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Review

Meat tenderness: advances in biology, biochemistry, molecular mechanisms and new technologies

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ABSTRACT

Meat tenderness is an important quality trait critical to consumer acceptance, and determines satisfaction, repeat purchase and willingness-to-pay premium prices. Recent advances in tenderness research from a variety of perspectives are presented. Our understanding of molecular factors influencing tenderization are discussed in relation to glycolysis, calcium release, protease activation, apoptosis and heat shock proteins, the use of proteomic analysis for monitoring changes, proteomic biomarkers and oxidative/nitrosative stress. Each of these structural, metabolic and molecular determinants of meat tenderness are then discussed in greater detail in relation to animal variation, postmortem influences, and changes during cooking, with a focus on recent advances. Innovations in postmortem technologies and enzymes for meat tenderization are discussed including their potential commercial application. Continued success of the meat industry relies on ongoing advances in our understanding, and in industry innovation. The recent advances in fundamental and applied research on meat tenderness in relation to the various sectors of the supply chain will enable such innovation.

1. Introduction

Tenderness is an important quality trait which determines satisfaction, repeat purchase and willingness-to-pay premium prices. Historically, over the 1920-1960's, the effects of genetics, biochemistry and production factors on meat tenderness were identified utilizing physical, chemical, histological and sensory methods. These experiments, along with the research conducted in the 1970's formed the basis of much of our understanding of meat tenderness (see review in Warner et al., 2021), and the data remain valid today. This research over the last 70 years has been pivotal in understanding the mechanisms determining meat texture and tenderness, as well as for industry advances in quality assurance. Recent advances and understanding of mechanisms, including biology, biochemistry and bio-physics of meat in relation to tenderness, have occurred throughout the meat supply chain.

The major determinants of meat tenderness are; connective tissue and cross-links, myofibrillar integrity, sarcomere length, protein denaturation and intramuscular fat. Our understanding of molecular factors influencing tenderization has advanced and this is reviewed here in relation to glycolysis, calcium release, protease activation, apoptosis and heat shock proteins, the use of proteomic analysis for monitoring changes, proteomic biomarkers and oxidation/nitrosative stress. Each of these structural, metabolic and molecular determinants of meat tenderness are then discussed in greater detail in relation to animal variation, and changes during postmortem ageing and cooking, with a focus on recent advances. Finally, recent innovations in postmortem

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technologies and enzymes for meat tenderization are discussed including their potential commercial application.

Methods to measure tenderness can include the conduct of sensory panels, consumer panels, or through instrumental measures such as hardness, derived from Texture Profile Analysis or much more commonly, shear force, a measure of the force required to shear through a meat sample. Shear force is described in the literature either simply as shear force, peak shear force or Warner-Bratzler shear force (WBSF) and for a discussion of the definition and use of these terms as well as their relation to sensory measures the reader is referred to Warner et al. (2021). WBSF and other variations of shear force are the most often reported values to measure tenderness and thus are used throughout this review, as significantly less studies included sensory or consumer panel data.

This review examines meat tenderness across species and through the supply chain from a variety of perspectives. These perspectives include biology, molecular, biochemistry, industry and technological, allowing the sometimes divergent viewpoints to be examined more closely and hopefully enabling convergence and innovation.

2. Advances in molecular understanding of factors influencing tenderization

The general viewpoint that myofibrillar protein degradation by endogenous proteases plays an important role in meat tenderization has long been accepted (Davey & Gilbert, 1969). The nature of meat tenderization is the development of proteolysis of myofibrillar proteins by multi-enzyme systems during the conversion of muscle to meat and subsequent aging time. The biochemical and metabolic processes involved in this muscle-to-meat conversion are extremely intricate due to the complex interactions across different pathways during postmortem aging. In recent decades, the developing biochemical approaches and proteomics techniques have been applied to unravel the cellular and molecular mechanisms behind the variation in meat quality attributes. The primary outcome has been the identification of differential protein expression and modification across phenotypes with variable meat quality attributes, highlighting the importance of finding potential biomarkers to predict meat tenderness. Based on protein functions and the involved metabolic pathways, the biomarkers can be categorized into metabolic enzymes, structural proteins, oxidative stress-related proteins, heat shock proteins, proteases, apoptotic and signaling proteins. These proteins are key participants in the critical biochemical events including glycolysis and energy metabolism, calcium release, apoptosis, proteolysis and involvement of oxidative and nitrosative stress in postmortem muscle metabolism.

2.1. Glycolysis and energy metabolism

In postmortem muscle, the anoxic state of the muscle cell prevents the production of a large amount of ATP by the citric acid cycle and oxidative phosphorylation. The shuttle between creatine/phosphocreatine and glycolysis occurs and *gradually* glycolysis dominates in ATP generation, resulting in lactate accumulation and pH decline. The ultimate pH and the pH decline rate are indicators of metabolic potential and can influence the development of meat tenderness. Lomiwes, Farouk, Wu, and Young (2014) provided convincing evidence that beef tenderization was compartmentalized by ultimate pH, owing to the variable degradation rate of myofibrillar proteins by the regulatory protease activity of Calpain-1 (μ-calpain) and potentially cathepsin B. The extent of pH decline and the ultimate pH are influenced by the glycolytic potential, which depends on functioning glycolytic enzymes catalyzing glycogen to lactate and an excess of muscle glycogen at slaughter. Recently, the role of mitochondrial and aerobic metabolism, adenosine monophosphate (AMP) kinase and other pathways in determining rate and extent of pH fall has been researched and comprehensive reviews are available (Apaoblaza et al., 2020; Chauhan & England, 2018; England et al., 2016, 2018). Positive relationships have been reported between meat tenderness and the abundance of glycolytic enzymes, including phosphoglucomutase, glyceraldehyde 3-phosphate dehydrogenase, triose-phosphate isomerase, enolase, pyruvate kinase and lactate dehydrogenase (Picard & Gagaoua, 2017). Succinate dehydrogenase and succinyl Co-A synthase, belonging to the tri-carboxylic acid (TCA) cycle, were reported to be more expressed in tender meat (Ouali et al., 2013). It should be noted that the use of glycolytic proteins as potential biomarkers to predict meat tenderness outcomes will be different between species and muscle types (Picard & Gagaoua, 2017, 2020).

2.2. Calcium release

Consumption of ATP in the muscle cell allows relaxation in the actomyosin bond and is involved in the sequestration of Ca^{2+} and ion gradients (Geeves & Holmes, 2005). As postmortem muscle cells encounter less energy and more acidic conditions, this can lead to the dysfunction of sarcoplasmic reticulum (SR), causing Ca^{2+} to leak into the sarcoplasm (Bing et al., 2016; Küchenmeister, Kuhn, & Ender, 2000; Kuchenmeister, Kuhn, & Stabenow, 2002). Decreased ATP levels combined with elevated cytoplasmic calcium initially results in the formation of the permanent cross-bridge, also called the actomyosin bond. On the other hand, calcium is an important messenger in many cell signaling pathways. Calcium is involved in calpain system activation, and also in the initiation of apoptosis, leading to proteolysis and meat tenderization. The components of Ca^{2+} channels located in the membrane of sarcoplasmic reticulum are lined with the membrane proteins sarco-endoplasmic reticulum calcium-ATPase 1, ryanodine receptor and inositol 1, 4, 5-trisphosphate receptor, which are suggested to be involved in meat tenderization. Kim et al. (2008) reported that more expression of inositol 1,4,5-trisphosphate receptor was detected in a tough meat group (Warner-Bratzler shear force, WBSF, 79±5.9 N) with a high Ca^{2+} level in beef *longissimus dorsi* compared to a tender meat group (WBSF, 36±2.9 N). Dysregulation and different expressions of Ca^{2+} channel proteins were reported in pale, soft, exudative (PSE, a quality defect) meat in pork (Guo et al., 2016; Wang et al., 2019) and PSE-like meat in broiler (Xing et al., 2017), relative to non-PSE normal meat. Recently, Dang et al. (2020) reported that the incubation of DS16570511, a cell-permeable inhibitor of the mitochondrial calcium uniporter, into bovine *longissimus thoracis et lumborum* within 20 min of exsanguination significantly increased the sarcoplasmic calcium concentration at 24 h and subsequently enhanced Calpain-1 autolysis, calpastatin degradation, myofibrillar protein proteolysis, and meat tenderness over a 14 d aging period. Collectively, it is suggested that sarcoplasmic calcium levels can be collectively modulated by mitochondria and sarcoplasmic reticulum and exhibit a crucial role in the development of meat tenderness during postmortem aging.

2.3. Protease activation and proteolysis

Accumulated evidence supports the predominant role of Calpain-1 in the proteolysis of myofibrillar proteins as the major contribution to meat tenderization (Camou, Marchello, Thompson, Mares, & Goll, 2007; Geesink, & A.H., & Koohmaraie, M., 2006; Koohmaraie, 1992) . The Calpain-2 (m-calpain), another member of calpain family, was thought to be inactive postmortem, due to insufficient calcium concentration in muscle and acidic conditions in post-rigor muscle (Maddock, Huff-Lonergan, Rowe, & Lonergan, 2005). However, Colle and Doumit (2017) found that Calpain-2 was responsible for the improvement of beef tenderness after 14 d of aging while Calpain-1 was mainly active in the first 14 d. The activity of Calpain-2 was shown to increase early postmortem by the injection of calcium chloride, or freezing (Wheeler, Koohmaraie, & Shackelford, 1997). The underlying mechanism through which calcium chloride improves meat tenderness is via modulation of calpain and calpastatin activities. Calcium chloride injection/infusion is

particularly beneficial for meat from tougher muscles or breeds, e.g. *Bos indicus*. For further information on the role of calcium on the activation and inactivation of calpains and calpastatin, refer to a comprehensive review by Nowak (2011). Proteolysis during the meat tenderization process may be the synergistic effects of multi-enzymes including calpains, cathepsins, and caspases, but the predominant role of calpains (Uytterhaegen, Claeys, & Demeyer, 1994) remains unchallenged in the literature. In particular, lysosome cathepsins are a large family of exo- and endo-peptidases and would be activated at low pH conditions which are favored by postmortem muscle cell with ultimate pH of 5.3-5.7. Zhang et al. (2019) found that cathepsin B and D released from destabilized lysosomal membrane in postmortem bovine *longissimus* activated the pro-apoptotic proteins Bid and Bax in the mitochondria. The mitochondrial membrane permeability was triggered by activated Bid and Bax and further induced caspase-9 and caspase-3 activation, leading to apoptosis and contributing to meat tenderness.

Extensive degradation of myofibrillar and cytoskeletal proteins, including troponin-T, tropomyosin, desmin, titin and nebulin, can occur while minor changes in actin, myosin and CapZ have been reported during postmortem aging (Lana & Zolla, 2016). Gradual degradation of myofibrillar proteins can cause the breakdown of the Z-line, thus weakening the longitudinal structure of the myofibrillar sarcomere (Huff-Lonergan et al., 2010). Recently, plectin, a scaffold protein traversing the periphery of Z-discs, costameres, mitochondria and nuclear membranes, was found to be gradually degraded in pork *longissimus thoracis* during 7 d of postmortem aging, predominantly by Calpain- 1 (Tian et al., 2019).

Protein phosphorylation has been reported to be involved in calpain activation and degradation of myofibrillar and cytoskeletal proteins. Li et al. (2017) found that *in vitro* phosphorylation of ovine myofibrillar proteins, especially desmin and troponin T, by protein kinase A prevented their degradation by Calpain-1. In addition, both phosphorylation of Calpain-1 by protein kinase A and dephosphorylation by alkaline phosphatase promoted the catalytic activity of Calpain-1 (Du et al., 2018; Du et al., 2019). It was also found that phosphorylated Calpain-1 was more sensitive to inhibition by calpastatin.

The basic components and mechanisms of tenderization postmortem are similar in poultry in comparison with mammalian muscle, such as the roles of actin-myosin interaction and Calpain-1 and -2 induced degradation of cytoskeletal proteins (Tomaszewska-Gras et al., 2011; Zhao et al., 2017). Dransfield (1994b) showed that 80% of maximum tenderness could be reached only 0.3 h after slaughter in chicken while 4.2, 7.7, 9.5, and 10 d were needed in pig, sheep, rabbit, and cattle muscles, respectively, suggesting a much more rapid tenderization process in chicken compared to other species such as beef, pork and mutton. This has been attributed to the greater calcium sensitivity and the activation of the calpain system (Lee et al., 2008). In addition, the thinness of the perimysium and endomysium, relative to mammalian muscle, is also thought to be a contributor to the high levels of tenderness in poultry muscle (An et al., 2010), likely partially associated with the young age at which poultry are slaughtered.

2.4. Apoptosis and heat shock proteins (HSPs)

Apoptosis in the postmortem cell is generally acknowledged to occur, based on the occurrence of typical characteristics including cell shrinkage, phosphatidylserine externalization and mitochondria alteration (Becila et al., 2017; Ouali et al., 2013). One of the representative pathways to induce apoptosis is the release of cytochrome C from mitochondria, promoted by the calcium-activated Bax in turn activating the caspases (Wang et al., 2018). The most profound effect of apoptosis on the muscle cell is the mediation of proteolysis executed by caspases (Kemp & Parr, 2012). Regulation of caspase activity has been shown to affect the degradation of myofibrils (Chen et al., 2011; Huang et al., 2014). Caspase-3 activity was reported to be negatively correlated with WBSF ($r = -0.49$ at 24 h of postmortem aging; $r = -0.61$ at 48 h of postmortem aging) in bull *longissimus*, and the authors speculated that caspase-3 was associated with advanced proteolysis (Cao et al., 2013; Zhang et al., 2013). A putative mechanism for the participation of caspases in proteolysis is the interaction with the calpain system, in particular the calpain endogenous inhibitor calpastatin, which is a substrate of caspases (Kemp & Parr, 2012). The interaction between caspases and the calpain system seems to be multifaceted and complex in postmortem muscle, hence warranting further research.

Heat shock proteins (HSPs) are synthesized in response to cell stress, acting as protectors, chaperones and restorers of cellular homeostasis. According to their monomeric molecular size, HSPs can be categorized into five conserved classes, including HSP60, HSP70, HSP90 and HSP100 as well as the small HSPs (12-43 kDa, e.g., HSP27, HSP20 and αβ-crystallin) (Gusev, Bogatcheva, & Marston, 2002). The initial role of HSPs is to activate an anti-apoptotic process in muscle cells, possibly by the following pathways; i) formation of a complex with active caspases to block their activity and function, ii) binding with substrates of effector caspases to delay or inhibit proteolysis and iii) restoration of damaged proteins to restrain the initiation of apoptosis (Lomiwes, Farouk, Wiklund, & Young, 2014). Heat shock proteins are reported to be biomarkers for the prediction of meat tenderness across a wide range of proteomic studies (see reviews in Ouali et al., 2013; Picard & Gagaoua, 2017). An *in vitro* myofibrillar protein digestion model conducted by Ding et al. (2018) showed that HSP27 might directly or indirectly interact with caspase-3 and Calpain-1 to decrease their activity and decrease the proteolysis of myofibrillar proteins. However, the individual contribution of HSPs to meat tenderization is difficult to elucidate and more investigations on the underlying mechanisms are needed.

2.5. Exploration of protein biomarkers for meat tenderness

Research has been carried out to identify potential protein biomarkers to predict meat tenderness and reviews on the topic have been conducted (Ouali et al., 2013; Picard & Gagaoua, 2020). Guillemin et al. (2011) conducted a functional interactome analysis of 24 proteins and showed that apoptosis, heat shock protein functions and oxidative stress resistance were associated with tenderness although this varied between muscle types. However, HSP's beta-1 and beta-6 were identified as robust biomarkers regardless of muscle type, breed and evaluation method of tenderness (Picard & Gagaoua, 2020). Similarly, MyHC-I (myosin heavy chain isoforms I), MyHC-IIa and cis-peroxiredoxin showed negative, but MyHC-IIx, parkinson disease protein 7 and Calpain-1 showed positive, association with tenderness regardless of breed, the end-point cooking temperature or the country origin of the panelist (Gagaoua, Terlouw, Richardson, Hocquette, & Picard, 2019). Picard and Gagaoua (2020) conducted meta-proteomics to integrate data across 12 studies. They identified variation between muscles and candidate biomarkers for beef tenderness could be grouped into proteins of structure and contraction, protection against oxidative stress and apoptosis, energy metabolism, 70 family HSPs and proteasome subunits in the *longissimus* and candidate bio-markers were identified which were consistent across muscles including several heat shock proteins.

Despite extensive research over more than a decade, accurate tenderness prediction using these biomarkers remains a challenge and has not been adopted by the meat industry, partly because meat tenderization is a complex biological process that depends on many intrinsic and extrinsic factors along the supply chain (Gagaoua, Monteils, & Picard, 2018). At present, while being of value in expanding our understanding of the tenderization process, the value of any of these biomarkers for predicting meat tenderness in a commercial environment remains to be seen. This is particularly because before any consideration of industry implementation, these potential biomarkers require extensive validation not only across species but also across different carcasses and muscles and also in terms of their accuracy of prediction for both instrumental and sensory measurements. Furthermore, Warner et al. (2021) discuss that in order to use proteomics as a tool for identifying

biomarkers for meat quality, there is a need for hypothesis-driven proteomics studies, rather than the current *post-hoc* explanations.

2.6. Oxidative and nitrosative stress

Reactive oxygen species (ROS) accumulate in postmortem muscle due to oxidative stress and altered mitochondrial activity. Oxidation of the amino acid side chains and backbone of proteins causes protein fragmentation and protein-protein cross-linkages which affects protein function and activity (Estevez, 2011; Zhang, Xiao, & Ahn, 2013). Meat tenderness can be promoted via ROS-mediated myofibrillar protein fragmentation (D'Alessandro & Zolla, 2013). Moreover, moderate oxidation of myofibrillar protein can enhance its susceptibility to Calpain-1 and caspases and then promote its degradation (Fu, Q.-q., Liu, R., Zhang, W., Ben, A., & Wang, R., 2020; Smuder, Kavazis, Hudson, Nelson, & Powers, 2010). However, ROS also cause the inactivation of Calpain-1, thus decreasing the proteolysis of myofibrillar proteins and inversely regulating meat tenderization (Lametsch et al., 2008). Antioxidant enzymes including superoxide dismutase, catalase, glutathione dismutase, protein DJ-1 and peroxiredoxins are guardians against ROS, balancing the redox state of muscle cell. A range of antioxidant proteins and enzymes have been identified to vary within postmortem muscles, some of which are reported as biomarkers for the prediction of meat tenderness (Hwang, Park, Kim, Cho, & Lee, 2005; Jia et al., 2007). Specifically, superoxide dismutase had higher expression in tender meat (Guillemin, Jurie, et al., 2011; Guillemin, Bonnet, Jurie, & Picard, 2011) while peroxiredoxin 2 and 6 were more abundant in tough meat (Carlson et al., 2017; Jia et al., 2009). Protein DJ-1 is an antioxidant protein playing a protective role against oxidative stress, and in proteomic studies its expression has been found to gradually increase during postmortem aging in pork, beef and lamb (Jia et al., 2007; Picard, Gagaoua, Micol, Cassar-Malek, & Hocquette, J. F. o., & Terlouw, C. E., 2014). Picard et al. (2014) used principal component analyses to demonstrate a relationship between protein DJ-1 and tenderness, which varied substantially between muscles; DJ-1 concentration was negatively correlated with tenderness in ST but positively correlated with tenderness in LT muscle. In contrast, Jia et al. (2009) found that there was no difference in protein DJ-1 expression between bovine *longissimus* muscles with variable meat tenderness, demonstrating that clarification of whether there is any relationship between DJ-1 expression and meat tenderness is required.

The origin of nitrosative stress in postmortem muscle is the production of nitric oxide (NO) presumably by the activation of the enzyme nitric oxide synthase (NOS), induced by the hypoxic conditions (Liu et al., 2015; Man, Tsui, & Marsden, 2014) and the reduction of nitrite and nitrate in the acid postmortem muscle environment (Lundberg, Weitzberg, & Gladwin, 2008). Manipulation of NO levels pre-slaughter and postmortem could significantly affect meat tenderness, although the results have been inconsistent across studies, as extensively discussed in the review of Liu et al. (2018). Recently, Hou et al. (2020) reported that shear force was decreased by NOS inhibitors and increased by NO donors, indicating NO could suppress meat tenderization. NO and protein S-nitrosylation are involved in postmortem metabolism which might account for the variation in meat tenderization. A large number of proteins including glycolytic enzymes, antioxidant proteins and enzymes, myofibrillar proteins, Ca^{2+} channel components and heat shock proteins were identified to be S-nitrosylated in pork muscle (see Table 1; Liu, Fu et al., 2018). Those proteins were proposed to be involved in biochemical processes including glycolysis and pH decline, calpain autolysis and proteolysis and Ca^{2+} release from SR in postmortem muscle (Fig. 1). A well-elucidated mechanism is the inhibition of Calpain- 1 autolysis leading to decreased myofibrillar protein degradation by NO-induced S-nitrosylation modification (Zhang, Pan, & Wu, 2018a) and the combination with calpastatin (Liu et al., 2019a). Glycolysis and pH decline were altered postmortem by manipulating NO levels in pork *longissimus thoracis* corresponding to decreased glycogen phosphorylase, glyceraldehyde-3- phosphate dehydrogenase and pyruvate kinase activities with their improved modification of S-nitrosylation (Zhang et al., 2019). Recently, significant differences in NOS activity, Ca^{2+} content, expression and S-nitrosylation modification of RyR1 and SERCA1 were observed between PSE and normal pork, suggesting NO and protein S-nitrosylation can putatively play a crucial role in regulating Ca^{2+} homeostasis (Wang et al., 2019). Moreover, myofibrillar proteins can also be S-nitrosylated which has been found to affect the susceptibility to Calpain-1 proteolysis *in vitro* (Liu et al., 2019b). Hou et al. (2020) utilized a NO donor (S-nitrosoglutathione, GSNO) and NOS inhibitor (N_{ω}-nitro-L-arginine methyl ester hydrochloride, L-NAME) and incubated them with beef *semimembranosus* muscle immediately post-slaughter for 24 h. Results showed that apoptosis-related morphological changes including more chromatin condensation, nucleus fragmentation, apoptotic body formation, and mitochondrial swelling were observed in L-NAME groups accompanied by higher caspase-3 and -9 activities while these changes in the GSNO group were retarded compared to the control. It was suggested that NO may play a negative role in beef apoptosis during postmortem aging. Taken together, NO and protein S-nitrosylation could exert an important role in the development of meat tenderness via pleiotropic pathways.

3. Advances in animal and pre-slaughter effects

Meat tenderness is affected by complex interactions of multiple antemortem and postmortem factors and in this section we review the pre-slaughter factors, with a focus on the animal. Fig. 2 illustrates the interactions between the antemortem factors and the affected metabolic, molecular, and enzymatic processes and systems.

3.1. Breed effects

Breed and genotype determine an animal's potential for producing tender meat, and the interaction of genetics with ante- and postmortem environment and management will determine the ultimate tenderness of the meat from an animal. Palatability trait differences have been characterized among cattle breeds (Koch, Dikeman, & Crouse, 1982; Wheeler, Cundiff, Shackelford, & Koohmaraie, 2001, 2004, 2005) and are considered in cross breeding programs. On average, aged *longissimus* from Jersey, Pinzgauer, Piedmontese, Red Poll, South Devon, Angus, and Wagyu tends to be more tender and *longissimus* from the *Bos indicus* breeds tend to be less tender, while a majority of breeds produce *longissimus* that is intermediate in tenderness. Cattle with *Bos indicus* inheritance are commonly used in tropical and subtropical environments (Cole, Ramsey, Hobbs, & Temple, 1964). The heat tolerance and insect resistance possessed by these breeds, coupled with their maternal characteristics and advantages from increased heterosis, have made them a valuable part of beef production in the tropical and subtropical environments (Cole et al., 1964; Crockett, Baker Jr, Carpenter, & Koger, 1979; Cundiff, Gregory, Koch, & Dickerson, 1986). However, *Bos indicus* cattle, especially Brahman and Nellore, have been repeatedly reported to produce tougher meat than *Bos taurus* cattle (Crouse, Cundiff, Koch, Koohmaraie, & Seideman, 1989; Johnson, Huffman, Williams, & Hargrove, 1990; Koch et al., 1982; Peacock, Koger, & Hodges, 1982; Wheeler, Cundiff, et al., 2001; Wheeler, Cundiff, Koch, & Crouse, 1996; Wheeler, Savell, Cross, Lunt, & Smith, 1990a, 1990b) due to less calpastatin inactivation and thus increased calpastatin levels at later postmortem times (Pringle, Williams, Lamb, Johnson, & West, 1997; Wheeler et al., 1990a; Whipple et al., 1990), resulting in less proteolytic degradation and slower improvements in tenderness with aging (O'Connor, Tatum, Wulf, Green, & Smith, 1997; Wheeler et al., 1990a, 1990b; Whipple et al., 1990). However, numerous other metabolic differences also may contribute to the reduced tenderness of *Bos indicus*-influenced cattle (Wright et al., 2018). The use of composite breeds comprised of 3/8 or 5/8 *Bos indicus* inheritance is common among beef producers to incorporate the positive attributes of *Bos indicus* cattle, but

Table 1
S-nitrosylated proteins and SNO-modified cysteine sites identified in pork *longissimus thoracis* during postmortem aging. The data shows evidence of nitrosylation of cysteine sites within key specific proteins postmortem after 0 days of ageing and also an increase in nitrosylation of these cysteine sites after 3 days ageing (adapted from Liu et al. (2018)).

Protein	Accession	Peptide sequence	Cys-site[1]	A0[2]	A3[2]	SD A0[2]	SD A3[2]	P-value
Aldolase C	F1RJ25	KGVVPLAGTDGETTTQGLDGLSERC[1]AQYKKD	135	1.005	1.744	0.046	0.066	0.0058
Alpha-Actinin-1	I3LIK6	R.LHKPPKVQEKC[1]QLEINFNTLQTKL	112	0.618	0.946	0.013	0.043	0.0002
ATP-dependent 6-phosphofructokinase	Q2HYU2	RLPLMEC[1]VQVTKD	351	0.844	1.097	0.117	0.069	0.0325
ATP-dependent 6-phosphofructokinase	Q2HYU2	RIFANTPDSGC[1]VLGMR.K	709	0.935	1.297	0.007	0.071	0.0010
Beta-Enolase	Q1KYT0	KFGANAILGVSLAVC[1]KAGAAEKG	119	0.595	0.638	0.107	0.120	0.6703
Beta-Enolase	Q1KYT0	KTGAPC[1]RSER.L	399	1.392	2.174	0.084	0.189	0.0028
Beta-Enolase	Q1KYT0	KVNQIGSVTESIQAC[1]]KL	357	0.968	1.338	0.006	0.061	0.0005
Glucose-6-phosphate isomerase	F1RNU9	KMIPC[1]DFLIPVQTQHPIR.K	404	0.786	1.038	0.036	0.030	0.0008
Glutathione reductase	F1RX66	RKTKC[1]VMKM	432	0.565	0.720	0.012	0.045	0.0047
Glyceraldehyde-3-phosphate dehydrogenase	Q0QES9	KIVSNASCTTNC[1]LAPLAKV	131	0.789	1.563	0.009	0.124	0.0004
Glyceraldehyde-3-phosphate dehydrogenase	Q0QES9	RVPTPNVSVVDLTC[1]RL	222	0.864	1.502	0.077	0.222	0.0093
Heat shock protein HSP 90-alpha	O02705	KKTKFENLC[1]KL	573	0.603	0.793	0.051	0.090	0.0355
L-lactate dehydrogenase A chain	P00339	KNRVIGSGC[1]NLDSARF	163	0.989	1.940	0.057	0.174	0.0008

L-lactate dehydrogenase C chain	Q9TSX5	RVIGSGC[1]NLDSARF	163	0.912	1.853	0.016	0.045	<0.0001
Malate dehydrogenase	P11708	KAIC[1]DHVR.D	251	0.771	1.141	0.013	0.056	0.0004
Malate dehydrogenase	P11708	KVIVVGNPANTNC[1]LTASKS	137	0.913	1.514	0.054	0.006	<0.0001
Phosphoglycerate kinase1	Q7SIB7	KAAIPSIKFC[1]LDNGAKS	50	0.926	1.720	0.053	0.181	0.0019
Phosphoglycerate kinase1	Q7SIB7	KIGQATVASGIPAGWMGLDC[1]GP ESSKKY	316	0.912	1.383	0.003	0.07	0.0003
Phosphoglycerate kinase1	Q7SIB7	KAC[1]ADPAAGSVILLENLRF	108	0.677	0.846	0.068	0.088	0.0590
Protein DJ-1	F1RII4	KVTVAGLAGKDPVQC[1]SR.D	46	0.806	1.504	0.030	0.037	0.0024
Sarcoplasmic\endoplasmic reticulum calcium ATPase1	F1RFH9	RANAC[1]NSVIRQ	471	0.831	2.221	0.021	0.130	<0.0001
Titin	/	KKTTC[1]KLKM	2352	0.652	0.862	0.049	0.010	0.0019
Triosephosphate isomerase	Q29371	KIAVAAQNC[1]YKV	67	0.787	1.548	0.042	0.206	0.0033
Triosephosphate isomerase	Q29371	RIIYGGSVTGATC[1]KE	218	0.919	1.267	0.012	0.046	0.0002
Aldolase C	F1RJ25	KGVVPLAGTDGETTTQGLDGLSER C[1]AQYKKD	135	1.005	1.744	0.046	0.066	0.0058

[1] The cysteine (C) in red indicates that this is modified by S-nitrosylation and the specific site of the cysteine is also shown.
[2] The proportion of modification of the specific SNO-sites in A0 and A3 samples was relative to that of G100 samples, where G100 represents 1.0. A0 and A3 represent the average of three replicate samples for 0 and 3 d ageing of pork *longissimus thoracis*, respectively. G100 refers to the A0 sample incubated with 100 μM S-nitrosoglutathione (GSNO), which is an NO donor. SD A0 and SD A3 represent the standard deviation for the three replicate samples.

breeds with 3/8 or 5/8 *Bos indicus* such as Brangus, Beefmaster and Santa Gertrudis still tend to have tougher *longissimus* on average than *Bos taurus* breeds (Bidner, Wyatt, Humes, Franke, & Blouin, 2002; Crouse et al., 1989; Johnson et al., 1990; O'Connor et al., 1997; Wheeler, Cundiff, Shackelford, & Koohmaraie, 2010). For this reason, the Australian Meat Standards Australia eating quality assurance system for beef predicts lower consumer scores for any cattle with *Bos Indicus* content greater than 25% (Polkinghorne, Philpott, Gee, Doljanin, & Innes, 2008). However, there have been three tropically-adapted *Bos taurus* breeds (Tuli, Bonsmara, and Romosinuano) identified that do not have reduced tenderness (Wheeler et al., 2005; Wheeler, Cundiff, et al., 2001). Since there is as much or more variation within breeds (6 genetic standard deviations) as between the most extreme breed averages (5 genetic standard deviations) for tenderness, the opportunity for improving tenderness by selecting seedstock within a breed may be as great, or greater, than by changing breeds (Wheeler et al., 1996). Differences in meat tenderness among lamb breeds also have been described (Hopkins & Fogarty, 1998; Warner, Greenwood, Pethick, & Ferguson, 2010). Shackelford, Leymaster, Wheeler, and Koohmaraie (2012) reported that among 10 sheep breeds, Finnsheep, Romanov, and Katahdin sired lambs had more tender *longissimus* at 7 days postmortem than did Dorset, Suffolk and composite (Columbia, Hampshire, Suffolk) sired lambs. Hopkins and Mortimer (2014) include an overview of the subtle sheep breed effects on eating quality.

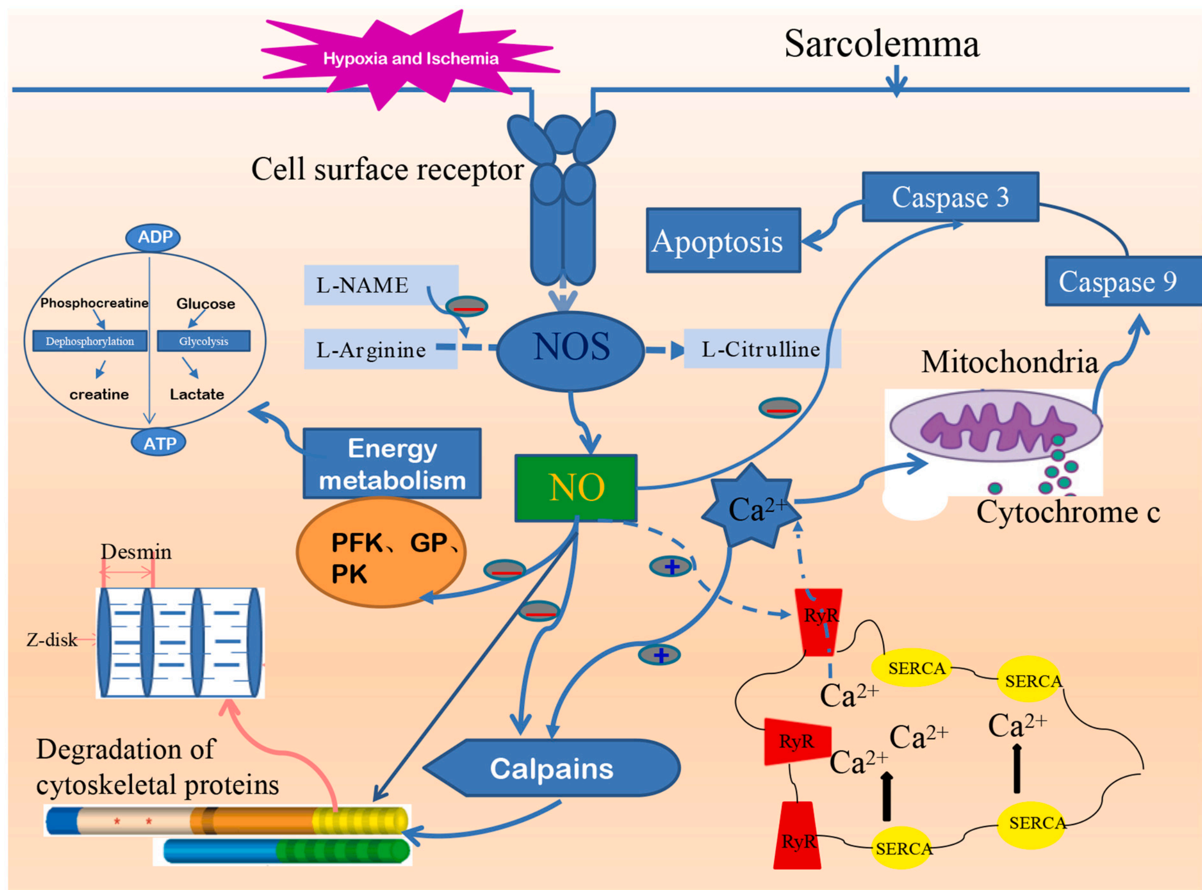

Fig. 1. Proposed pathways of nitric oxide involvement in postmortem aging including energy metabolism, glycolysis, calpains, calcium release, apoptosis and proteolysis via protein S-nitrosylation.
Abbreviation: NOS: nitric oxide synthase, NO: nitric oxide, RyR: ryanodine receptor, SERCA: Sarcoplasmic\endoplasmic reticulum calcium ATPase, PFK: phosphofructokinase, GP: glycogen phosphorylase, PK:pyruvate kinase.

3.2. Major genes

A mutation in the myostatin gene has been associated with the condition in cattle known as "double muscling" (Arthur, 1995; Grobet et al., 1998; Kambadur, Sharma, Smith, & Bass, 1997; McPherron & Lee, 1997; Smith, Lopez-Corrales, Kappes, & Sonstegard, 1997). Carcasses of double muscled cattle yield a greater percentage of retail product than carcasses of normal cattle (Wheeler et al., 2001) and meat from these animals is more tender, predominantly due to reduced collagen concentration (Ngapo et al., 2002; Wheeler et al., 2001). The myostatin mutation found in the Limousin cattle (F94L) results in improved meat tenderness, but to a lesser extent than those in Piedmontese and Belgian Blue cattle (Bennett et al., 2019; Lines, Pitchford, Kruk, & Bottema, 2009). Furthermore, F94L interacts with CAPN1 (see section below) polymorphisms such that the CAPN1 effect on increased tenderness is less pronounced.

Callipyge is a muscle hypertrophy condition in sheep that causes dramatic toughening of the resulting meat, but with variation among muscles (Carpenter, Rice, Cockett, & Snowder, 1996; Cockett et al., 1994, 2005; Freking, Keele, Nielsen, & Leymaster, 1998; Koohmaraie, Shackelford, Wheeler, Lonergan, & Doumit, 1995). It is associated with increased calpastatin activity and hence decreased protein degradation postmortem by Calpain-1 (Freking et al., 1998; Koohmaraie et al., 1995; Lorenzen et al., 2000).

3.3. Genomic markers

Measures of beef tenderness have been reported to be moderately heritable, with estimates ranging from 0.30 to 0.53 (Shackelford et al., 1994; Wheeler et al., 1996, Wheeler, Cundiff, et al., 2001; Wheeler et al., 2004, 2005; Dikeman et al., 2005). Smith et al. (2003) estimated that 46% of the variation in beef tenderness is genetic and 54% is environmental. In Australia, *Bos indicus* or tropically adapted breeds have a higher heritability for tenderness (*longissimus* WBSF h^2=0.30; consumer panel tenderness score h^2=0.31) and phenotypic variance compared to *Bos taurus* breeds (WBSF h^2=0.09; consumer panel tenderness score h^2=0.1) (Johnston, Reverter, Ferguson, Thompson, & Burrow, 2003). Whereas heritability of WBSF in pork in the Canadian pig population is 39% (Miar et al., 2014) and in the Australian sheep population is 20 and 36% for *longissimus* and *semitendinosus* respectively for sensory assessments and 24% for WBSF in the *longissimus* (Mortimer, Swan, Pannier, Ball, & Jacob, 2015). These data indicate that improving tenderness via genetic selection is possible. However, the degree to which a trait is influenced by genes versus environment will depend on the particular environment and genes of each specific situation (Warner et al., 2010).

Historically, in order to improve tenderness, breeding animals with superior genetic potential must be identified either through progeny testing or by direct measurements on the breeding animals themselves. The costs and time requirements associated with accurate collection of tenderness data has limited the use of progeny testing for tenderness traits in commercial practice. The use of genetic marker-assisted selection would allow greater efficiency in genetic progress with regard to tenderness. The development and implementation of genetic markers has been described in some detail (Allan & Smith, 2008; Smith et al., 2003; Warner et al., 2010). Single nucleotide polymorphism (SNP) markers with significant utility for marker-assisted selection have been

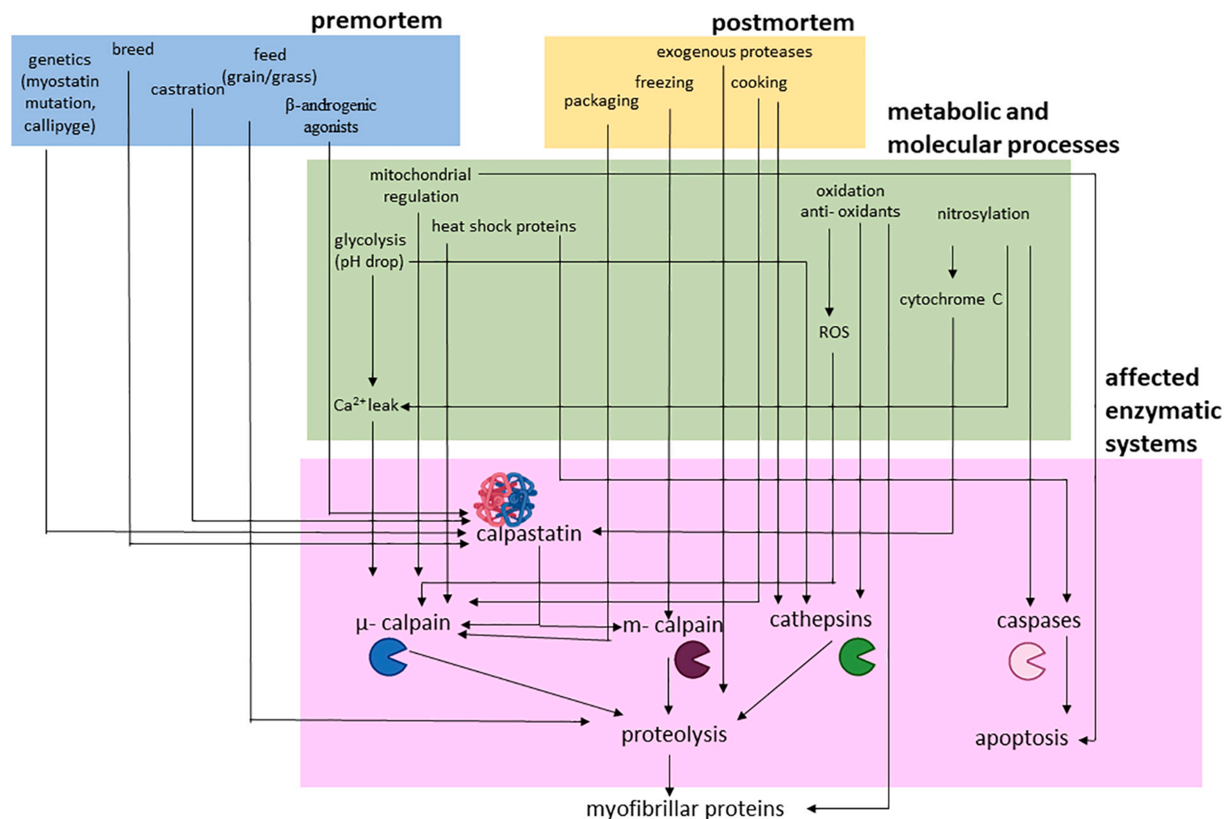

Fig. 2. Overview of the interactions between antemortem factors, postmortem factors, metabolic and molecular processes, and the affected enzymatic systems relevant for meat tenderization.

identified in beef in the calpain system for the CAPN1 gene (Page et al., 2002, 2004; White et al., 2005) and the CAST gene that codes for the inhibitor of calpains, calpastatin (Casas et al., 2005, 2006; Schenkel et al., 2006) and in pork (Lindholm-Perry et al., 2009; Nonneman et al., 2011, 2013; Rohrer, Thallman, Shackelford, Wheeler, & Koohmaraie, 2005). In the last 15 years or so, the association of multiple SNPs in both calpain and calpastatin genes in a wide variety of breeds of cattle, goats, sheep and pigs with variations in meat tenderness and other aspects of meat quality has been a very active area of research. Leal-Gutiérrez, Elzo, Johnson, Hamblen, and Mateescu (2019) reviewed the effects of 3 CAPN SNPs (Capn4751, Capn316, Capn530) and three CAST SNPs (UoG-Cast, Cast2959, Cast2832) in some detail. Therefore, it appears that markers for both of these genes (CAPN1, CAST) can be used simultaneously in breeding programs to improve tenderness. Some of these research population-developed markers (CAPN1 316 and 4751; CAST-T1) have been validated on independent beef *longissimus* samples from USA commercial meat processors (Shackelford, personal communication) and their value in offsetting some of the negative impact of aggressive implant strategies on *longissimus* tenderness has been demonstrated (King et al., 2012). Additional SNPs have been identified with significant association with pork tenderness (Ji et al., 2018), but need to be validated for commercial pigs. Genetic markers for tenderness are now available in commercial SNP chip assays in a variety of formats for high density genotyping (50K and 770K for beef, 60K for pork, and 50K for lamb) using HD bead-chip assays. This technology has allowed development of genomically enhanced expected progeny differences (EPDs). However, further improvements in the accuracy of reference genomes and continued improvement in next generation sequencing technology at progressively lower cost have made genotyping by sequence a feasible option with some advantages. These advancements will lead to improved accuracy of whole genome sequence imputation that increases the ability to identify causal genetic variants and improve genomic selection for traditional and novel traits like tenderness (Butty, 2019).

3.4. *Growth promotants*

Improving the rate and efficiency of growth in market animals, and carcass leanness, are important economic considerations for livestock producers. Therefore, the administration of agents that partition nutrients towards muscle deposition is a common practice in many countries. The most common metabolic modifiers used in meat production include anabolic steroids and β-adrenergic agonists (BAA). At least 90% of steers and heifers fed in the USA receive anabolic steroid implants (Dikeman, 2007), which can be classified according to their active ingredient (estrogens, progestins, androgens, or combination). Of these, the combination implants at multiple timepoints are considered to be more "aggressive", because they generally provide greater increases in growth rate and feed efficiency (Dikeman, 2007). A wide variety of products are available commercially and the impact on meat tenderness depends on the kind and number of implants. For example, a meta-analysis was used to show that the application of anabolic steroids reduces consumer tenderness scores by 5 units and increases WBSF by 4.1 N (Dunshea, D'Souza, Pethick, Harper, & Warner, 2005). However, these effects are largely dependent on the implanting strategy used. As implanting strategies increase in aggressiveness (use of combination and/or multiple implants), the negative effect on tenderness is amplified, particularly when used within 70 days of the harvest date (Dikeman, 2003; Platter, Tatum, Belk, Scanga, & Smith, 2003). Anabolic steroid implants are not used in pig, sheep and poultry production in Australia/New Zealand and USA and in many other countries. Anabolic steroid implants are also not used in cattle production in many countries, particularly in Europe; about 40% of Australian cattle are free of anabolic steroid implants whereas only a small percentage of USA cattle production are implant

free.

Use of BAA's, such as ractopamine and zilpaterol, in pigs and cattle, dramatically increases lean growth. However, numerous reports indicate that administration of BAA's has negative effects on the tenderness of beef and pork (Dikeman, 2003, 2007; Dunshea et al., 2005; Lean, Thompson, & Dunshea, 2014). Feeding BAA's has been reported to increase calpastatin activity which results in greater muscle hypertrophy and decreased tenderness primarily from the inhibition of postmortem proteolysis (Koohmaraie, Shackelford, Muggli-Cockett, & Stone, 1991; Koohmaraie, Shackelford, & Wheeler, 1996). These negative effects on tenderness may be even greater when combined with aggressive anabolic steroid implant strategies. In August 2013, the manufacturer of zilpaterol withdrew it from the USA and Canadian markets after the USA Food and Drug Administration (FDA) received reports of lameness or lying down of cattle fed zilpaterol (Dunshea, D'Souza, & Channon, 2016). Thus, some jurisdictions have a zero tolerance level for certain BAA's and this is likely to impact export markets and may limit in-country use of a BAA, in order to protect export markets (Centner, Alvey, & Stelzleni, 2014). Aroeira, Feddern, Gressler, Contreras Castillo, and Hopkins (2020) recently reviewed the impact of growth promoting compounds in cattle and pigs including minor negative effects on eating quality.

3.5. Animal age

Production systems vary throughout the world, and therefore animals are harvested at different points in their life-cycle. Animals harvested at very young ages will generally be very lean, and smaller than those of mature animals. Therefore, their carcasses may chill more rapidly, potentially resulting in cold-induced toughening (Cross, Crouse, & MacNeill, 1984). In addition, as animals mature, intermolecular cross-links stabilize the connective tissue matrix of muscle and increased collagen stability is associated with increased toughness (Purslow, 2018). However, animals undergoing rapid growth will have a higher proportion of newly synthesized, heat-labile collagen (Aberle, Reeves, Judge, Hunsley, & Perry, 1981). Therefore, age effects can be partially mitigated by feeding mature animals a high-energy diet (Boleman, Miller, Buyck, Cross, & Savell, 1996; Miller, Cross, Crouse, & Jenkin, 1987). However, Purslow (2018) concludes that although heat-soluble collagen explains some of the tenderness differences among muscles and ages of animals, there is considerable variation in the strength of this effect. He further concludes that the future focus should be on the heat-insoluble fraction of collagen to develop strategies to reduce cooked meat toughness of some muscles (Purslow, 2018). Such strategies are most likely to involve manipulation of the turnover of intramuscular connective tissue in the live animal by stimulation of collagen degradation and collagen resynthesis (Purslow, Archile-Contreras, & Cha, 2012) even though collagen turnover in muscle is slower than in some other tissues (Laurent, 1987). This may include supplements of vitamins C and E (Archile-Contreras, Cha, Mandell, Miller, & Purslow, 2011) and use of selected growth promotants (Roy, Sedgewick, Aalhus, Basarab, & Bruce, 2015 or selection of animals for single nucleotide polymorphisms in the matrix metalloproteinase-1 collagenase that is known to reduce the strength of raw perimysium in cattle (Christensen, Monteavaro, & Purslow, 2020).

3.6. Castration effects on meat tenderness – focus on cattle and pigs

The castration of male domestic animals of most species, with the exception of breeding stock, has been practiced for centuries. Historically, the main reasons for castration were to control the reproductive status of females (as often males and females were kept together), to reduce negative and aggressive behaviors and to fatten animals. However, in some parts of the world bull calves from dairy production are sometimes not castrated, and in some countries entire male pigs are raised to take advantage of the lean and rapid growth. It should be noted that in Australia, where traditionally male pigs are not castrated, immuno-castration is used on 65% of the male pig population, to reduce the risk of boar taint (Dunshea et al., 2016). Castration of pigs will likely decrease particularly in the EU, as castration without the use of anaesthetics increasingly becomes an animal welfare issue (Prunier et al., 2006). In 2014, the EU passed a resolution banning surgical castration without anesthetic but as this is voluntary, some countries in 2020 are still castrating pigs without pain relief (Aluwé et al., 2020).

Young, intact males produce more rapid and efficient growth and result in leaner carcasses than their steer/wether (castrated sheep and goats) counterparts, but are associated with management problems, most notably behavior (Goetsch, Merkel, & Gipson, 2011; Nagamine & Sunagawa, 2017; Sales, 2014; Seideman, Cross, Oltjen, & Schanbacher, 1982). In a literature review on the use of intact males for beef production, Seideman et al. (1982) concluded that meat from bull carcasses was less tender and more variable than the meat produced by steer carcasses. Using a meta-analysis, Sales (2014) demonstrated that rams had higher WBSF values (tougher meat) than wether castrates and Nagamine and Sunagawa (2017) showed that castrated goats had lower WBSF and the meat had lower odour/taint scores than uncastrated billy goats. In the case of cattle, Cross et al. (1984) suggested that higher concentrations of less-soluble collagen could contribute to these differences. Morgan et al. (1993) reported *longissimus* steaks from bull carcasses have higher shear force values and less myofibril fragmentation than *longissimus* steaks from steer carcasses due to higher calpastatin activity in muscle from bull carcasses. Higher incidence of DFD meat in entire male cattle (Tarrant, 1989) and pigs (D'Souza, Warner, Dunshea, & Leury, 1999) could contribute to decreased tenderness, as intermediate pH is known to often have increased toughness relative to normal and high pH meat (Purchas & Aungsupakorn, 1993). The use of intact boars for pork production has some impacts on tenderness measured by sensory tenderness, but these are relatively small, being of the order of 3 units on a 100 point hedonic scale (Channon et al., 2018; Channon, Hamilton, D'Souza, & Dunshea, 2016; Seideman et al., 1982; Warner, Dunshea, & Channon, 2018). The magnitude of these differences in tenderness are similar to those observed with equivalent increases in carcass leanness obtained through genetic selection for lean growth and may be an inherent consequence of the production of leaner meat (Warner et al., 2021) . However, there is always a risk of boar taint with raising intact males, which can be overcome with immuno-castration (Channon et al., 2018). Carcasses can be selected for boar taint using a variety of chemical or sensory techniques but tainted pork still needs to be used and further processing does not necessarily eliminate the boar taint issue (Tørngren, Claudi-Magnussen, Støier, & Kristensen, 2011).

3.7. Grain feeding

In many countries, cattle, sheep, and goats are commonly placed in feedlots to produce rapid, efficient growth from a high energy diet. This practice has been reported to produce heavier, fatter, and more muscular carcasses, with higher intramuscular fat, compared to forage feeding (Aberle et al., 1981; Bowling, Smith, Carpenter, Dutson, & Oliver, 1977; Warner, Dunshea, Gutzke, Lau, & Kearney, 2014). Grain-fed animals also generally produce steaks that are more tender than steaks from grass -fed animals, except that the increased mass and fat thickness in grain-fed carcasses, along with higher body temperature, slows chilling, which can sometimes result in heat-toughening (Warner et al., 2014). But the improved tenderness of grain fed animals is likely attributable to increased growth rate associated with increased protein turnover (Koohmaraie, Kent, Shackelford, Veiseth, & Wheeler, 2002), postmortem proteolysis (Aberle et al., 1981; Purchas, Sobrinho, Garrick, & Lowe, 2002), collagen solubility (Aberle et al., 1981), increased marbling and reduced incidence of high pH DFD meat (Warner, Truscott, Eldridge, & Franz, 1988).

Vitamin D supplementation to improve tenderization has increasingly attracted research attention. The use of vitamin D is thought to

result in increased mobilization of calcium ions and thus more calpain activity. Indeed, supplementation of vitamin D3 or its metabolite 25-hydroxyvitamin D3 was reported to lead to increased muscle calcium concentration and calpain-induced degradation of troponin-T (Carnagey et al., 2008; Foote et al., 2004; Montgomery et al., 2004). Feedlot supplementation with vitamin D3 and its metabolites has been shown to reduce the shear force of meat from heifers and steers (Duffy et al., 2017; Montgomery et al., 2004), but not cull cow (Sell, Mikel, Xiong, & Behrends, 2004), lamb (Boleman, McKenna, Ramsey, Peel, & Savell, 2004), pork (Duffy et al., 2018; Wiegand et al., 2002) or *Bos indicus* cattle (Lawrence et al., 2006). It is worth noting that reports on the effectiveness of vitamin D3 on shear force and sensory tenderness vary in these studies, likely due to differences in level and type of supplementation, species and breed, carcass characteristics, muscle and aging time. Thus, vitamin D3 and its metabolite supplementation for the purpose of improved tenderization requires further research.

It is also worth mentioning that carcass weight has been steadily increasing in most animal production systems due to various factors, including changes in genetics, animal husbandry, nutrition, slaughter age and growth promotants. Heavier carcasses present challenges in chilling and pH-temperature decline management. A substantial amount of research has been conducted to optimize different chilling technologies (e.g. blast chilling, rapid chilling, very fast chilling, cryogenic chilling, spray chilling, Rinse&Chill®) (Zhang et al., 2019). Studies examining the effect of carcass weight on quality of feedlot steers reported heavier carcasses had a faster pH decline, a slower temperature decline, and passed through the heat shortening window (>35 °C at pH 6) (Agbeniga & Webb, 2018; Warner et al., 2014). However, in the study of Agbeniga and Webb (2018), the sarcomere length was not affected by carcass weight, nor was the shear force after 14 days of aging. Using regression analysis, Okeudo and Moss (2005) found a significant correlation between carcass weight and shear force of different lamb muscles. On the other hand, a meta-analysis found no relationship between beef carcass weight and sensory tenderness (Trefan, Doeschl-Wilson, Rooke, Terlouw, & Bunger, 2013). The mechanism through which increased carcass weight may influence meat tenderness is multifaceted due to the compounding effects of other carcass characteristics such as growth rate (potential effect on calpains), subcutaneous fat , intramuscular fat, collagen content, muscle type and aging. Although it is tempting to recommend further research, these compounding/confounding factors suggest that accurate description of all these attributes for carcass and quality phenotypes is critical. This is particularly evident in the lack of reporting of these critical attributes in the methodology section of many journal publications.

4. Advances in postmortem factors influencing tenderization, including cooking

Postmortem changes in muscle involve complex biological processes which are influenced by intrinsic and extrinsic factors. An understanding of postmortem physical and biochemical changes that impact meat tenderness, including during the cooking process, is therefore crucial. There are a wide variety of postmortem treatments and conditions that affect the tenderness of the final product, and a comprehensive review of all of these is not possible here. In this section, we focus on those which have greatest relevance to two of the molecular mechanisms discussed above, namely oxidation and postmortem proteolysis, as well as those that have direct effects on the integrity of the structure of muscle tissue. Freezing and thawing of meat disrupts structures and may release calcium ions and affect proteolysis. Several postmortem treatments of raw meat, including pulse electric field and ultrasonic treatments, have a primary effect of enhancing endogenous proteolysis, whereas hydrostatic and dynamic high-pressure treatments appear to primarily disrupt meat microstructure without enhancing proteolysis. Treatment of meat by exogenous (mainly plant-based) enzymes is another postmortem treatment with an obvious focus on tenderization by proteolysis. The final step of the production to consumption chain is the cooking of meat, which brings about its own structural effects, and in its initial stages may also promote proteolysis. Fig. 2 demonstrates the interactions between some of the postmortem factors, metabolic and molecular processes and enzymatic systems involved in meat tenderization.

4.1. Oxidation

An important postmortem change during meat aging, or during frozen storage, is the potential for increased levels of oxidation. Postmortem oxidation occurs in both lipid and protein components, and the link between lipid and protein oxidation has been established (Faustman, Sun, Mancini, & Suman, 2010). The negative effects of lipid oxidation on sensory traits are well recognized but the focus here is on protein oxidation and its effects on tenderization. Oxidation of myofibrillar and sarcoplasmic proteins has been shown to result in the formation of carbonyl derivatives and disulfide cross-links. These chemical changes lead to (i) inactivation of calpains which are essential for the tenderization process and (ii) an increase in toughness due to myofibrillar protein aggregation. Multiple reviews have focused on the causes, mechanism and effect of oxidation on meat quality, including tenderness (Bao & Ertbjerg, 2018; Estevez, 2011; Estevez et al., 2020; Warner, Dunshea, Ponnampalam, & Cottrell, 2005; Zhang, Xiao, & Ahn, 2013). Minimizing postmortem protein oxidation is therefore an important approach to improve meat tenderness.

4.1.1. Oxidation during aging and storage

Postmortem oxidation of meat proteins can occur within 24 hours following slaughtering, if conditions are inducive to oxidation (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004a). Xue, Huang, Huang, and Zhou (2012) showed that *in vitro* exposure of beef myofibrillar proteins to H_20_2 and Fe^{2+} led to a reduction in troponin-T degradation, demonstrating that oxidative modifications of myofibrillar proteins changed their susceptibility to Calpain-1. A similar study on pork *longissimus* showed that OH·-induced oxidation of myosin leads to protein polymerization and aggregation, resulting in a reduced proteolytic susceptibility (Morzel, Gatellier, Sayd, Renerre, & Laville, 2006). In addition, oxidation has been shown to decrease activity of Calpain-1, and inactivate calpastatin (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004b). Thus, industry-adoptable approaches, such as supplementing animal feeds with antioxidants, have been developed to increase protection of myofibrillar proteins against oxidation during meat aging. A decrease in calpastatin activity and a significant increase in Calpain-1 activation and proteolysis of troponin-T in steaks from vitamins E and C fed steers was observed compared to steers fed conventional feedlot diets (Pogge, Lonergan, & Hansen, 2015; Rowe et al., 2004b). Recent research with bovine fibroblasts from *longissimus* and *semitendinosus* suggests vitamins E and C can modulate collagen synthesis and degradation which have implications for postmortem meat tenderness (Archile-Contreras et al., 2011; Archile-Contreras & Purslow, 2011).

4.1.2. Oxidation in packaging

The effect of packaging on oxidation status of meat protein has been well established. Application of high oxygen modified atmosphere packaging (hiOxMAP) in retail display has been shown to result in a dramatic reduction in both instrumental and sensory tenderness of different muscles from beef, pork, lamb and poultry meats (Bao & Ertbjerg, 2015; Frank et al., 2017; Fu et al., 2015; Geesink, Robertson, & Ball, 2015; Jongberg, Wen, Tørngren, & Lund, 2014; Lorenzo & Gomez, 2012; Peng et al., 2019). The negative impact of hiOxMAP on eating quality, including tenderness, of meat, is believed to be a direct result of oxygen-induced oxidation. Meat packed in hiOxMAP has been shown to have both a loss of free thiol groups and an increase in total carbonyl content compared to those of meat packed in vacuum (Bao & Ertbjerg, 2015; Chen, Zhou, & Zhang, 2015; Lund, Lametsch, Hviid, Jensen, &

Skibsted, 2007). These chemical modifications of meat proteins are linked to reduced proteolysis measured by myofibril fragmentation index (Clausen, Jakobsen, Ertbjerg, & Madsen, 2009) and desmin degradation (Fu et al., 2015) and increased cross-linking between myosin heavy chains (Bao & Ertbjerg, 2015; Kim, Huff-Lonergan, Sebranek, & Lonergan, 2010; Lund, Luxford, Skibsted, & Davies, 2008; Zakrys-Waliwander, O'Sullivan, O'Neill, & Kerry, 2012), cross-linking between myosin heavy chains and titin (Kim et al., 2010), and decreased Calpain-1's catalytic activity (Fu et al., 2015; Lindahl, Lagerstedt, Ertbjerg, Sampels, & Lundstrom, 2010).

In order to reduce the negative impact of high oxygen modified atmosphere packaging on meat tenderness, various approaches have beed tested, with varying success. These include lowering the oxygen content (Bao & Ertbjerg, 2015; Resconi, Escudero, & ¡n, J., Olleta, J., Ýudo, C., & Mar Campo, M. a. d., 2012; Spanos, Torngren, Christensen, & Baron, 2016), injection of calcium lactate/phosphate (Cruzen et al., 2015), modification of the gas content and headspace (Murphy, O'Grady, & Kerry, 2013; Spanos et al., 2016), use of carbon monoxide and sodium nitrite (Djenane & Roncalés, 2018; Roberts et al., 2017), feeding diets high in anti-oxidants (Ripoll, Joy, & Munoz, 2011), and development of active and smart packaging materials (Arvanitoyannis & Stratakos, 2012). While studies on these packaging methods report varying levels of success in suppressing oxidation, their adoption in industry will depend on further research in cost-benefit analysis, adaptability to the current supply chain, and food regulations. It is worth noting that oxidation-induced chemical modifications of proteins differ across different meat types and cuts. For example, desmin degradation was reduced as a result of hiOxMAP for beef *longissimus* (Fu et al., 2015) but not for pork *longissimus* (Lund et al., 2008; Bao & Ertbjerg, 2015). Similarly, a study on packaging of chicken breast (*pectoralis major*) and thigh (*peroneus longus*) showed that a similar increase in oxidation measured by thiol loss and protein cross-linking in both muscles due to hiOxMAP did not result in the same reduction in sensory tenderness score for the two muscles (Jongberg et al., 2014). Thus, optimization of MAP packaging for meat retail display will need to be species- and muscle-specific. While further developments in packaging technologies are on-going, extensive evidence has shown that vacuum packaging and vacuum skin packaging are ready-to-adopt alternatives to MAP which can ensure optimal tenderization and eliminate oxidation-induced toughening of meat. These low/no oxygen packaging systems are reported to result in more degradation of troponin-T and desmin, less myosin cross-linking, reduced WBSF, and increased consumer sensory acceptability (Holman, Kerry, & Hopkins, 2018).

4.1.3. Oxidation in other meat processing methods

Other postmortem methods for processing of meat, such as freezing/thawing, irradiation, pressure treatment and cooking, also influence the oxidation status of meat proteins and hence meat tenderness (Bao & Ertbjerg, 2018; Guyon, Meynier, & de Lamballerie, 2016; Leygonie, Britz, & Hoffman, 2012; Yu, Morton, Clerens, & Dyer, 2017). Specific settings of the parameters in these processes, e.g. rate and number of freezing/thawing cycles; magnitude of pressure; and cooking temperature, have been shown to result in varying levels of protein oxidation. For instance, a significant increase in protein oxidation, measured as carbonyl content, in pork *longissimus,* was observed at 100 °C and 140 °C compared to pork cooked at 70 °C (Bax et al., 2013). Oxidation of meat proteins due to these processes not only affect tenderization of fresh meat but also protein functionality during subsequent processing, e.g. processed meat products (Buckow, Sikes, & Tume, 2013; Utrera & Estevez, 2012). Thus, further research in innovative technologies aiming at mitigating the impact of protein oxidation in meat is needed to improve both meat quality and subsequent usage.

4.2. Meat tenderization using exogenous proteases

Traditionally, use of certain plant parts (leaves, stems, seeds, latex, fruits, roots, and pulps), such as *Artocarpus integer*, pineapple, papaya, ginger, figs and others (Table 2), to tenderize meat has been considered important. Although the modern meat industry has been able to reduce variability in meat tenderness, by implementing accelerated conditioning and aging and use of electrical stimulation, inherent variation in meat tenderness, means that less than 10% of the carcass meat is suitable for grilling (Polkinghorne et al., 2008b). Proteases which break-down myofibrillar proteins can be endogenous (eg. calpains and cathepsins) and exert their effects in the animal and during aging (see Warner et al., 2021 for review) or exogenous, with application to the meat postmortem. Many of the meat cuts obtained from slaughtered animals could benefit from the use of exogenous enzymes to reduce the toughness and add-value (Bekhit, Hopkins, Geesink, Bekhit, & Franks, 2014).

Proteases can be classified as acidic, neutral, or alkaline proteases on the basis of optimal pH for their activity, and also as animal, plant, bacterial, fungal, yeast, or marine proteases on the basis of their source (Table 2); or as endopeptidases and exopeptidases on the basis of their cleavage position. Comprehensive accounts of protease classification, characteristics, regulation, and the level of investigation in meat research can be found in Bekhit, Hopkins, et al. (2014), Bekhit, Carne, Ryder, Ha, & Kong (2017) and Tantamacharik, Carne, Agyei, Birch, and Bekhit (2018). Therefore, the following section will provide information on recent trends for the use of exogenous proteases to tenderize meat and make general comments in relation to the potential commercial application.

4.2.1. Plant proteases

Proteases are widely distributed in plants (Tantamacharik et al., 2018) but most research on meat tenderization has focused on a few cysteine proteases such as papain (papaya latex), bromelain (pineapple stem), ficin (figs), actinidin (kiwifruit) and zingibain (ginger rhizome).

Papain and bromelain lack substrate specificity towards meat proteins and the extensive and non-selective hydrolysis of myofibrillar and connective tissue protein results in mushy texture and generation of 'off' sensory notes such as 'grainy' texture and 'bitter' flavour (Bekhit, Hopkins, et al., 2014). The process needs to be strictly regulated to achieve the right level of tenderness but can be used to generate tender meat (Barekat & Soltanizadeh, 2018; Ma et al., 2019) and beef products for older consumers (Botinestean et al., 2018). Actinidin has attracted much interest (Zhang, Sun, Liu, Li, & Jiang, 2017; Zhu, Kaur, Staincliffe, & Boland, 2018; Bekhit, Ha, Carne, Hopkins, & Geesink, 2018; Bekhit et al., 2018; Gong, Morton, Bhat, Mason, & Bekhit, 2019), as has zingibain (Naqvi et al., 2021) due to the mild and effective tenderization (Han, Morton, Bekhit, & Sedcole, 2009). A very effective tenderization process involved an actinidin-containing preparation which was infused pre-rigor and led to early activation of Calpain-2 and very tender meat at 5 hrs postmortem (Han et al., 2009). Less known plant proteases with potential tenderizing effects include extracts of asparagus (Ha, Bekhit, Carne, & Hopkins, 2013; Yonezawa, Kaneda, & Uchikoba, 1998), *Sarcodon aspratus* (mushroom species; Kim, Lee, & Ryu, 2015), crude mango peel (Dhital & Vangnai, 2019) and *Spondias cytherea* roots (plum tree species; Ahmad et al., 2019).

Plant proteases have been extensively studied, however according to the best knowledge of the authors, these enzymes are not used in meat products commercially. This is likely due to various issues related to formulation, stability and control of the enzymes post-treatment which are discussed in full detail in Bekhit, Suwandy, Carne, Ali, and Wang (2017) and need to be addressed in order for future uptake in the meat industry. Many of these issues are related to the fact that commercial protease preparations contain multiple complex proteins and proteases (Ha et al., 2013; Ha, Bekhit, Carne, & Hopkins, 2012) that exhibit variable hydrolytic activities and can lead to over-tenderization and production of 'off' sensory notes, as mentioned above for papain and bromelain. The variability in purity of the proteases in commercial preparations would result in different tenderization outcomes. Another issue with plant protease extracts is that they can carry some flavor of

Table 2
Plant, microbial and animal proteases potentially useful in meat tenderization. Derived from Tantamacharik et al. (2018).

Origin and enzymes	Source
ANIMAL ORIGIN	
Placental protease; Pancreatin; Pepsin; Chymotrypsin, Trypsin, Elstase, Carboxypeptidase	Pancreas and stomach of mammals
BACTERIAL ORIGIN	
Alkaline elastase, alkaline protease, collagenase (Sigma type VII)	*Alkalophilic Bacillus sp; Bacillus polyfermenticus; Clostridium histolyticum*
Subtilisin (EC 3.4.21.62) and subtilisin-like cold active proteases	*Serratia marcescens; Bacillus* sp.; *Pseudomonas lundensis; Enterococcus faecalis; Stenotrophomonas maltophilia; Curtobacterium. Lutium; Pseudoalteromonas* sp.; *Aspergillus ustus; Pedobacter cryoconitis; Bacillus cereus; Colwellia* sp.; *Bacillus amyloliquefaciens; Flavobacterium psychrophilum; Leucosporidium antarcticum; Pseudomonas; Pseudoaltermonas* sp.
FUNGAL ORIGIN	
Acid, alkaline, serine and neutral proteases	*Aspergillus Sojae; A.flavus, A. fumigatus; A. niger; Chrysosporium keratinophilum; Conidioboluscoronatus; Paecilomyces lilacinus; Rhizopus oligosporus; Debaryomyces hansenii; Mrakia frigida; Candida parapsilosis; Penicillium restrictum; Penicillium roqueforti; Mucor circinelloides; Debaryomyces castellii; Kluyveromyces marzianus; Aspergillus candidus; Aspergillus. Oryzae Fusariumeumartii*
YEAST ORIGIN	*Saccharomyces cerevisiae, Candida lipolytica* (NRRL Y-1094)
PLANT ORIGIN	
Zingibain (EC 3.4.22.67)	Ginger *(Zingiber officinale)*
Papain (EC 3.4.22.2)	Papaya latex
Bromelain (EC 3.4.22.4)	Pineapple stem
Ficin (EC 3.4.22.3)	Fig latex
Capparin	Caper *(Capparis spinosa)*
serine-type endopeptidase (EC 3.4.21.92)	Asparagus
Actinidin (EC3.4.22.14)	Kiwifruit (*Actinidia deliciosa*)
Cucumisin (EC 3.4.21.25)	Kachri *(Cucumis trigonus Roxb); Cucumis sativus L.*
Subtilisin-like/serine protease	*Taraxacum officinale; Heliantus annas; Machira pomifera; Cucumis melo; Cucurbita ficifolia; Benincasa cerifera; Benincasa hispida; Trichosantus cucumeroides; Trichosantus kirrilowi; Trichosanthes bracteata; Euphorbia supine*
MARINE ORIGIN	
Pepsin, pepsinogen, gastricsin, trypsin, chymostrypsin, elastase, collagenase	Northern Shrimp *(Pandalus borealis)* heads; marine by products

their own that may be acceptable to some and unacceptable to others, such as occurs with ginger extracts containing zingibain.

4.2.2. Proteases from bacteria and fungi

Proteases from bacterial and fungal sources have been extensively used in food and biotechnological applications. The microbial-derived proteases have several advantages compared to plant-derived proteases. The microbes can be cultured relatively quickly under strict conditions that allow more control over the production of the proteases. The expression and activity of the proteases can be manipulated using modified production conditions or cloning. The cloning of an aspartic protease gene (RmproA) in *Rhizomucor miehei* CAU432 fungi is an example which resulted in a protease with the same efficacy as papain for tenderizing pork (Sun et al., 2018).

Microbial-derived proteases are commercially available from non-pathogenic sources and many have been approved by regulatory authorities. Many of these microbial-derived proteases have higher specificity and are easier to control than plant proteases (Ashie, Sorensen, & Nielsen, 2002). However, many consumers are uncomfortable with the concept of bacterial or fungal additives to food products. A good strategy to overcome this negative perception is to target probiotic bacteria as sources of effective proteases, which could be used for the dual function of gut health, and meat tenderization (Chanalia, Gandhi, Attri, & Dhanda, 2018).

4.2.3. General comments

It is difficult to achieve controlled proteolysis with broad substrate specificity proteases (Schaller, 2004) and this has resulted in undesirable over-tenderized product. This may not be a problem if the final product is designed for infants, seniors or patients who may find chewing difficult. Mild tenderizing proteases (microbial-derived proteases, zingibain and actinidin) are probably easier to control and more available compared to plant proteases which are often limited by geographical or production issues. Pre-rigor infusion has not been a commercial reality until recently. The development of Rinse & Chill® technology makes the application of compounds such as actinidin to pre-rigor carcass meat a viable option. Recent studies have combined proteases and emerging technologies, such as ultrasound (Barekat & Soltanizadeh, 2018) and high pressure processing (Ma et al., 2019), and show promise for new strategies to improve distribution within the muscle, facilitate better interaction between proteases and ultrastructural proteins, and hence allow greater control of tenderization.

4.3. Freezing/thawing effects on tenderness

The freezing of meat produces ice crystals, the size and location of which depend on freezing rate and temperature. Rahelić, Gawwad, and Puač (1985) showed that ice crystals formed in the extracellular space at slow freezing to -10°C, intracellularly and extracellularly at -20°C, and intracellularly at temperatures between -33°C and -196°C. In their experiments, lower temperatures were accompanied by faster freezing rates. Ultrastructural studies on these frozen specimens (Rahelić et al., 1985) revealed lateral separation of muscle fibers at -10 and -20°C and disruption of intracellular structures below -33°C. Dobraszczy, Atkins, Jeronimidis, and Purslow (1987) demonstrated that the mechanical properties of beef *semitendinosus* muscle frozen to -21°C and then aged at temperatures between -5°C and -30°C undergo various transitions, with a peak of work to fracture at temperatures between -10 and -15°C, indicating that the varying location of ice crystals and the plasticity due to unfrozen water affect the properties of the frozen material. Thawing rates and methodologies (ambient temperature, chilled temperature, ohmic heating, acoustic, high-pressure, microwave, etc.) can also vary greatly and slow rates of thawing produce higher drip losses (Akhtar, Khan, & Faiz, 2013), with the possibility of reformation of larger ice crystals in slow thawing. Zhang and Ertbjerg (2018) interpreted the reduction in water-holding of frozen versus non-frozen pork loin as evidence of myofibrillar protein denaturation during the freeze/thaw process.

Locker and Daines (1973) found small increments of tenderization in beef *sternomandibularis* after repeated freeze-thaw cycles. Winger and Fennema (1976) used the same muscle to demonstrate that reductions in shear force with aging occurred more rapidly in frozen samples than non-frozen samples. Crouse and Koohmaraie (1990) found that meat aged after freezing had lower cooked shear force values than meat frozen after the same aging times. While Hergenreder et al. (2013) reported decreases in WBSF in beef *longissimus* but not *gluteus medius* due to freezing, no significant effects of freezing on sensory tenderness were found. Similarly, Lagerstedt and Johansson (2008) concluded that freezing and aging decreased peak shear force values, but sensory panelists perceived meat chilled for a similar aging period to be more tender, possibly due to a higher perception of juiciness in the chilled versus frozen samples. Grayson, King, Shackelford, Koohmaraie, and Wheeler (2014) concluded that freezing, or freezing and aging, does decrease slice shear force measures of toughness by 10-20% in beef *longissimus*, although the effect is less pronounced for beef *semitendinosus*, with an increase in proteolysis (as measured by desmin degradation) matching the decrease in shear force. In addition, Kim, Kim, Seo, Setyabrata, and Kim (2018), examined pork loins subjected to different ageing/freezing/thawing regimes and reported that ageing prior to a fast freeze/thaw cycle was an effective method to improve tenderness. Thus, some structural damage caused by ice crystals in frozen meat followed by enhanced proteolysis after thawing does seem to weaken the muscle structure, although the effects can vary greatly with freezing rate, temperature, thawing rate and method, and also between muscles and breeds (Aroeira et al., 2016). However, the effects on sensory tenderness may be confounded by decreased perception of juiciness. Emerging technologies to assist with freezing and thawing, including the use of high pressure, electrical and magnetic fields, ultrasound, microwave, and antifreeze protein, have shown promising results (Cheng, Sun, Zhu, & Zhang, 2017; Zhan, Sun, Zhu, & Wang, 2018). By utilizing these physical factors during the freezing and thawing processes, ice crystal formation, migration and distribution in meat are manipulated to minimize the impact on water holding capacity and texture. Our understanding of the effect of these technologies on the tenderness of frozen/thawed meat is limited, compared to other supply chain factors, thus further research is required. Such research should be targeted towards intrinsic meat factors that are known to influence the rate of freezing and thawing, e.g. species, muscles, intramuscular fat, post-mortem biochemistry and ageing status of the meat.

4.4. Selected technologies for tenderization

In recent years, much interest has been paid to developing more efficient and sustainable technologies to tenderize meat, or accelerate the tenderization process (Warner et al., 2017). The potential use of pulsed electric fields, ultrasound, muscle stretching techniques (Tenderstretch, Smartstretch™ and PiVac™, see Warner et al., 2017 for review) and pressure-inducing techniques (high pressure processing, hydrodynamic and shockwave) have been investigated for their potential meat tenderizing effects. Comprehensive reviews on the topics that describe principles, mode of action, effect on meat quality and future prospects of the various technologies are available (Alarcon-Rojo, Carrillo-Lopez, Reyes-Villagrana, Huerta-Jiménez, & Garcia-Galicia, 2019; Bhat, Morton, Mason, & Bekhit, 2018; Bhat, Morton, Mason, & Bekhit, 2019a; Troy, Ojha, & J. P., & Tiwari, B., K., 2016; Warner et al., 2017). A meta-analysis of literature on emerging technologies demonstrated that, across a number of studies, HPP was the most effective technology to reduce the WBSF of meat (Warner et al., 2017). The only cautionary note was that many of the technologies only had a limited number of studies, whereas HPP technology had 23 studies, compared to, for example, PEF, which had only 12 studies.

4.4.1. High pressure – hydrostatic and hydrodynamic

A recent meta-analysis of 23 experiments and 216 treatments on high pressure processing (HPP) applied to beef, sheepmeat, pork and chicken showed that the maximum tenderization occurred using 68-80 °C at 100-150 MPa, and significant tenderization also occurred under HPP conditions of 35-60 °C and 100-150 MPa (Warner et al., 2017). Recent studies have focused on exploring the mechanism of action for the tenderizing effect of HPP (high hydrostatic pressure) (Morton et al., 2017; Morton, Lee, Pearson, & Bickerstaffe, 2018; Zhang, Pan, & Wu, 2018b; Zhang et al., 2018). Beef hot-boned within 1 h of slaughter, at a temperature of 30-35°C, treated with HPP (175 MPa, for 2 min) and chilled to -1°C for 1 day, resulted in 60% and 43% lower WBSF in *longissimus thoracis* and *gluteus medius*, respectively and better sensory scores compared to controls (Morton et al., 2017). These results were similar to the effect of chiller aging for 28 days. The tenderizing effect of HPP was subsequently confirmed using the same HPP conditions (175 MPa, for 2 min) for *longissimus thoracis* samples from prime beef and bulls and resulted in 63% and 70% lower WBSF, respectively, and better sensory scores (Morton et al., 2018). Electron microscopy revealed that HPP had caused significant disruption to the sarcomere structure and led to a loss of network integrity, but this did not appear to be related to proteolysis, as HPP resulted in less activation of Calpain-1, shorter sarcomeres and lower myofibrillar fragmentation (MFI) (Morton et al., 2018). This suggested a lack of involvement of Calpain-1 in the observed tenderizing effect of HPP. Contrary to these findings, Zhang, Pan, & Wu (2018b) reported that pork subjected to HPP treatment (range 0-400 MPa, for 10 min at 20°C and kept at 4°C before treatment) within 2 h of slaughter showed higher MFI, an indication of increased proteolysis. HPP treatment of Calpain-2 and Calpain-1 and calpastatin in saline resulted in a small decrease in the Calpain-1 activity and a substantial decrease in calpastatin activity, suggesting a role for the calpain system in pork tenderization by HPP (Zhang, Pan, & Wu, 2018b) which is in contrast to previous findings. Furthermore, the authors reported that HPP prevented rigor development and thus it appears that mechanical and biochemical factors may explain the tenderizing effects of HPP of pork. In both studies, it is likely that exposing bone-less meat samples to low temperatures during either sampling or post-treatment storage would induce cold shortening, which may have been more severe in beef stored at -1°C compared to pork that was stored at 4°C. Assuming sarcomere shortening occurred (due to cold-induced shortening), this would potentially hinder access of calpain to its substrates (Weaver, Bowker, & Gerrard, 2008) in beefand thus may explain the low proteolysis observed in the samples. Although Wheeler and Koohmaraie (1999) did not find any evidence for this in sheep *longissimus*. The important information from these studies is that HPP is capable of tenderizing meat, either mechanically or through other systems, without the involvement of calpains. A 30% to 80% reduction in WBSF has been found with the application of HPP to post-rigor meat, but this required a processing temperature above 50-60°C (Warner et al., 2017).

Compared to high hydrostatic pressure (high pressure processing) for which there are numerous references, there are very few references on the application of high hydrodynamic pressure (shockwave) for meat tenderization (see review by Warner et al., 2017 for the references for both high hydrostatic and hydrodynamic pressure). Chian et al. (2019) reported that shockwave treatment caused an 11% reduction in the WBSF of beef brisket. Earlier research on shockwaves by Bolumar, Bindrich, Toepfl, Toldrá, and Heinz (2014) reported 18% reduction in the WBSF of beef loin steaks and reported it was caused by physical disruption.

4.4.2. Ultrasonication

High intensity ultrasound (HIU) at frequencies typically between 20-40 KHz produces cavitation in the intramuscular fluid when applied to raw meat, and this is thought to have two possible effects: (i) direct disruption of myofibrillar, cell membrane and connective tissue structures, and (ii) potentiation of proteolysis through the release of enzymes and effects on calcium release. These mechanisms have been reviewed at length by Alarcon-Rojo and colleagues (Alarcon-Rojo et al., 2019; Alarcon-Rojo, Janacua, Rodriguez, Paniwnyk, & Mason, 2015). Chang, Wang, Tang, and Zhou (2015) reported that HIU disrupted intramuscular connective tissue, reducing the thickness of perimysium and disrupting endomysium. However, the study did not reveal the length of time of storage at 4°C of specimens between application of ultrasound and the time of testing. Similarly, Chang, Xu, Zhou, Li, and Huang (2012) reported that HIU weakened the thermal denaturation of collagen in meat, but not its heat-solubility. However, their measurements of thermal stability were taken after storage of meat samples at 4°C for up to one week after ultrasonication, so that accelerated proteolysis was a possible contributor and the reported effects cannot be ascribed to connective tissue disruption alone. Other studies focus on ultrasonic disruption of myofibrillar structures. (Kang et al., 2017) and Stadnik, Dolatowski, and Baranowska (2008) reported disrupted Z-discs and swollen myofibrils after HIU treatment, but both of these studies also stress the acceleration of proteolysis during the aging process. As Alarcon-Rojo et al. (2019) pointed out, the numerous studies on the effects of ultrasound on meat tenderness are difficult to interpret due to the wide range of ultrasonic intensities and treatment times employed, as well as the variable times between ultrasonic treatment and measurement of biochemical, structural and tenderness parameters, However, a mix of physical weakening of muscle structures and accelerated proteolysis by release of cathepsins and calcium ions that activate calpains was likely (Alarcon-Rojo et al., 2019).

4.4.3. Pulsed electric field

Pulsed electric field (PEF) technology has been the subject of considerable recent research activity and has been critically reviewed by Bekhit, Carne, et al. (2017) and Bhat et al. (2019a). The first study to document a tenderizing effect of PEF in beef (Bekhit et al., 2014) reported an average of 19% reduction in WBSF relative to untreated samples. A subsequent study (Bhat et al., 2019a) documented the tenderizing effect but highlighted it was dependent on the muscle type and the status of the meat (pre- or post-rigor). A major concern for PEF use in pre-rigor meat is the heat generation that could lead to a cooking and toughening effect if high PEF intensity is used. Recent studies demonstrated PEF led to early activation of Calpain-2 and increased the proteolysis of desmin and troponin-T (Bhat, Morton, Mason, & Bekhit, 2019b, 2019c). However, the tenderizing effect of PEF is much lower compared with that achieved by HPP (Warner et al., 2017). Interestingly, PEF treatment has been shown to affect connective tissue and cause a reduction in the denaturation temperature of connective tissue and increased collagen solubilization at 60°C and 70°C (Alahakoon, Oey, Silcock, & Bremer, 2017). Although PEF has promise in tenderizing meat, there are several obstacles that need to be addressed. According to Bekhit, Carne, et al. (2017), heat generation during the treatment of fresh meat could negatively affect important quality attributes such as color, color stability, and water holding capacity. Commercial application will need a balance between the effective use of PEF and excessive heating. Furthermore, all reported studies have used isolated muscle tissue and no research on intact composite samples (containing muscle, connective tissue, fat and bone) has been reported. It is conceivable that non-uniform and uneven treatment distribution in non-homogenous material, such as meat, would occur and the effectiveness of the treatment would vary with the composition of the sample. The upscaling of PEF technology to suit meat applications is another technological hurdle required for commercial use of the technology. Most PEF experiments have used parallel plates less than 10 cm apart and small portions of meat. Processing of larger cuts would require higher voltages to generate sufficient electric field strength, with increased risk of heating.

4.4.4. Stretching and summary

Stretching is another technology designed to improve meat tenderness. Stretching can be applied at the carcass level (tenderstretch and

tendercut) or at the primal/cut level (PiVac® and Smartstretch™). The basic principle behind stretching of meat is to minimise sarcomere shortening during rigor mortis. Several reviews are available with good summaries of different stretching methods and usage (Bekhit, Hopkins, et al., 2014; Sørheim & Hildrum, 2002; Warner et al., 2017). While tenderstretch has been more widely adopted by selected meat processors compared to other stretching methods, most likely due to its easier adoptability, some of the issues commonly raised by processors include chiller space limitation, boning efficiency, primal shape changes and yield (Condon, 2019). Tenderstretch has been incorporated in Meat Standards Australia grading scheme to improve eating quality of beef and sheepmeat.

In summary, there are a range of postmortem treatments of meat that impact tenderness either through direct disruption of myofibrillar structure or accelerated proteolysis, or a combination of both. Fig. 3 shows an estimation from the meta-analysis of Warner et al. (2017) of the relative benefits of a subset of these techniques, compared to treatments administered to live animals, in terms of changes to cooked meat tenderness.

4.5. Changes in tenderness during cooking

Cooking is the final step prior to consumption and has a significant effect on sensory qualities. This section examines the impact of cooking on tenderness, with a focus on changes in protein conformation and degradation. Extensive research has been conducted on heat-induced denaturation of major meat proteins. These changes in the secondary structure can be observed by differential scanning calorimetry (DSC) and spectroscopic methods, such as Raman and Fourier Transform spectroscopy. DSC thermograms of meat consist of three or more major peaks, also known as transition temperatures, which are usually associated with the denaturation of major proteins and changes in meat. When conducting DSC, care should be taken when interpreting transition temperatures of major meat proteins that overlap and the process of denaturation should be regarded as a continuous process (Vaskoska et al., 2021). Denaturation of actin and myosin has been associated with tougher meat, and collagen denaturation has been linked to a decrease in firmness (Martens, Stabursvik, & Martens, 1982). The extent of collagen denaturation is dependent on heating temperature and heating rate. Lattore, Velazquez, and Purslow (2018) showed that the temperature, at which collagen denatured (transition temperature), increased with increasing heating rate (Fig. 4). About 5 % denaturation of collagen can be achieved through long-time, low-temperature (LTLT) cooking method in beef cooked at 60°C for 24 hours (Latorre, Palacio, Velázquez, & Purslow, 2019; Purslow, 2018). Similarly, increased tenderness in pork can be achieved with LTLT cooking which is related to solubilized collagen and reduced perimysial thickness (Li et al., 2019). Spectroscopic methods have been used to link meat tenderness to specific changes in the secondary conformation of proteins (Beattie, Bell, Borggaard, & Moss, 2008; Beattie, Bell, Farmer, Moss, & Patterson, 2004; Schmidt, Scheier, & Hopkins, 2013). While α- helices in muscle protein conformation are associated with greater toughness in bovine *semitendinosus* and ovine *longissimus* (Beattie et al., 2004; Schmidt et al., 2013), an increase in aggregated β-sheets has also been related to greater WBSF in porcine *longissimus* (Beattie et al., 2008). It is noteworthy that changes in content of α- helix and aggregated β- sheet are continuous with an increase in temperature. On the other hand, the level of tenderness fluctuates along the course of cooking as shown in Christensen, Purslow, and Larsen (2000) and Vaskoska, Ha, Naqvi, White, and Warner (2020). Thus, protein conformational change alone cannot fully explain the tenderness of cooked meat.

Another possible factor contributing to tenderness of meat is proteolysis during cooking. The role of calpains in tenderness of cooked meat remains largely unreported, most likely due to calpain inactivation at high temperature. However, desmin (whose degradation by Calpain-1 is a well-established marker of meat tenderization during aging) has been shown to be further degraded during cooking of porcine *longissimus thoracis et lumborum* (Ertbjerg, Christiansen, Pedersen, & Kristensen, 2012, suggesting involvement of cathepsins in proteolysis occurring during cooking of meat. Cathepsins are endogenous carboxyl proteases in muscle which have generally been considered to have no contribution, or a minor contribution, to tenderization during aging (Warner et al., 2021). However, recent studies have suggested cathepsins remain active during cooking, with increased activity between 53 and 63 °C (Christensen, Ertbjerg, Aaslyng, & Christensen, 2011). Injecting prerigor lamb with aspartyl protease inhibitor pepstatin, and aspartic protease inhibitor 1,2-epoxy-3-nitrophenoxypropane (EPNP), resulted in increases in WBSF (from 57 to 64 N, and from 60 to 80 N, respectively) of lamb *longissimus* cooked at 60 °C (King & Harris, 1982a, 1982b). Similarly, the activity of cathepsins B+L was negatively correlated (r =-0.50) with the WBSF of cooked porcine *longissimus* (Christensen et al., 2011). In addition, Vaskoska et al. (2021) showed that inhibition of cathepsins during heating of muscle fibre fragments causes a change in longitudinal and transverse shrinkage, both of which were related to

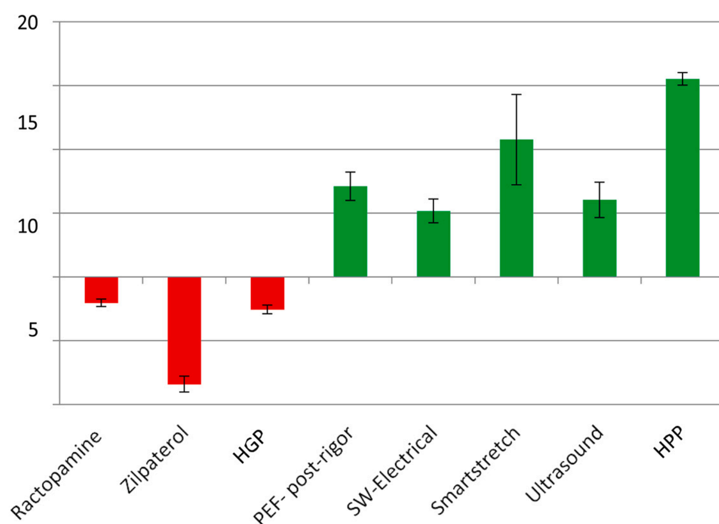

Fig. 3. Results of meta-analyses of Warner et al. (2017) predicting the change in peak shear force (N) in response to various treatments. Positive changes (green bars) are predicted reductions in shear force, whereas negative changes (red bars) are predicted increases. Pre-rigor treatments of Smartstretch , post-rigor pulsed electric field (PEF-post-rigor), electrical shock wave (SW-electrical), ultrasound and high-pressure processing (HPP) are compared to predicted effects of applications of ractopamine, zilpaterol and hormonal growth promotants (HGP) to beef cattle. The mean effect is shown and the vertical bar is the least significant difference (2 x SED). Reproduced from Warner et al. (2017) with the permission of Elsevier Ltd.

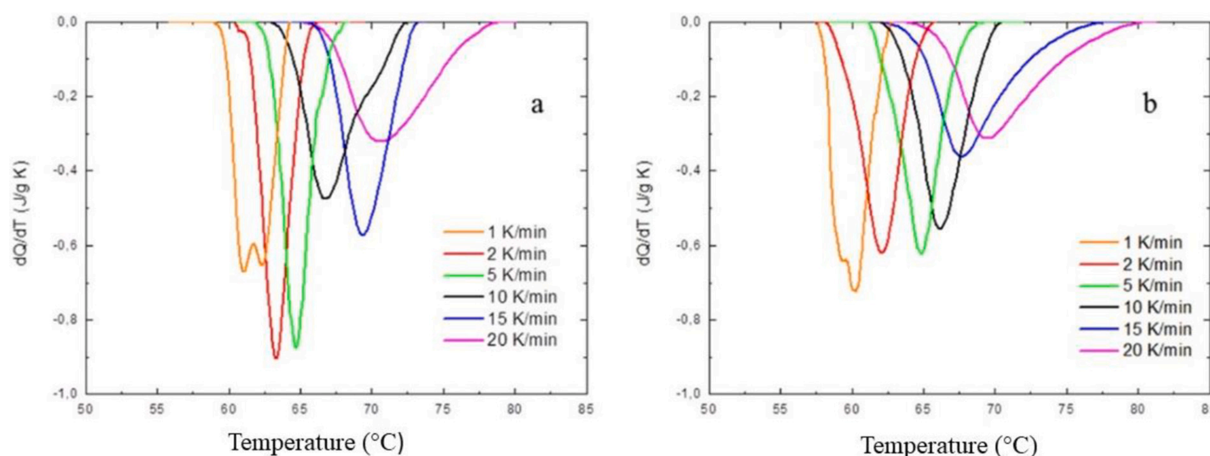

Fig. 4. Differential scanning calorimetry thermograms of a) perimysium from *pectoralis profundus* and b) perimysium from *semitendinosus* at variable heating rates (1, 2, 5, 10, 15 and 20 K/min), reproduced from Latorre et al. (2018)

meat tenderness. These studies together indicate that cathepsins may contribute to tenderness of meat, particularly when cooked under conditions that are conducive to their proteolytic activity, e.g. LTLT cooking.

5. Summary and further research

The importance of tenderness to the sustainability of the meat industry is recognized because it has a strong influence on the consumers acceptance of the quality of the meat they purchase, thus determining repeat purchase. There have been many advances in knowledge since the 1970's, on the factors affecting meat tenderness from a structural, muscle protein, biochemical and technological point of view.

The value of identifying biomarkers for prediction of meat tenderness from proteomic studies at this stage appears to be mainly in expanding our understanding of the tenderization process. This is partly because the complex processes associated with tenderisation postmortem rely on many factors in the supply chain. For this reason, some have predicted that single protein biomarkers will not be likely to accurately or reliably predict meat tenderness (Starkey, Geesink, Collins, Hutton Oddy, & Hopkins, 2016) whereas we suggest potential biomarkers still need extensive validation across species, carcasses and muscles. In addition, the role of collagen in tenderness has been overlooked in proteomic studies, likely because it is very challenging to isolate and purify (Warner et al., 2021).

Collagen has not only been overlooked in recent proteomic studies, but also there is a general lack of research on the contribution of collagen to meat tenderness. This is particularly in light of the data showing the postmortem degradation of collagen (Sylvestre, Balcerzak, Feidt, Baracos, & Bellut, 2002), the possible role of Vitamins C and E in collagen synthesis (Archile-Contreras et al., 2011) and potential for manipulation of the pools of heat-labile collagen in the animal and postmortem (Purslow, 2014, 2018). Hence future research on tenderness should include a focus on the changes in collagen in the animal, postmortem during ageing and also during cooking. This will assist in developing strategies to reduce cooked meat toughness of some animals and muscles.

Many hypothesis-driven studies have been conducted on effects of genetic, nutritional and environmental and molecular factors influencing meat tenderization whereas proteomic studies have focused on generating *post-hoc* hypotheses for the role of proteins in meat quality (Purslow, Gagaoua, & Warner, 2021. These molecular studies have been useful in identifying the important role of energy metabolism and new insights of apoptosis and proteases other than calpain in protein breakdown postmortem. Recent research has highlighted the importance of considering the interaction between different proteases including between caspases, cathepsins and the calpain system which seems to be multifaceted and complex in postmortem muscle. Recent data shows that proteolysis, which is initiated in the meat during ageing, continues during heating and cooking (Vaskoska, Ha, Ong, Kearney, et al., 2021), which challenges some of the traditional thinking that proteolysis ceases once cooking occurs. Further research on the interaction between the protease systems in animals, during processing and storage and also during cooking warrants further research.

The application of processing technologies and enzymes for advanced meat tenderization has been ongoing. Critically, evidence for substantial tenderization of very tough muscles has had most success with high hydrostatic pressure processing and also with plant-derived enzymes such as ginger and kiwifruit. Importantly, these technologies and enzymes are far more effective in tenderizing than any toughness arising to hormonal growth promotants, genetics or nutrition of the animal. The research on processing technologies and enzymes require further validation on muscles other than the longissimus and also in a wider range of carcasses and species. In addition, investigation of the molecular and biological mechanisms underpinning these technologies and enzymes will enable advances in understanding in addition to industry application.

The research conducted on meat tenderness has allowed eating quality assurance programs to be developed around the world and in some countries, this has resulted in premium prices for 'quality assured tenderness'. Future research should continue to advance the field to enable innovations in the meat industry.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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