1		1	1	
Q2HJH2	RAB1B	Ras-related protein Rab-1B						1		1	1	
Q6EWQ7	EIF5A	Eukaryotic translation initiation factor 5A-1		1						1	1	
Q0VCY0	ATP2A1	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1						1		1	1	
P81287	ANXA5	Annexin A5					1			1	1	
P13696	PEBP1	Phosphatidylethanolamine-binding protein 1	Proteolysis		1			1		2	2	
Q2YDE4	PSMA6	Proteasome subunit alpha type-6						1		1	1	
E1BP91	NPEPPS	Puromycin-sensitive aminopeptidase						1		1	1	
Q3T141	CMBL	Carboxymethylenebutenolidase homolog						1		1	• 0	
		Total identified proteoforms		12	7	5	12	49	2	67		

Legend
UP ES
DOWN ES
Both directions

(caption on next page)

Fig. 3. A simplified overview of the compendium of the 67 proteins (proteoforms) identified from the six ES proteomics studies given in Table 4 [Study 1: Bjarnadóttir et al., 2011, Study 2: Li et al., 2012, Study 3: Shen et al., 2012, Study 4: Kim et al., 2013, Study 5: Li et al., 2015 and Study 6: Contreras-Castillo et al., 2016]. The proteins are those detected by gel-based approaches coupled to mass spectrometry and database searches to change as a result of ES from bovine *Longissimus* muscle. The proteins given with their bovine Uniprot ID, gene names and full protein names were categorized by manual annotation into seven biological pathways that are given by their corresponding colors. The number of proteoforms identified for each protein in a given study are depicted with a number and highlighted in blue (down regulated in ES), red (up-regulated in ES), in orange (when a protein is in both directions (up and down-regulated) depending on the number of proteoforms or sampling time). This condition of being up and down-regulated for the same protein can be exemplified by ALDOA in study 4. The total number of identifications across studies is given on the right of study 6 along of the total number of proteoforms. The proteins within each biological pathway were ranked based on the number of identifications, and those reported in more than one study ($\geq$ 2 times) are highlighted with black cells. From the "Metabolic pathways and ATP process", the proteins (n = 14/21) belonging to glycolysis are indicated with bold characters. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

example three times higher than that reported for dark-cutting beef proteomics studies (Gagaoua, Warner, et al., 2021). Half of the common proteins were enzymes from the "Metabolic pathways and ATP process", from which PKM (Pyruvate kinase), ALDOA (Fructose-bisphosphate aldolase A), ENO3 (Beta-enolase), TPI1 (Triosephosphate isomerase), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) and PGK1 (Phosphoglycerate kinase 1) (Figs. 3 and 5B) are all glycolytic enzymes (Fig. 5C and D). Interestingly, all these enzymes are from the payoff phase of glycolysis. Creatine kinase M-type (CKM) is the seventh and top protein biomarker that did not belong to glycolysis (Figs. 3 and 5C). The other proteins were three structural and contractile proteins: ACTA1 (α-actin), MYL2 (Myosin regulatory light chain 2, ventricular/cardiac muscle isoform) and MYLPF (Myosin regulatory light chain 2, skeletal muscle isoform), and three small heat shock proteins: CRYAB (Alpha-crystallin B chain), HSPB1 (Heat shock protein beta-1) and HSPB6 (Heat shock protein beta-6). The PEBP1 (phosphatidylethanolamine-binding protein 1) from proteolysis and related regulating pathways was the only protein identified as more abundant in two studies in ES muscle. The 14 proteins were also found to be interrelated in a unique protein-protein interaction network (Fig. 5B), thereby shedding light on the

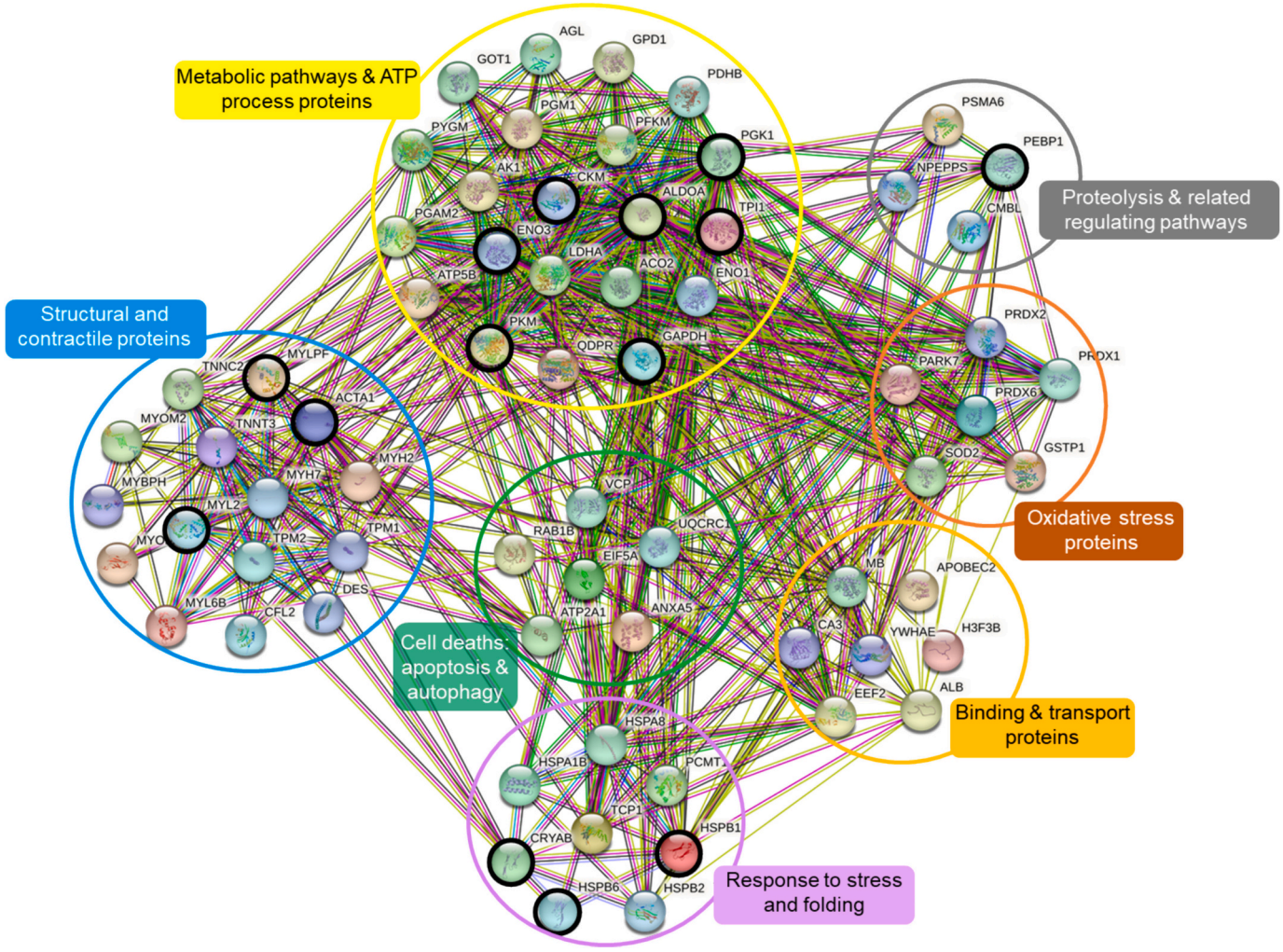
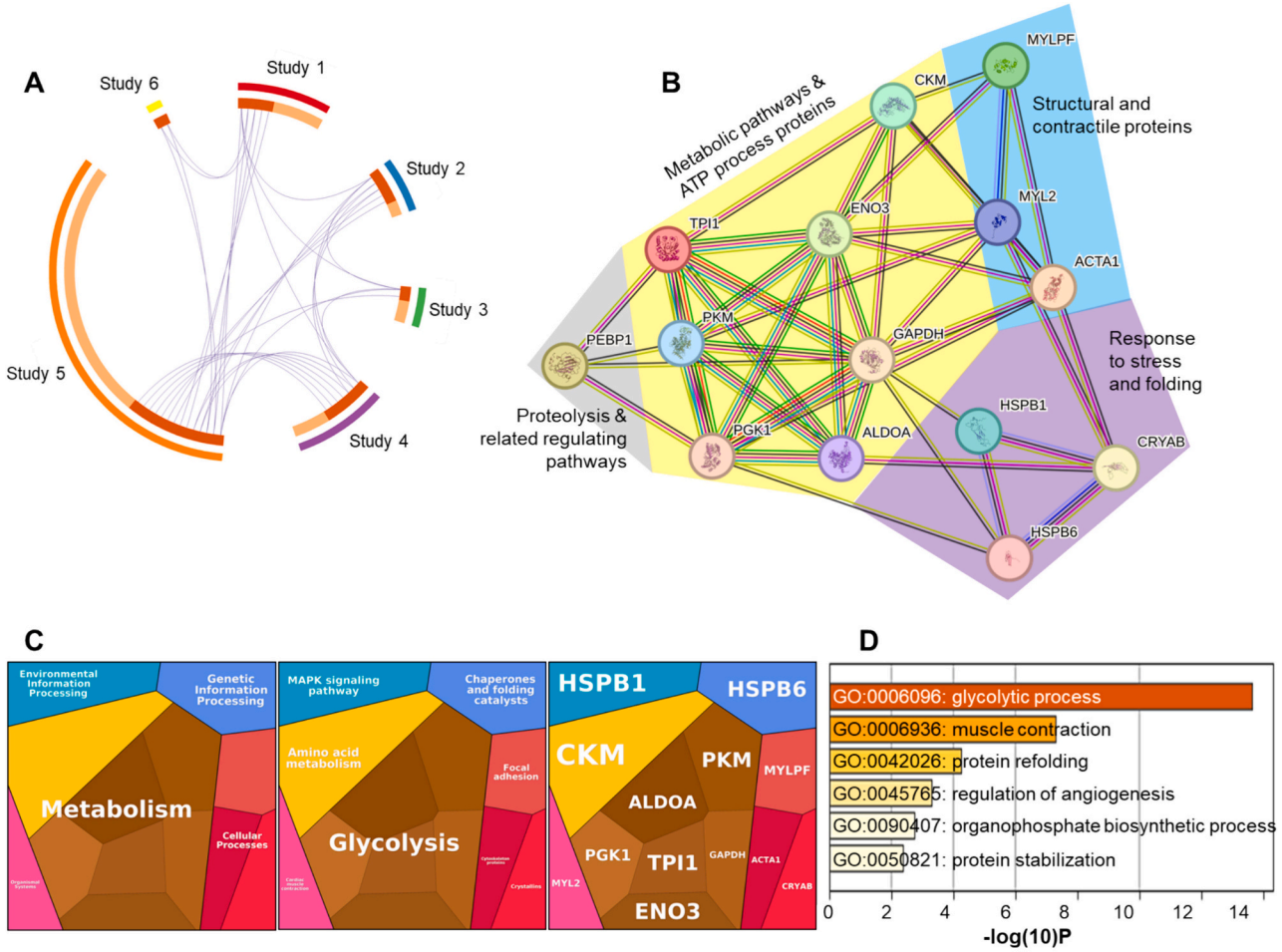


Fig. 4. Protein-protein interaction network highlighting the interconnectedness of the 7 biological pathways related to the impact of electrical stimulation on muscle proteins. The proteins as categorized in Fig. 3 are grouped with their corresponding colors. The 14 proteins that were identified in more than one study ($\geq$ 2 times) are highlighted with black circles, these being CKM, PKM, ALDOA, ENO3, TPI1, GAPDH and PGK1 from "Metabolic pathways and ATP process proteins"; ACTA1, MYL2 and MYLPF from "Structural and contractile proteins"; CRYAB, HSPB1 and HSPB6 from "Response to stress & folding"; and PEBP1 from "Proteolysis & related regulating pathways". For the full names of the proteins, given with their gene names retrieved from Uniprot database, refer to Fig. 3. Default settings of confidence of 0.6 and 4 criteria for linkage were used to generate the interaction map from the web-based search STRING database (https://string-db.org/).



(caption on next page)

Fig. 5. Distribution of the proteins and enriched molecular pathways among the six ES beef proteomics studies given in Table 4. A) Circos plot highlighting the degree of overlap across the studies. B) Protein-protein interaction network generated by the web-based search STRING database (https://string-db.org/) using the 14 proteins that overlap among the studies and identified in more than one study (≥ 2 times). C) Proteomaps pathway analysis of the 14 common proteins based on their number of identifications as described in Fig. 3. Proteins with larger polygons depict high number of identifications. Each polygon corresponds to a single Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and the size correlates with the number of proteins (genes) involved in the pathway. D) Metascape® Gene Ontology (GO) analysis. All the enriched GO terms are depicted and colored according to *P*-values. E) Hierarchical heatmap clustering based on the top 20 significantly enriched GO terms comparing the molecular pathways among the six studies. The enrichment of GO pathways were analyzed by Metascape® (https://metascape.org/). Colors from grey to brown indicate *P*-values (derived by a hypergeometric test) from high to low; and grey cells indicate no enrichment. The GO terms enriched in ≥3 studies are shown with red solid circles, and those enriched in at least two studies are with green solid circles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

crosstalk among the four pathways and, hence, depicting complex and sophisticated pathways occurring after ES intervention.

Besides the low number of proteins shared across the six studies in this integrative analysis, common features emerged regarding the related molecular pathways (as indicated by their GO terms) underlying the effects of LVES on bovine muscle (Fig. 5D, E). The most enriched GO term is "ATP metabolic process" found to be common to 4 studies, followed by "muscle system process", "regulation of muscle contraction" and "protein refolding", all enriched in half of the studies. Seven other, but complimentary pathways, were identified in at least two studies (details in Fig. 5E). The most enriched pathways in the entire compendium depicted in Fig. 6 align with the four molecular signatures depicted in Fig. 5B. These different results and analyses revealed for the first time, i) the main molecular signatures triggered by ES along with ii) the proteins changing in response to ES, from which the most important proteins and significant GO terms based on both the Benjamini−Hochberg *P*-value correction algorithm and hypergeometric test are discussed in the following sections.

5.2.1. ATP metabolic process including glycolysis are the primary pathways triggered by electrical stimulation

The dominance of energy and ATP-producing pathways, particularly glycolysis involving 14 out of 21 proteins undergoing changes, with all 6 top proteins belonging to the payoff phase of glycolysis (Fig. 3), aligns with the primary goals of ES to manipulate early post-mortem metabolism and hasten the onset of *rigor mortis* by accelerating muscle metabolism (Clarke, Shaw, & Morton, 1980; Fabiansson & Laser Reuterswärd, 1985; Hwang et al., 2003). There are, however, a few studies in the literature dealing with the early post-mortem energy metabolites of bovine *Longissimus* muscle following ES (Hollung et al., 2007; Jia et al., 2007). Concomitant with the increase in the intensity of glycolytic enzymes, an earlier report evidenced the effect of ES post-mortem of bovine *Psoas major* muscle to induce their binding to actin filaments (Clarke et al., 1980), hence, impacting their protein abundance, as evidenced by the multiple LVES proteomics studies we gathered in this work. Such interactions have been confirmed in the protein-protein network (Fig. 4), especially on the significant number of proteins from "binding and transport" pathway that were detected (Figs. 3 and 4).

The results of this integrative study confirm a shift in post-mortem energy metabolism after ES toward a glycolytic pathway. Accelerated glycolysis induces a fast pH decline (Eilers, Tatum, Morgan, & Smith, 1996), which induces extensive proteolysis (Gagaoua, Troy, & Mullen, 2021), thereby contributing positively to meat tenderization (Purchas, Yan, & Hartley, 1999). The enrichment of "GO:0046034: ATP metabolic process" is followed by that of "GO:0006094: gluconeogenesis" (3rd top pathway, Fig. 6), a GO term synonymous of "glucose biosynthesis" related to the formation of glucose from non-carbohydrate precursors (*e.g.,* pyruvate, amino acids and glycerol). Early post-mortem, the energy metabolism is significantly, but not totally, switched from aerobic to anaerobic metabolism (England, Scheffler, Kasten, Matarneh, & Gerrard, 2013; Lamri, della Malva, Djenane, Albenzio, & Gagaoua, 2023; Ouali et al., 2013) to sustain the different processes at interplay by

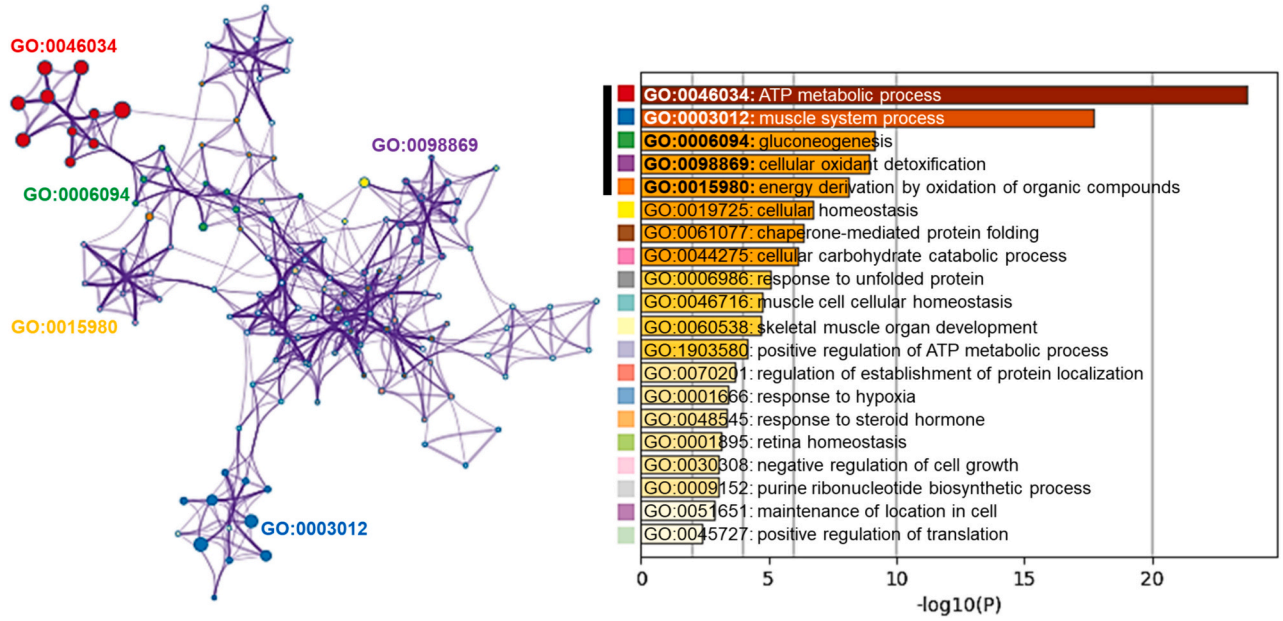


Fig. 6. Metascape® Gene Ontology (GO) analysis on the repertoire of 67 proteins given in Fig. 3 and the generated enriched ontology network. The top 20 GO terms are given at the right of the network and their enriched clusters are depicted with different colors in the network. Each enriched cluster term is presented with the corresponding color, where nodes that share the same cluster ID are typically close to each other. The sizes of the nodes reflect the degree of significance. The bar graphs on the right highlight the enriched terms and colored according to *P*-values. The five most enriched terms are highlighted in the network layout with a black line and their GO numbers are given in the network.

maintaining the homeostasis of cellular energy in post-mortem muscle (Wang, Matarneh, Gerrard, & Tan, 2022). The primary contributors to ATP production at this stage include the breakdown of glycogen into lactic acid and the transfer of phosphate from creatine phosphate to ADP (Wang et al., 2022). The higher rate of glycogenolysis in fast-twitch muscles is caused by a high content of phosphorylase, a greater activation of phosphorylase and a higher content of creatine phosphate.

In agreement with the foregoing, the noteworthy outcome reveals a consistent alteration in the abundance of metabolic enzymes due to ES, more interestingly CKM (Fig. 3). CKM was identified in 5 studies to be consistently down-regulated in electrically stimulated muscle and to interact with contractile and muscle structure proteins (Figs. 4 and 5B). CKM, a cellular energy sensor, is an enzyme used by striated muscles for ATP regeneration by creatine phosphate (Robergs, Ghiasvand, & Parker, 2004), hence, allowing maintenance of ATP levels without a net production of hydrogen ions during rapid energy demands through the rephosphorylation of ADP to ATP before switching to ATP production *via* glycolysis. Therefore, lower abundances of CKM in ES carcasses is unsurprising, considering the substantial increase in energy consumption during and post-stimulation (Fabiansson & Laser Reuterswärd, 1985). From the energy metabolism perspective, ATP produced through CKM-mediated reactions lasts only for a short period, followed by a subsequent decrease in ATP levels (Wang et al., 2022). Accordingly, earlier targeted studies reported that around 50 % of creatine phosphate stored in the muscle might be consumed during LVES and it has been depleted after 6 h post-mortem (Fabiansson & Laser Reuterswärd, 1985). The authors further evidenced half-life of ATP was significantly lowered in LVES samples compared to unstimulated carcasses.

The consistent identification of CKM can also be related to its characteristic of being an indicator of muscle damage (Daroit & Brandelli, 2008; Rochester, Chandler, Johnson, Sutton, & Miller, 1995). In fact, CKM is known to be degraded early post-mortem (Jia et al., 2007; Laville et al., 2009) and has been reported as a robust positive biomarker of both beef tenderness (Gagaoua, Terlouw, et al., 2021) and color-stable beef muscles (Gagaoua et al., 2020). In addition, protein fragments of CKM increased in abundance in post-mortem muscle protein extracts of beef (Bjarnadottir, Hollung, Faergestad, & Veiseth-Kent, 2010) and pork (Lametsch et al., 2003). In beef tenderization, the role of CKM has been further postulated to be specific to mixed fast oxido-glycolytic skeletal muscle like *Longissimus* (Gagaoua, Terlouw, et al., 2021), known to have more fast-twitch muscle fibers and, thus, higher amounts of CKM and creatine phosphate (Joseph, Suman, Rentfrow, Li, & Beach, 2012) compared to slow oxidative muscles (Okumura et al., 2005). The location of CKM in the M-band, where it binds to the central domains of M-protein and myomesins, would support its fragmentation and consistent decrease in its abundance. In co-operation with other soluble muscle proteins, for instance metabolic enzymes, certain proteins may also assist in the creation of cross-links or aggregates between adjacent actin filaments or in binding troponin to the thin filaments, to enhance energy provision where it is actively needed during contraction, and this binding or cross-linking can affect the distance between myofibrils (Hughes, Clarke, Li, Purslow, & Warner, 2019). These align with the reports that demonstrated ultrastructural alteration in beef muscle following ES (Hwang et al., 2003; Hwang & Thompson, 2002). Targeted studies on the meat degradome (protein degradation during muscle-to-meat conversion) as previously proposed (Gagaoua et al., 2021) are essential to better explore the underlying mechanisms, not only with respect to ES intervention and its impact on post-mortem muscle fragmentation, but also on meat tenderization under normal conditions or following other post-mortem processing interventions. In this context, proteomics and peptidomics can be effective tools to capture the degraded proteins, beyond myofibrillar proteins, during muscle-to-meat conversion. Last, but not least, a decrease in the solubility of certain proteins such as CKM, may occur following ES due to the rapid decline in intramuscular pH, as evidenced by an earlier study on pork (Boles, Parrish Jr., Huiatt, & Robson, 1992). Although this evidence pertains to pork muscle, the authors suggested that a decline in pH, when associated with high carcass temperatures, causes proteins to become denatured, insoluble, and aggregated/precipitated onto myofibrils. Such aggregates that are a consequence of conformational changes can be partly related to the extent of oxidative stress and protein oxidation, which can lead to either activation or inactivation of the protein function, but mostly to the loss of their biological properties. These aspects need targeted studies to be profoundly explored on ES cattle muscle.

5.2.2. Is there any possible link between electrical stimulation and the activation of apoptotic pathways and consequences on tenderization?

The high intensity and concomitant identification of glycolytic enzymes as a result of ES intervention could be attributed to an enhanced glycolytic rate, hence maintaining ATP production, especially for the energy-dependent and consuming processes such as apoptosis and autophagy (Elmore, 2007; Love, Palee, Chattipakorn, & Chattipakorn, 2018; Ouali et al., 2013). Referring to the applications of ES to human cells to manipulate cell apoptosis as a method to treat certain diseases (for review: (Love et al., 2018)), we propose that apoptosis is also likely to be more active in electrically stimulated bovine muscles. To the best of our knowledge, the existing literature on bovine post-mortem muscle did not yet explore the link between ES and apoptosis. However, in light of the following factors: i) an observed increase in the release of free calcium from the sarcoplasmic reticulum after ES (Hwang et al., 2003), ii) the documented high abundance of calcium in the sarcoplasm and the inter-myofibrillar space of beef muscle at 4 h post-mortem (Vignon, Beaulaton, & Ouali, 1989), iii) the calcium intake by mitochondria and related apoptotic mechanisms (Ouali et al., 2013), iv) the significant influx of calcium into the matrix leading to mitochondria fission (Purslow et al., 2021), thereby triggering the apoptosis onset (Orrenius, Gogvadze, & Zhivotovsky, 2015), v) the higher rate of caspase-3 activation in fast pH decline (Ma, Yu, & Han, 2022), and vi) the regulation and pivotal role in the modulation of cellular apoptosis pathways, particularly at the mitochondrial level, by heat shock proteins (Beere, 2004), there may be avenues for further investigation into the proposed hypothesis. It is important to note that proteins involved in apoptosis (and autophagy) were identified to undergo changes under ES intervention (Fig. 3). Furthermore, two GO terms, these being "GO:2001242: regulation of intrinsic apoptotic signaling pathway" and "GO:2001234: negative regulation of apoptotic signaling pathway" were significantly enriched (Fig. 5). These proteins were interacting with several molecular pathways, including those related to energy and ATP metabolic processes (Fig. 4). Relationships between intracellular pH, bioenergy metabolism, and caspase-3 activation in cells undergoing apoptosis have been reported (Sergeeva et al., 2017). The cytosolic acidification seemed to precede the activation of caspase-3, a condition that is likely to be favored by ES intervention. The gradual depletion of oxygen in post-mortem muscle due to the anoxia situation caused by the sudden loss of blood circulation (Ouali et al., 2013) may further trigger and accelerate the shift in the energy metabolism toward other metabolic pathways (*e.g.,* more oxidative energy metabolism) to produce ATP. Although studies employing other high-throughput omics, likely metabolomics to decipher these mechanisms in ES bovine muscles are needed, earlier research demonstrated that LVES accelerated the glycolytic processes, leading to an immediate increase in muscle lactate and about 30 % reduction in ATP (Kondos & Taylor, 1987). Conclusively, these processes are intricately tied to the modulation of energy metabolism pathways, which govern the sophisticated mechanisms underlying the transformation of the post-mortem muscle into meat and more likely through the acceleration of apoptosis onset.

5.2.3. Interplay among proteolysis, muscle structure, responses to cellular and oxidative stress in electrically stimulated muscle

In electrically stimulated post-mortem muscle, the hastened proteolysis of myofibrillar and cytoskeletal proteins by endogenous enzymes such as calpains, caspases and proteasomes, along of a possible increase

in the onset of apoptotic processes early post-mortem involves, as emphasized earlier, the utilization of free calcium released from the sarcoplasmic reticulum and mitochondria (Gagaoua et al., 2015; Ouali et al., 2013; Purslow et al., 2021). These mechanisms are essentially orchestrated by the rate and extent of the drop in pH and temperature. In light of the results revealed by this study (Fig. 4), we suggest that these mechanisms are interrelated with other sophisticated complex mechanisms, likely the triggering of apoptosis, the response to cellular stress and oxidative stress and their consequences on the degradation of myofibrillar proteins by endogenous muscle proteolytic systems. The regulation of proteolysis may represent an additional layer within these intricate pathways, as notably evidenced by the sustained up-regulation of phosphatidylethanolamine-binding protein 1 (PEBP1, Figs. 3 and 5B). The PEBP1, is known to interact with multifunctional proteins along with the coordination of cytoskeleton changes and energy metabolism (Schoentgen & Jonic, 2020), and is a key target of calpains and proteasomes (Chen, Wang, Thompson, Hall, & Guttmann, 2006). In this context, proteins involved in structural and contractile functions were changing and constituted the second major enriched molecular pathway responding to ES (Fig. 6), with consistent change in the intensity of three proteins (ACTA1, MYL2 and MYLPF: Figs. 3 and 5B). Among these, ACTA1 (actin), a known biomarker of beef tenderness (Gagaoua, Terlouw, et al., 2021), can influence positively meat tenderization through the pivotal role it plays in muscle structure and proteolysis. We postulate that changes in ACTA1, a hallmark of apoptosis and the first target of caspases (Ouali et al., 2013), could significantly influence meat tenderization in response to ES *via* the alteration of muscle structure, thereby affecting susceptibility to proteolysis and impacting the disruption of muscle fibers during post-mortem processes. In the gel of Fig. 7, we can observe a fragment of actin (spot 668, molecular weight = 37.2 kDa) detected at 24 h post-stimulation. We suggest that the balance between actin abundance and proteolytic activity is crucial in determining the final beef tenderness, which appears to be affected by ES, but this warrants further investigation given its high resistance to degradation in normal post-mortem muscle.

The early post-mortem involvement of cytoskeletal proteins as a result of ES may also support the onset of apoptotic processes (Ouali et al., 2013). The key cellular event in post-mortem muscle apoptosis includes the release of reactive oxygen species (ROS), which aligns with the observation of a higher oxidative stress response within the first 24 h post-mortem (Lamri et al., 2023). The generation and accumulation of ROS early post-mortem, due to the intense activity of the energy metabolism pathways identified as key players (Fig. 3), could activate endogenous muscle antioxidant defence systems (Zhang, Pan, Cao, & Wu, 2013). Interestingly, all the oxidative stress proteins identified ($n = 6$) were up-regulated in electrically stimulated muscle (Fig. 3). In the aftermath of exsanguination, skeletal muscle cells are predisposed to apoptosis through the intrinsic pathway, triggered by both anoxic and oxidative stress resulting from the abrupt depletion of oxygen and nutrients (Rønning, Andersen, Pedersen, & Hollung, 2017). Consistent with the detrimental conditions associated with the lack of oxygen, oxidative modifications may occur in myofibrillar proteins. This process can increase the degradation of myofibrillar proteins, facilitated by the activation of calpains and caspases (Smuder, Kavazis, Hudson, Nelson, & Powers, 2010), which provides an explanation for the inclusion of "GO:0098869: cellular oxidant detoxification" among the top 5 significantly enriched GO terms (Fig. 6). Moreover, it is worthy to note the enrichment of a GO term characterizing hypoxia (GO:0001666: response to hypoxia), thereby highlighting the impact of ES on oxygen level and, probably, its deeper penetration into the muscle tissue.

The enhanced tenderization ascribed to ES was found to be concomitant with a significant expression level of several heat shock proteins (HSPs) along with their enrichment across several studies (Fig. 5C). Within the global GO analysis of the compendium (Fig. 6), three GO terms were enriched: "GO:0061077: chaperone-mediated protein folding", "GO:0006986: response to unfolded protein" and

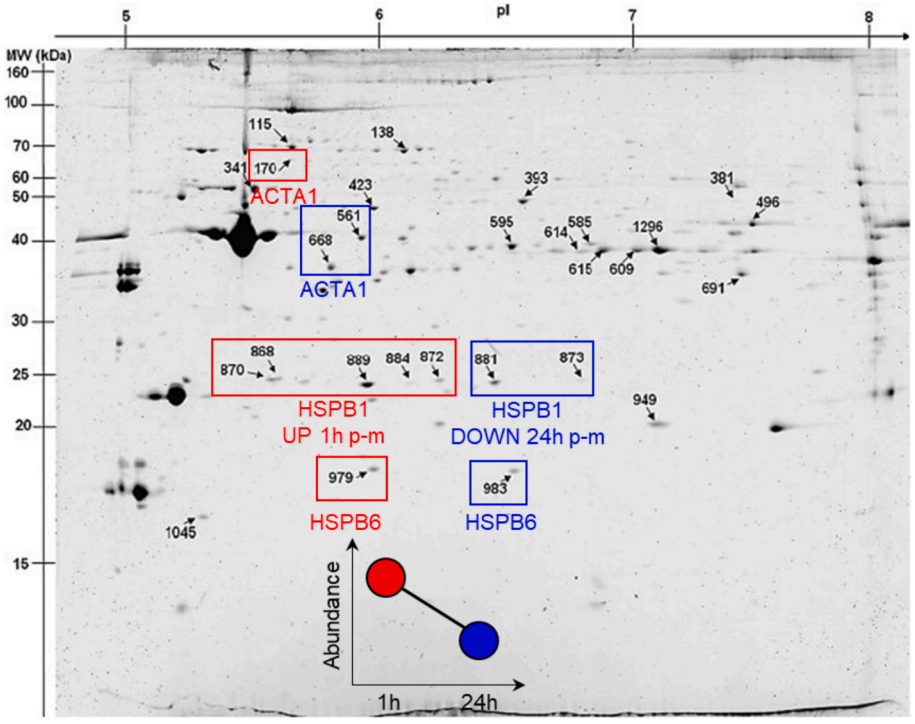


Fig. 7. Representative two-dimensional gel electrophoresis of the insoluble protein extracts from bovine *Longissimus thoracis* muscle specifically highlighting the spots of small heat shock proteins (sHSP) and actin (ACTA1) changing in their abundances as a result of electrical stimulation (ES) at either 1 (red) or 24 h (blue) post-LVES (study 1 from Bjarnadóttir et al. (2011). The gel has been modified to highlight the changes in the direction of the abundances and number of proteoforms (isoforms) of HSPB1 (Hsp27), HSPB6 (Hsp20) and ACTA1 (actin), that are up-regulated at 1 h and down-regulated at 24 h post-mortem. Figure modified and reproduced with permission. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

"GO:0070201: regulation of establishment of protein localization". More interestingly and irrespective of the study, the results depicted higher abundances of small HSPs early post-mortem (Fig. 3). The increase in small HSPs levels might indicate a role in the stabilization of myofibrillar proteins in response to stress (Lomiwes, Farouk, Wiklund, & Young, 2014; Picard & Gagaoua, 2017, 2020a), as well as a prevention of the degradation and structural damage of proteins caused by apoptosis (Beere, 2004). Small HSPs, mainly HSPB1 (HSP27), are known to protect actin filaments along with other cytoskeletal proteins from fragmentation (Lomiwes et al., 2014; Pivovarova et al., 2005), which we assume are enhanced in electrically stimulated muscles. The importance of small HSPs in meat tenderization, including CRYAB, HSPB1 and HSPB6, has been evidenced in earlier integromics studies as being key biomarkers for the prediction of beef tenderness and color variability (Gagaoua et al., 2020; Gagaoua, Terlouw, et al., 2021). More specifically, an earlier study found that HSPB1 was related to higher actin fragmentation during storage and suggested that HSPB1 may promote proteolysis by preventing actin aggregation (Morzel, Terlouw, Chambon, Micol, & Picard, 2008). To exemplify this, in Fig. 7 an aggregate of actin is observed 1 h post-mortem at around 66.4 kDa compared to its intact form (41.4 kDa), which is concomitant with the up-regulation of HSPB1 proteoforms at the same post-mortem time. In this integrative analysis study, it is interesting to observe that small HSPs were consistently more abundant early post-mortem, but decreased at 24 h (Figs. 3 and 7). This shift in the direction, depicted in Fig. 7 for both HSPB1 and HSPB6, underscores the highly dynamic response of the muscle to ES as an external stimulus (elevated cellular stress level) shortly following the post-mortem intervention and, on the contrary, decreases in their abundances a few hours after *rigor mortis* and within 24 h post-mortem. A correlation between the degradation of both small HSPs at 24 h post-mortem and indicators of proteolysis (desmin and troponin-T) has also been reported (Balan, Kim, & Blijenburg, 2014). The degradation of small HSPs post-mortem results in loss of their anti-apoptotic function, which facilitates proteolysis of myofibrillar proteins (Balan et al., 2014; Ding, Wei, Zhang, Zhang, & Huang, 2021; Lomiwes et al., 2014; Ma & Kim, 2020). Increased abundances of small HSPs early post-mortem aim to preserve muscle homeostasis and restore the initial and active structure of proteins (Beere, 2005; Ouali et al., 2013), mainly cytoskeletal (Fig. 4) that have experienced structural damage, whether caused by stress itself or apoptosis onset. Interestingly, 5 spots were identified as HSPB1 at 1 h post-mortem (Fig. 7), which were detected at similar molecular weights (25 kDa), but with different p*I* values, suggesting modifications or isoforms of the protein, most likely phosphorylated forms. The phosphorylation of HSPB1 has been reported to modulate actin filament dynamics in response to external stimulus (Lavoie, Lambert, Hickey, Weber, & Landry, 1995), mainly caused by alternative splicing (Scheler, Li, Salnikow, Dunn, & Jungblut, 1999) or some different protein kinases (Mymrikov, Seit-Nebi, & Gusev, 2011). The importance of the dynamics of small HSPs early post-mortem in triggering meat tenderization and their potential as markers for ES monitoring is evident. It is, however, of interest to explore in further studies the response of HSP proteins using other ES methods and within the various conditions discussed as impacting the efficiency of ES.

Despite the small number of studies and weak overlap observed in the identified proteins among LVES proteomics studies, the new findings at the proteome level revealed valuable insights into the major biological pathways underlying the conversion of stimulated beef muscle into meat. These findings allowed thanks to advanced bioinformatics analyses contributing to a better understanding of the complex biochemical processes involved in post-mortem muscle metabolism and their impact on meat tenderization, promoting evidence-informed strategies for optimizing meat processing techniques. Utilizing proteomics platforms to monitor protein abundances and degradation has helped to further our understanding of muscle biochemistry and the conversion of muscle to meat, including the mechanisms behind ES as evidenced in this review. However, the development of multi-omics and systems biology approaches would allow us to decipher further mechanisms and extend our understanding and control of the factors at interplay. New and improved methods for the detection and quantification of proteins are being continually developed. For example, label-free MS-based quantification, a form of shotgun proteomics, is a method we suggest for measuring peptide concentrations in complex samples using a combination of high-performance liquid chromatography (HPLC) and MS, which are currently successfully applied in meat research (for details: Gagaoua et al. (2024)).

6. Conclusion and future perspectives

In this manuscript, we provided an updated overview of the effects of ES in cattle carcasses as well as the different factors and settings impacting ES efficiency. Overall, ES accelerates glycolysis and the rate of pH drop, leading to earlier rigor onset, thereby preventing cold shortening and reducing time to *rigor mortis*. Thanks to the enhanced free calcium release from the sarcoplasmic reticulum, ES further induces early activation of proteolytic enzymes, ultimately favoring meat tenderization. However, improvements in beef tenderness, extensively evaluated by Warner-Bratzler shear force, have generally been observed in studies that used a few cohorts of electrically stimulated carcasses. There are limited studies that have used trained sensory panels but, to the best of our knowledge, none have yet incorporated untrained consumers. It is evident that the improvement of tenderness by ES would depend on the conditions and type of ES systems applied. We believe that conducting a meta-analysis on ES bovine studies is necessary to address this point. While ES affects meat color by decreasing fiber diameter and increasing light scattering, it also enhances redness through improved oxygenation and structural changes. Despite concerns about reduced water-holding capacity, evidence suggests minimal impact on drip.

The analysis of the proteome profiling in ES carcasses provided novel insights into the dynamic mechanisms and changes in the abundances of proteins occurring in post-mortem muscle in response to ES intervention. Using an integrative proteomics approach based on six published LVES proteomics studies, we created the first compendium of changing proteins and thanks to in-depth bioinformatics, we revealed part of the underpinning molecular pathways at interplay in stimulated bovine carcasses. As a proof-of-concept within the realm of data reuse, we demonstrated the interconnectedness in the molecular signatures triggered by ES, even though our analysis was limited to a small number of studies and partially overlapping proteins. The biochemical pathways included the ATP metabolic process, muscle structure and contraction, as well as responses to cellular and oxidative stresses, along with protein folding. These pathways elucidate the intricate biochemical mechanisms underlying ES intervention, likely amplifying apoptosis onset and its consequences on beef determination. ES accelerates the rate of glycolysis, which might speed up the onset of apoptosis, and prompts the expression of small heat shock proteins; thus, the process of protein degradation occurs earlier and would explain why tender meat *in Bos indicus* cattle based on mean values is generally reported at least in the early post-mortem period. Notwithstanding, the validity of the mechanisms and molecular signatures identified is limited to LVES proteomics studies and the experimental conditions applied, hence, exploration to other ES systems and conditions is warranted to extend and compare the findings. In addition, we believe that understanding how early post-mortem interventions, for instance ES, impact the apoptosis onset, is key for successful meat tenderization and guaranteed beef tenderness. The application of different powerful methods such as shotgun proteomics on high number of animals, targeted mass spectrometry such as MRM (Multiple Reaction Monitoring) and PRM (Parallel Reaction Monitoring) as well as multi-omics approaches (for instance proteomics and metabolomics) would be very helpful to identify novel proteins at interplay and for validating those reported in our compendium. These approaches would further expand our knowledge on the underlying mechanisms at interplay.

Consent form

All authors consent to the publication of this manuscript. This manuscript is a review and didn't involve human subjects for experimentation.

CRediT authorship contribution statement

Mohammed Gagaoua: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Nuria Prieto:** Data curation, Writing – original draft, Writing – review & editing. **David L. Hopkins:** Data curation, Writing – original draft, Writing – review & editing. **Welder Baldassini:** Data curation, Writing – review & editing. **Yimin Zhang:** Data curation, Writing – review & editing. **Oscar López-Campos:** Data curation, Writing – review & editing. **Marzia Albenzio:** Writing – review & editing. **Antonella della Malva:** Data curation, Visualization, Formal analysis, Writing – review & editing.

Declaration of competing interest

There is no conflict of interest involved in this work.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meatsci.2024.109663.

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