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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.

Key words

genetics, proteomics, oxidation, molecular, cooking, proteases, nitrosative, high pressure processing, connective tissue, collagen

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, Miller, Ha, Wheeler, Dunshea, Li, Vaskoska, & Purslow, 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 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 and 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 (Küchenmeister, Kuhn, and Ender, 2000; Küchenmeister, Kuhn, and Strabenow, 2002; Bing et al., 2016). 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). 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 (Koohmaraie, 1992; Geesink et al., 2006; Camou et al., 2007) . 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 et al., 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

167 favored by postmortem muscle cell with ultimate pH of 5.3-5.7. Zhang, Li, Yu, Han, & Ma
168 (2019) found that cathepsin B and D released from destabilized lysosomal membrane in
169 postmortem bovine longissimus activated the pro-apoptotic proteins Bid and Bax in the
170 mitochondria. The mitochondrial membrane permeability was triggered by activated Bid and
171 Bax and further induced caspase-9 and caspase-3 activation, leading to apoptosis and
172 contributing to meat tenderness.

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

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

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

200 *2.4 Apoptosis and heat shock proteins (HSPs)*

201 Apoptosis in the postmortem cell is generally acknowledged to occur, based on the occurrence
202 of typical characteristics including cell shrinkage, phosphatidylserine externalization and
203 mitochondria alteration (Becila et al., 2017; Ouali et al., 2013). One of the representative
204 pathways to induce apoptosis is the release of cytochrome C from mitochondria, promoted by
205 the calcium-activated Bax in turn activating the caspases (Wang et al., 2018). The most
206 profound effect of apoptosis on the muscle cell is the mediation of proteolysis executed by
207 caspases (Kemp & Parr, 2012). Regulation of caspases activity has been shown to affect the
208 degradation of myofibrils (Chen et al., 2011; Huang et al., 2014). Caspase-3 activity was
209 reported to be negatively correlated with WBSF (r = –0.49 at 24 h of postmortem aging; r = –
210 0.61 at 48 h of postmortem aging) in bull *longissimus*, and the authors speculated that caspase-3
211 was associated with advanced proteolysis (Cao et al., 2013; Zhang, Wang, et al., 2013). A
212 putative mechanism for the participation of caspases in proteolysis is the interaction with the
213 calpain system, in particular the calpain endogenous inhibitor calpastatin, which is a substrate of
214 caspases (Kemp & Parr, 2012). The interaction between caspases and calpain system seems to
215 be multifaceted and complex in postmortem muscle, hence warranting further research.

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

231 *2.5 Exploration of protein biomarkers for meat tenderness*

232 Research has been carried out to identify potential protein biomarkers to predict meat tenderness
233 and reviews on the topic have been conducted (Ouali et al., 2013; Picard & Gagaoua, 2020).
234 Guillemin, Bonnet, Jurie, and Picard (2011b) conducted a functional interactome analysis of 24
235 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 consistent across muscles were 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, Purslow, Gagaoua and Warner (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, Liu, Ben & Wang, 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, Lonergan, & Huff-Lonergan, 2008). Antioxidant enzymes including superoxide

dismutase, catalase, glutathione dismutase, protein DJ-1 and peroxiredoxins are guardians against ROS to balance 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 et al., 2011a, b) 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 et al., 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, Weitzber, & 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. (2018a). 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 et al., 2018b). 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 (Figure 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 et al., 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., 2019a). 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 accompanying with 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 ante- mortem and post- mortem factors and in this section we review the pre-slaughter factors, with a focus on the animal. Figure 2 illustrates the interactions between the ante- mortem 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, Dikerman, & Crouse, 1982; Wheeler et al., 2001a, 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, 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 (Koch et al., 1982; Peacock, Koger, & Hodges,

1982; Crouse, Cundiff, Koch, Koohmaraie, & Seideman, 1989; Johnson, Huffman, Williams, & Hargrove, 1990; Wheeler et al., 1990a, b, 1996, 2001) due to less calpastatin inactivation and thus increased calpastatin levels at later postmortem times (Wheeler et al., 1990a; Whipple et al., 1990; Pringle, Williams, Lamb, Johnson, & West, 1997), resulting in less proteolytic degradation and slower improvements in tenderness with aging (Whipple et al., 1990; Wheeler et al., 1990a, b; O'Connor, Tatum, Wulf, Green, & Smith, 1997). 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 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 (Crouse et al., 1989; Johnson et al., 1990; O'Connor et al., 1997; Bidner, Wyatt, Humes, Franke, & Blouin, 2002; Wheeler, Cundiff, Shackelford, & Koomaraie, 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 et al., 2008a, b). However, there have been three tropically-adapted *Bos taurus* breeds (Tuli, Bonsmara, and Romosinuano) identified that do not have reduced tenderness (Wheeler et al., 2001, 2005). 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, Cundiff, Koch, & Crouse, 1996). Differences in meat tenderness among lamb breeds also have been described (Hopkins & Fogarty, 1998; Warner, Greenwood, Pethick, & Ferguson, 2010). Shackelford, Leymaster, Wheeler, & 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.

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 (Cockett et al., 1994, 2005; Koohmaraie, Shackelford, Wheeler, Lonergan, & Doumit, 1995; Carpenter, Rice, Cockett, & 1996; Freking, Keele, Nielsen, & Leymaster, 1998). It is associated with increased calpastatin activity and hence decreased protein degradation post-mortem by μ-calpain (Lorenzen et al., 2000; Freking et al.,1998; Koohmaraie et al., 1995).

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, 2001, 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 et al., 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 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-

417 Perry et al., 2009; Nonneman et al., 2011, 2013; Rohrer, Thallman, Shackelford, Wheeler, &
418 Koohmaraie, 2005). In the last 15 years or so, the association of multiple SNPs in both calpain
419 and calpastatin genes in a wide variety of breeds of cattle, goats, sheep and pigs with variations
420 in meat tenderness and other aspects of meat quality has been a very active area of research.
421 Leal-Gutiérrez, Elzo, Johnson, Hamblen, & Mateescu (2019) reviewed the effects of 3 CAPN
422 SNPs (Capn4751, Capn316, Capn530) and three CAST SNPs (UoG-Cast, Cast2959, Cast2832)
423 in some detail. Therefore, it appears that markers for both of these genes (CAPN1, CAST) can
424 be used simultaneously in breeding programs to improve tenderness. Some of these research
425 population-developed markers (CAPN1 316 and 4751; CAST-T1) have been validated on
426 independent *longissimus* samples from USA commercial meat processors (Shackelford,
427 personal communication) and their value in offsetting some of the negative impact of aggressive
428 implant strategies on *longissimus* tenderness has been demonstrated (King et al., 2012).
429 Additional SNPs have been identified with significant association with pork tenderness (Ji et al.,
430 2018), but need to be validated for commercial pigs. Genetic markers for tenderness are now
431 available in commercial SNP chip assays in a variety of formats for high density genotyping
432 (50K and 770K for beef, 60K for pork, and 50K for lamb) using HD bead-chip assays. This
433 technology has allowed development of genomically enhanced expected progeny differences
434 (EPDs). However, further improvements in the accuracy of reference genomes and continued
435 improvement in next generation sequencing technology at progressively lower cost have made
436 genotyping by sequence a feasible option with some advantages. These advancements will lead
437 to improved accuracy of whole genome sequence imputation that increases the ability to
438 identify causal genetic variants and improve genomic selection for traditional and novel traits
439 like tenderness (Butty, 2019).
440
441 *3.4 Growth promotants*
442 Improving the rate and efficiency of growth in market animals, and carcass leanness, are
443 important economic considerations for livestock producers. Therefore, the administration of
444 agents that partition nutrients towards muscle deposition is a common practice in many
445 countries. The most common metabolic modifiers used in meat production include anabolic
446 steroids and β-adrenergic agonists (BAA). At least 90% of steers and heifers fed in the USA
447 receive anabolic steroid implants (Dikeman, 2007), which can be classified according to their
448 active ingredient (estrogens, progestins, androgens, or combination). Of these, the combination
449 implants at multiple timepoints are considered to be more "aggressive", because they generally
450 provide greater increases in growth rate and feed efficiency (Dikeman, 2007). A wide variety of
451 products are available commercially and the impact on meat tenderness depends on the kind and
452 number of implants. For example, a meta-analysis was used to show that the application of
453 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). Swine, poultry, and a small percentage of USA cattle production as well as many other countries (particularly in Europe), do not use anabolic steroid implants.

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 et al., 1991, 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 et al. (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 (Miller, Cross, Crouse, & Jenkin, 1987; Boleman, Miller, Buyck, Cross, & Savell, 1996). 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 (Seideman, Cross, Oltjen, & Schanbacher, 1982; Sales et al., 2014; Goetsch et al., 2011; Nagamine et al., 2017). 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. Dikeman 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; Warner, Dunshea, & Channon, 2018; Seideman, et al., 1982). The magnitude of these differences in tenderness are similar to those observed with similar 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., 2020). 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 a 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 feed lots 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 (Bowling, Smith, Carpenter, Dutson, & Oliver, 1977; Aberle et al., 1981; Warner, Dunshea, Gutzke, Lau, & Kearney, 2014). Concentrate-fed animals also generally produce steaks that are more tender than steaks from forage-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 (Purchas, Sobrinho, Garrick, & Lowe, 2002; Aberle et al., 1981), 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

563 the shear force of meat from heifers and steers (Duffy et al., 2017; Montgomery et al., 2004),
564 but not cull cow (Sell, Mikel, Xiong, & Behrends, 2004), lamb (Boleman, Mckenna, Ramsey,
565 Peel, & Savell, 2004), pork (Duffy et al., 2018; Wiegand et al., 2002) or *Bos indicus* cattle
566 (Lawrence et al., 2006). It is worth noting that reports on the effectiveness of vitamin D3 on
567 shear force and sensory tenderness vary in these studies, likely due to differences in level and
568 type of supplementation, species and breed, carcass characteristics, muscle and aging time.
569 Thus, vitamin D3 and its metabolite supplementation for the purpose of improved tenderization
570 requires further research.

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

592

593 ## 4. Advances in postmortem factors influencing tenderization, including
594 cooking

595

596 Postmortem changes in muscle involve complex biological processes which are influenced by
597 intrinsic and extrinsic factors. An understanding of postmortem physical and biochemical

598 changes that impact meat tenderness, including during the cooking process, is therefore crucial.
599 There are a wide variety of postmortem treatments and conditions that affect the tenderness of
600 the final product, and a comprehensive review of all of these is not possible here. In this section,
601 we focus on those which have greatest relevance to two of the molecular mechanisms discussed
602 above, namely oxidation and post-mortem proteolysis, as well as those that have direct effects
603 on the integrity of the structure of muscle tissue. Freezing and thawing of meat disrupts
604 structures and may release calcium ions and affect proteolysis. Several post-mortem treatments
605 of raw meat, including pulse electric field and ultrasonic treatments, have a primary effect of
606 enhancing endogenous proteolysis, whereas hydrostatic and dynamic high-pressure treatments
607 appear to primarily disrupt meat microstructure without enhancing proteolysis. Treatment of
608 meat by exogenous (mainly plant-based) enzymes is another postmortem treatment with an
609 obvious focus on tenderization by proteolysis. The final step of the production-to consumption
610 chain is the cooking of meat, which brings about its own structural effects, and in its initial
611 stages may also promote proteolysis. Figure 2 demonstrates the interactions between some of
612 the post- mortem factors, metabolic and molecular processes and enzymatic systems involved in
613 meat tenderization.
614

615 *4.1 Oxidation*

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

629 *4.1.1 Oxidation during aging and storage*

630 Postmortem oxidation of meat proteins can occur within 24 hours following slaughtering, if
631 conditions are inducive to oxidation (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004a).
632 Xue, Huang, Huang, and Zhou (2012) showed that in-vitro exposure of beef myofibrillar
633 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 also been shown to decrease activity of Calpain-1, and inactivation of 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).

Various approaches have been trialed with varying success to reduce the negative impact of high oxygen modified atmosphere packaging on meat tenderness. These include lowering the oxygen content (Bao & Ertbjerg, 2015; Resconi et al., 2012; Spanos, Torngren,

669 Christensen, & Baron, 2016), injection of calcium lactate/phosphate (Cruzen et al., 2015),
670 modification of the gas content and headspace (Murphy, O'Grady, & Kerry, 2013; Spanos et al.,
671 2016), use of carbon monoxide and sodium nitrite (Djenane and Roncalés, 2018; Roberts et al.,
672 2017), feeding diets high in anti-oxidants (Ripoll, Joy, & Munoz, 2011), and development of
673 active and smart packaging materials (Arvanitoyannis & Stratakos, 2012). While studies on
674 these packaging methods report varying levels of success in suppressing oxidation, their
675 adoption in industry will depend on further research in cost-benefit analysis, adaptability to the
676 current supply chain, and food regulations. It is worth noting that oxidation-induced chemical
677 modifications of proteins differ across different meat types and cuts. For example, desmin
678 degradation was reduced as a result of hiOxMAP for beef *longissimus* (Fu et al., 2015) but not
679 for pork *longissimus* (Bao & Ertbjerg, 2015). Similarly, a study on packaging of chicken breast
680 (*pectoralis profundus*) and thigh (*peroneus longus*) showed that a similar increase in oxidation
681 measured by thiol loss and protein cross-linking in both muscles due to hiOxMAP did not result
682 in the same reduction in sensory tenderness score for the two muscles (Jongberg et al., 2014).
683 Thus, optimization of MAP packaging for meat retail display will need to be species- and
684 muscle-specific. While further developments in packaging technologies are on-going, extensive
685 evidence has shown that vacuum packaging and vacuum skin packaging are ready-to-adopt
686 alternatives to MAP which can ensure optimal tenderization and eliminate oxidation-induced
687 toughening of meat. These low/no oxygen packaging systems are reported to result in more
688 degradation of troponin-T and desmin, less myosin cross-linking, reduced WBSF, and increased
689 consumer sensory acceptability (Holman et al., 2018).

690 *4.1.3 Oxidation in other meat processing methods*

691 Other postmortem methods for processing of meat, such as freezing/thawing, irradiation,
692 pressure treatment and cooking, also influence the oxidation status of meat proteins and hence
693 meat tenderness (Bao & Ertbjerg, 2018; Guyon, Meynier, & de Lamballerie, 2016; Leygonie,
694 Britz, & Hoffman, 2012; Yu, Morton, Clerens, & Dyer, 2017). Specific settings of the
695 parameters in these processes, e.g. rate and number of freezing/thawing cycles; magnitude of
696 pressure; and cooking temperature, have been shown to result in varying levels of protein
697 oxidation. For instance, a significant increase in protein oxidation, measured as carbonyl
698 content, in pork *longissimus,* was observed at 100 °C and 140 °C compared to pork cooked at 70
699 °C (Bax et al., 2013). Oxidation of meat proteins due to these processes not only affect
700 tenderization of fresh meat but also protein functionality during subsequent processing, e.g.
701 processed meat products (Buckow, Sikes, & Tume, 2013; Utrera & Estevez, 2012). Thus,
702 further research in innovative technologies aiming at mitigating the impact of protein oxidation
703 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 of many meat cuts 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, 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 et al. (2014; 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 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 et al., 2018a, b; Gong, Morton, Bhat, Mason, & Bekhit, 2019), as has zingibain (Naqvi, Thomson, Ha, Campbell, McGill, Friend, & Warner, 2021) due to their 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

739 Calpain-2 and very tender meat at 5 hrs postmortem (Han et al., 2009). Less known plant
740 proteases with potential tenderizing effects include extracts of asparagus (Ha, Bekhit, Carne, &
741 Hopkins, 2013; Yonezawa, Kaneda, & Uchikoba, 1998), *Sarcodon aspratus* (mushroom
742 species; Kim, Lee, & Ryu, 2015), crude mango peel (Dhital & Vangnai, 2019) and *Spondias*
743 *cytherea* roots (plum tree species; Ahmad et al., 2019).

744 Plant proteases have been extensively studied, however according to the best knowledge
745 of the authors, these enzymes are not used in meat products commercially. This is likely due to
746 various issues related to formulation, stability and control of the enzymes post-treatment which
747 are discussed in full detail in Bekhit et al. (2017) and need to be addressed in order for future
748 uptake in the meat industry. Many of these issues are related to the fact that commercial
749 protease preparations contain multiple complex proteins and proteases (Ha, Bekhit, Carne, &
750 Hopkins, 2012, 2013) that exhibit variable hydrolytic activities and can lead to over-
751 tenderization and production of 'off' sensory notes, as mentioned above for papain and
752 bromelain. The variability in purity of the proteases in commercial preparations would result in
753 different tenderization outcomes. Another issue with plant protease extracts is that they can
754 carry some flavor of their own that may be acceptable to some and unacceptable to others, such
755 as occurs with ginger extracts containing zingibain.

756 *4.2.2 Proteases from bacteria and fungi*

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

765 Microbial-derived proteases are commercially available from non-pathogenic sources
766 and many have been approved by regulatory authorities. Many of these microbial-derived
767 proteases have higher specificity and are easier to control than plant proteases (Ashie, Sorensen,
768 & Nielsen, 2002). However, many consumers are uncomfortable with the concept of bacterial or
769 fungal additives to food products. A good strategy to overcome this negative perception is to
770 target probiotic bacteria as sources of effective proteases, which could be used for the dual
771 function of gut health, and meat tenderization (Chanalia, Gandhi, Attri, & Dhanda, 2018).
772

773 *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, & 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, 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 on aging occurred more rapidly in frozen samples than non-frozen samples. Crouse & 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, Enfalt, Johansson, and Lundström (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 et al. (2018), examining pork loins subjected to different ageing/freezing/thawing regimes, reported 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 et al., 2017; Zhan et al., 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 extensive amount of 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 High pressure, ultrasonics and pulsed electric field 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 (Troy, Ojha, Kerry, & Tiwari, 2016; Alarcon-Rojo et al., 2019; Bhat, Morton, Mason, & Bekhit, 2018a, 2019a; 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 ^{o}C at 100-150 MPa, and significant tenderization also occurred under HPP conditions of 35-60 ^{o}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 et al., 2018b; Zhang, Pan, & Wu, 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 et al. (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 et al., 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, & 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, Carrillo-Lopez, Reyes-Villagrana, Huerta-Jiménez, & Garcia-Galicia, 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, & 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, Gao, Ge, Zhou, and Zhang (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 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, c). 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 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 fabricated meat. Processing of larger cuts would require higher voltages to generate sufficient electric field strength, with increased risk of heating.

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

954 stretching methods and usage (Bekhit et al., 2014; Sørheim & Hildrum, 2002; Warner et al.,
955 2017). While tenderstretch has been more widely adopted by selected meat processors
956 compared to other stretching methods, most likely due to its easier adoptability, some of the
957 issues commonly raised by processors include chiller space limitation, boning efficiency, primal
958 shape changes and yield (Condon, 2019). Tenderstretch has been incorporated in Meat
959 Standards Australia grading scheme.

960 In summary, there are a range of post-mortem treatments of meat that impact tenderness
961 either through direct disruption of myofibrillar structure or accelerated proteolysis, or a
962 combination of both. Figure 3 shows an estimation from the meta-analysis of Warner,
963 McDonnell, Bekhit, Claus, Vaskoska, Sikes, Dunshea, & Ha (2017) of the relative benefits of a
964 subset of these techniques, compared to treatments administered to live animals, in terms of
965 changes to cooked meat tenderness.

966

967 *4.5 Changes in tenderness during cooking*
968 Cooking is the final step prior to consumption and has a significant effect on sensory qualities.
969 This section examines the impact of cooking on tenderness, with a focus on changes in protein
970 conformation and degradation. Extensive research has been conducted on heat-induced
971 denaturation of major meat proteins. These changes in the secondary structure can be observed
972 by differential scanning calorimetry (DSC) and spectroscopic methods, such as Raman and from
973 Fourier Transform spectroscopy. DSC thermograms of meat consist of three or more major
974 peaks, also known as transition temperatures, which are usually associated with the denaturation
975 of major proteins and changes in meat. When conducting DSC, care should be taken when
976 interpreting transition temperatures of major meat proteins that overlap and the process of
977 denaturation should be regarded as a continuous process (Vaskoska et al., 2021a). Denaturation
978 of actin and myosin has been associated with tougher meat, and collagen denaturation has been
979 linked to a decrease in firmness (Martens, Stabursvik, & Martens, 1982). The extent of collagen
980 denaturation is dependent on heating temperature and heating rate. Lattore, Velazquez, and
981 Purslow (2018) showed that the temperature, at which collagen denatured (transition
982 temperature), increased with increasing heating rate (Figure 4). About 5 % denaturation of
983 collagen can be achieved through long-time, low-temperature (LTLT) cooking method in beef
984 cooked at 60°C for 24 hours (Latorre, Palacio, Velázquez, & Purslow, 2019; Purslow, 2018).
985 Similarly, increased tenderness in pork can be achieved with LTLT cooking which is related to
986 solubilized collagen and reduced perimysial thickness (Li et al., 2019). Spectroscopic methods
987 have been used to link meat tenderness to specific changes in the secondary conformation of
988 proteins (Beattie, Bell, Borggaard, & Moss, 2008, 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 et al. (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., 2020). However, recent studies have suggested cathepsins remain active during cooking, possibly with increased activity between 53 and 63 °C (Christensen, Ertbjerg, Aaslyng, & Christensen, 2011). Injecting pre-rigor 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, 1982). 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. (2021b) 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 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.

1023 The value of identifying biomarkers for prediction of meat tenderness from proteomic
1024 studies at this stage appears to be mainly in expanding our understanding of the tenderization
1025 process. This is partly because the complex processes associated with tenderisation post-
1026 mortem rely on many factors in the supply chain. For this reason, some have predicted that
1027 single protein biomarkers will not be likely to accurately or reliably predict meat tenderness
1028 (Starkey, Geesink, Collins, Oddy, & Hopkins, 2016) whereas we suggest potential biomarkers
1029 still need extensive validation across species, carcasses and muscles. In addition, the role of
1030 collagen in tenderness has been overlooked in proteomic studies, likely because it is very
1031 challenging to isolate and purify (Warner et al., 2021).

1032 Collagen has not only been overlooked in recent proteomic studies, but also there is a
1033 general lack of research on the contribution of collagen to meat tenderness. This is particularly
1034 in light of the data showing the post-mortem degradation of collagen (Sylvestre et al., 2002), the
1035 possible role of Vitamins C and E in collagen synthesis (Archile-Contreras et al., 2011) and
1036 potential for manipulation of the pools of heat-labile collagen in the animal and post-mortem
1037 (Purslow, 2014; 2018). Hence future research on tenderness should include a focus on the
1038 changes in collagen in the animal, post-mortem during ageing and also during cooking. This
1039 will assist in developing strategies to reduce cooked meat toughness of some animals and
1040 muscles.

1041 Many hypothesis-driven studies have been conducted on effects of genetic, nutritional and
1042 environmental and molecular factors influencing meat tenderization whereas proteomic studies
1043 have focused on generating *post-hoc* hypotheses for the role of proteins in meat quality
1044 (Purslow et al., 20201. These molecular studies have been useful in identifying the important
1045 role of energy metabolism and new insights of apoptosis and proteases other than calpain in
1046 protein breakdown post-mortem. Recent research has highlighted the importance of considering
1047 the interaction between different proteases including between caspases, cathepsins and the
1048 calpain system which seems to be multifaceted and complex in postmortem muscle. Recent data
1049 shows that proteolysis, which is initiated in the meat during ageing, continues during heating
1050 and cooking (Vaskoska et al., 2021b), which challenges some of the traditional thinking that
1051 proteolysis ceases once cooking occurs. Further research on the interaction between the
1052 protease systems in animals, during processing and storage and also during cooking warrants
1053 further research.

1054 The application of processing technologies and enzymes for advanced meat tenderization has
1055 been ongoing. Critically, evidence for substantial tenderization of very tough muscles has had
1056 most success with high hydrostatic pressure processing and also with plant-derived enzymes such
1057 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.

Conflicts of interest

The authors declare that they have no conflict of interest.

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2037

2038 **Tables and Figures**

2039

2040 Table 1. S-nitrosylated proteins and SNO-modified cysteine sites identified from pork
2041 during postmortem aging (adapted from Liu et al, 2018b).

Protein	Accession	Peptide sequence	Cys-site	A0	A3	Std A0	Std A3	P-value
Aldolase C	F1RJ25	KGVVPLAGTDGETTTQGLDGLSER C[1]AQYKKD	135[2]	1.005	1.744	0.046	0.066	0.0058
Alpha-Actinin-1	I3LIK6	R.LHKPPKVQEKCQLEINFNTLQTK L	112	0.618	0.946	0.013	0.043	0.0002
ATP-dependent 6-phosphofructokinase	Q2HYU2	RLPLMECVQVTKD	351	0.844	1.097	0.117	0.069	0.0325
ATP-dependent 6-phosphofructokinase	Q2HYU2	RIFANTPDSGCVLGMR.K	709	0.935	1.297	0.007	0.071	0.0010
Beta-Enolase	Q1KYT0	KFGANAILGVSLAVCKAGAAEKG	119	0.595	0.638	0.107	0.120	0.6703
Beta-Enolase	Q1KYT0	KTGAPCRSER.L	399	1.392	2.174	0.084	0.189	0.0028
Beta-Enolase	Q1KYT0	KVNQIGSVTESIQAC]KL	357	0.968	1.338	0.006	0.061	0.0005
Glucose-6-phosphate isomerase	F1RNU9	KMIPCDFLIPVQTQHPIR.K	404	0.786	1.038	0.036	0.030	0.0008
Glutathione reductase	F1RX66	RKTKCVMKM	432	0.565	0.720	0.012	0.045	0.0047
Glyceraldehyde-3-phosphate dehydrogenase	Q0QES9	KIVSNASCTTNCLAPLAKV	131	0.789	1.563	0.009	0.124	0.0004
Glyceraldehyde-3-phosphate dehydrogenase	Q0QES9	RVPTPNVSVVDLTCRL	222	0.864	1.502	0.077	0.222	0.0093
Heat shock protein HSP 90-alpha	O02705	KKTKFENLCKL	573	0.603	0.793	0.051	0.090	0.0355
L-lactate dehydrogenase A chain	P00339	KNRVIGSGCNLDSARF	163	0.989	1.940	0.057	0.174	0.0008

L-lactate dehydrogenase C chain	Q9TSX5	RVIGSG**C**NLDSARF	163	0.912	1.853	0.016	0.045	<0.0001
Malate dehydrogenase	P11708	KAI**C**DHVR.D	251	0.771	1.141	0.013	0.056	0.0004
Malate dehydrogenase	P11708	KVIVVGNPANTN**C**LTASKS	137	0.913	1.514	0.054	0.006	<0.0001
Phosphoglycerate kinase1	Q7SIB7	KAAIPSIKF**C**LDNGAKS	50	0.926	1.720	0.053	0.181	0.0019
Phosphoglycerate kinase1	Q7SIB7	KIGQATVASGIPAGWMGLD**C**GPESSKKY	316	0.912	1.383	0.003	0.07	0.0003
Phosphoglycerate kinase1	Q7SIB7	KA**C**ADPAAGSVILLENLRF	108	0.677	0.846	0.068	0.088	0.0590
Protein DJ-1	F1RII4	KVTVAGLAGKDPVQ**C**SR.D	46	0.806	1.504	0.030	0.037	0.0024
Sarcoplasmic\endoplasmic reticulum calcium ATPase1	F1RFH9	RANA**C**NSVIRQ	471	0.831	2.221	0.021	0.130	<0.0001
Titin	/	KKTT**C**KLKM	2352	0.652	0.862	0.049	0.010	0.0019
Triosephosphate isomerase	Q29371	KIAVAAQN**C**YKV	67	0.787	1.548	0.042	0.206	0.0033
Triosephosphate isomerase	Q29371	RIIYGGSVTGAT**C**KE	218	0.919	1.267	0.012	0.046	0.0002
Aldolase C	F1RJ25	KGVVPLAGTDGETTTQGLDGLSER **C**[1]AQYKKD	135[2]	1.005	1.744	0.046	0.066	0.0058

[1]The cysteine in red means that was modified by S-nitrosylation.

[2]The amount of specific SNO-sites modification in A0 and A3 samples was relative to that of G100 samples. A0 and A3 represent the samples for aging 0 and 3 d of pork longissimus thoracis muscle, respectively and G100 represents the sample for 100 μM S-nitrosoglutathione (GSNO, a NO donor) incubation with A0 protein sample.

2049 Table 2. Plant, microbial and animal proteases potentially useful in meat tenderization.

2050 Derived from Tantamacharik et al. (2018).

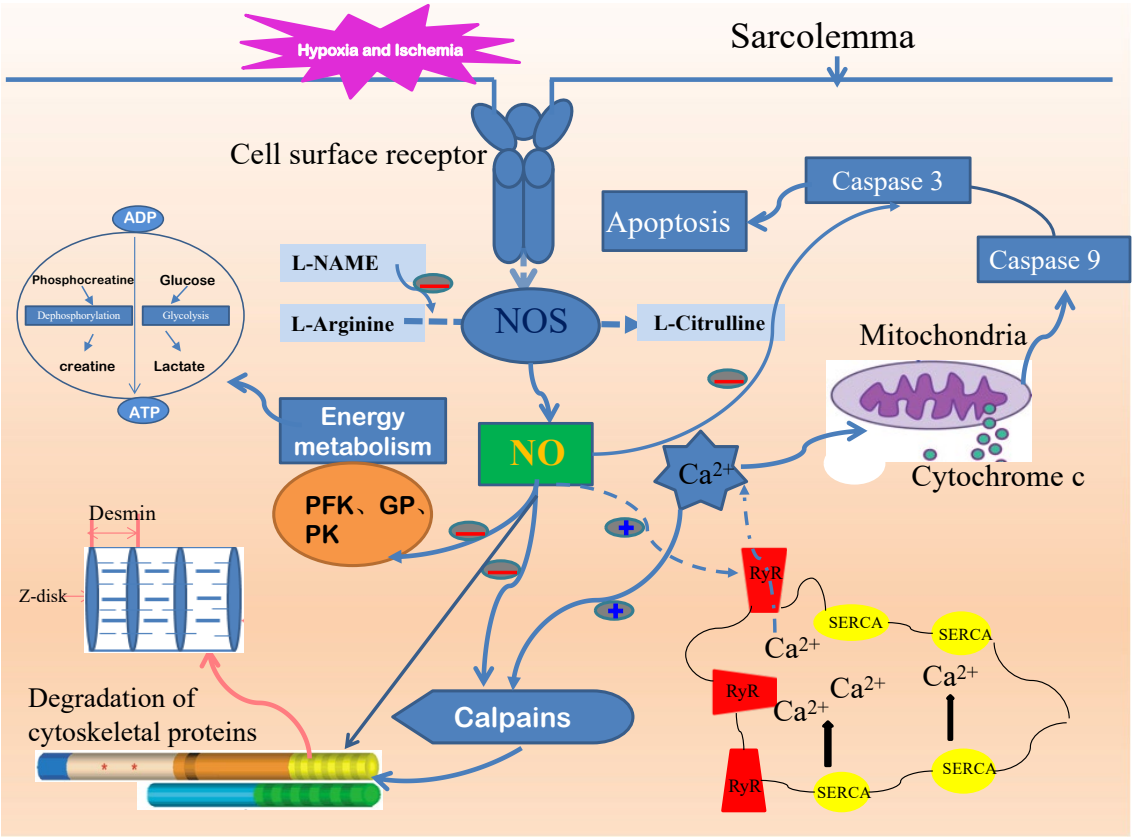
Origin and enzymes	Source
ANIMAL ORIGIN	
Placental protease; Pancreatin; Pepsin; Chymotrypsin, Trypsin, Elstase, Carboxypeptidase	Pancreas and stomach of mammals
BACTERIAL ORIGIN	
Alkaline elastase, alkaline protease, collagenase (Sigma type VII)	*Alkalophilic Bacillus sp. Bacillus polyfermenticus Clostridium 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) Subtilisin-like/serine protease	Kachri *(Cucumis trigonus Roxb); Cucumis sativus L. 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

2051

Figure 1. Proposal pathways of nitric oxide involved 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 ATPas, PFK: phosphofructokinase, GP: glycogen phosphorylase, PK：pyruvate kinase.



2060 Fugure 2. Overview of the interactions between ante- mortem, post- mortem factors, metabolic
2061 and molecular processes, and the affected enzymatic systems relevant for meat tenderization.

2062

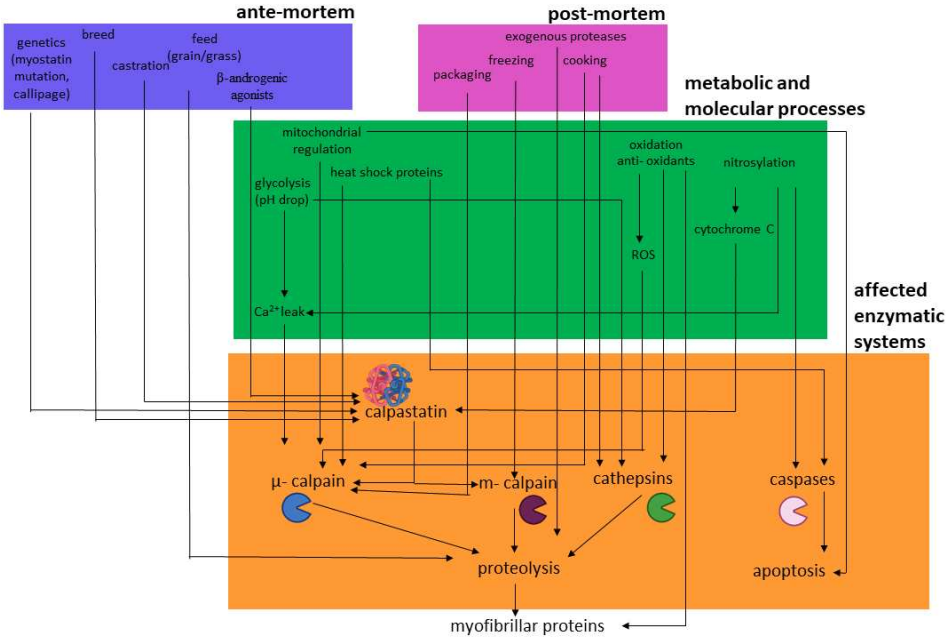


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Figure 3: Results of meta-analyses of Warner et al. (2017) predicting the change in peak shear force (N) in response to various treatments. Positive changes (green bars) are predicted reductions in shear force, whereas negative changes (red bars) are predicted increases. Pre-rigor treatments of Smartstretch™ lengthening, post-rigor pulsed electric field (PEF-post-rigor), electrical shock wave (SW-electrical), ultrasound and to both pre- and post-rigor meat of 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.

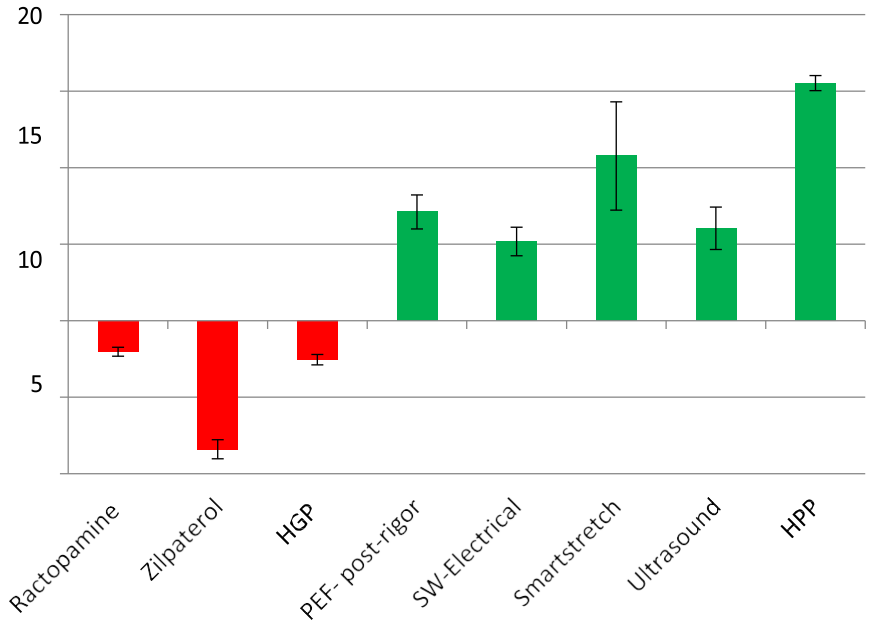

Figure 4. Differential scanning calorimetry thermograms of a) perimysium from *pectoralis profundus* and b) perimysium from *semitendinosus* at variable heating rates (1, 2, 5, 10, 15 and 20 K/min), reproduced from Latorre, Velazquez, and Purslow (2018)