Characterizing intramuscular connective tissue degradation by Matrix Metalloproteinase - 9 in extended aging of beef

by

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Abstract

Tenderness is negatively affected by intramuscular connective tissue (IMCT), which provides a "background toughness" in meat. In order to better utilize “lower quality” beef cuts, which contain a high amount of IMCT, meat scientists need to better understand connective tissue and come up with strategies to mitigate the effect of connective tissue on beef tenderness. A mechanism for increasing IMCT tenderness could be attributed to the activity of matrix metalloproteinases (MMPs), which is known for remodeling connective tissue components in the live animal. Hence, this research aims to characterize structural and property modifications of IMCT during extended aging and how MMPs participate in this process. For conducting the experiments, *Longissimus lumborum* (*LL*), *Gluteus medius* (*GM*), and *Gastrocnemius* (*GT*) were collected from 10 USDA choice carcasses, fabricated and assigned to one of four aging periods: 3, 21, 42, or 63 days (n = 120).

In chapter 2, tenderness of meat and structural modifications of the connective tissue were explored. Warner-Bratzler shear force decreased, and connective tissue texture softened after 21 days of postmortem aging ($P < 0.05$). Transition temperature of collagen decreased ($P < 0.01$) after 42 days of postmortem aging. Trained panelists identified steaks aged for only 3 days were with lower myofibrillar tenderness ($P < 0.05$), more connective tissue amount ($P < 0.05$) and less tender for overall tenderness ($P < 0.01$) compared to steaks from the other aging periods. Collagen content did not change throughout the aging periods ($P > 0.10$). However, collagen structure was altered where relative % of γ chain that decreased after 42 days of postmortem aging ($P < 0.05$), while α1 chain % increased at 63 days ($P < 0.01$). Finally, the *LL* and *GT* had a decrease in the 75 kDa aggrecan fragments from 3 to 21 to 42 days ($P < 0.05$).

In chapter 3, the effect of MMPs were explored in the extended aging utilizing the collagen zymography technique. MMP-9 was shown to be the active collagenase during aging of meat, and the highest activity was detected at 3 days of postmortem aging ($P < 0.05$). Moreover, when testing MMP-9 in refrigerated temperatures at 4 °C, activity was not detected prior to 42 days of storage in the cooler, where the activity was higher for the 63 than 42 days of aging ($P < 0.05$). In order to improve activity, different zinc concentrations were tested in MMP-9, and supplementation with 20 µM of $ZnCl_2$ showed the highest MMP-9 activity when compared to other levels of supplementation ($P < 0.01$). The findings from this research filled a knowledge gap on the IMCT structural modification during postmortem aging in beef. Lastly, we provided concrete proof that MMP-9 activity can be stimulated with low concentration of zinc in combination. Further research still needs to be done to better understand MMP-9 mechanism during postmortem aging of meat. With a better understanding of MMP-9 in the aging process, the beef industry can provide better connective tissue management strategies for lower quality beef cuts.

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List of Abbreviations

APS……Ammonium persulfate

CTSF……...Connective tissue shear force

DMBA.....Dimethylaminobenzaldehyde

DPD.....Deoxypyridinoline

ECM.....Extracellular matrix

GAGs.....Glycosaminoglycans

GM.....Gluteus medius

GT.....Gastrocnemius

HCl.....Hydrochloric acid

LL.....Longissimus lumborum

PVDF.....Polyvinylidene difluoride

PYD.....Pyridinoline

TEMED.....Tetramethylethylenediamine

TIMPs.....Tissue inhibitor of metalloproteinases

WBSF.....Warner-Bratzler shear force

α1.....Alpha 1

α2.....Alpha 2

β.....Beta

γ.....Gamma

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Dedication

I would like to dedicate this thesis to my mom and my dad. Without their endless love and encouragement, I would never have been able to complete my graduate studies. I love you both and I appreciate everything that you have done for me.

Chapter 1 - Connective tissue effect on tenderness and strategies to improve meat quality

1. Introduction

Whole beef carcasses are separated into primals, sub-primals, then retail cuts based on organoleptic attributes, particularly tenderness (Jung et al., 2016). Therefore, tenderness often dictates the values of the cuts, and tougher ones are frequently priced significantly lower than the more tender cuts. On average, tender cuts are preferred for consumers. However, with the current inflation in food prices, including meat, consumers begin to look for cheaper alternatives (Ortez et al., 2021). Tenderness is negatively affected by intramuscular connective tissue (IMCT), which provides a "background toughness" in meat (T. Nishimura, 2010). However, this component which is usually abundant in lower quality cuts, has not been the focus of meat research (Veiseth-Kent et al., 2018). In order to better utilize the “alternative” or “lower quality” beef cuts, meat scientists need to better understand connective tissue and come up with strategies to mitigate the effect of connective tissue on beef tenderness.

Strategies to decrease the toughness of IMCT in meat have been investigated (L. Christensen et al., 2013; S. Li et al., 2019), and a mechanism for increasing IMCT tenderness could be attributed to the activity of matrix metalloproteinases (MMPs) in the post-mortem aged meat (Sylvestre et al., 2002). This review will focus on understanding the impact of the connective tissue on meat tenderness, strategies to improve tenderness, and the possible influence of matrix metalloproteinases on the connective tissue texture.

2. Connective Tissue Structure and composition:

Connective tissue provides structural support for the organs and muscles of the body (Järvinen et al., 2002). Epimysium is the thick sheet of connective tissue surrounding the whole muscle. Each muscle is comprised of several muscle bundles surrounded by the perimysium. In each muscle bundle, there are several muscle fibers surrounded by the endomysium (Figure1; Boland et al., 2018). Although the epimysium is thick and tough, it is typically removed from meat prior to consumption (Listrat et al., 2016). Therefore, it only contributes minimally to meat texture. Approximately 90% of IMCT in meat is perimysium, which plays a significant role in determining the textural differences related to connective tissue (McCormick, 1999). Endomysium is a network of fine fibrils and represent a small amount of connective tissue in the muscle (An et al., 2010). No matter the location of the connective tissue, the connective tissue is generally composed of two main elements: extracellular matrix (ECM) and cells (Theocharis et al., 2016).

2.1. Extracellular Matrix

The ECM is a structural support network made up of diverse proteins, sugars, and other components known to play a part in numerous cellular processes, including cell proliferation, differentiation, and migration (Kular et al., 2014). Moreover, it is composed of fibers embedded in the ground substance, a gel-like component composed of water, extracellular proteins, glycosaminoglycans (GAGs), and proteoglycans (Stecco et al., 2015). The most common fibers in the connective tissue are collagen, elastin, and reticulin, which define the tissue's mechanical properties (Glynn, 1964; Stecco et al., 2015).

2.1.1. Proteoglycans

Proteoglycans are large molecules consisting of a core protein covalently attached to many GAGs, made up of repeating disaccharide units, and one of the sugars in each disaccharide unit is a glycosamine (Stecco et al., 2015). Three major classes of the core protein are chondroitin sulfate, heparan sulfate, and keratan sulfate (Olson & Esko, 2004). Proteoglycans participate in several cell functional properties, such as cell signaling, proliferation, migration, differentiation, apoptosis, and adhesion (Theocharis et al., 2016). Proteoglycans are structurally diverse, with more than 25 types identified in mammals that can be classified based on their location and homology (Olson & Esko, 2004). The aggrecan family shares the chondroitin sulfate as the core protein and includes aggrecan, versican, brevican, and neurocan (Olson & Esko, 2004), with aggrecan being the most well-known member because it is the major proteoglycan present in cartilage, providing resistance to compressive forces (Kiani et al., 2002).

2.1.2. Collagen

Collagen accounts for 30% of all the proteins in mammals and is the major protein in the connective tissue (Patino et al., 2002). The collagen formation starts with the transcription from DNA to messenger RNA inside the fibroblasts, which are the responsible cells for synthesizing most collagen (Mansell & Bailey, 2004). Polypeptide chains are formed and aggregates to form a triple-helix molecule, the procollagen, that is released from the fibroblast (J. Li & Kirsner, 2005). In the extracellular space, they form the tropocollagen, which later forms hydrogen bonds and covalent crosslinks to create collagen fibers (J. Li & Kirsner, 2005).

Collagen fibers receive mechanical and chemical stability from intramolecular and intermolecular cross-links (Sorushanova et al., 2019). Intermolecular crosslinks are first synthesized as divalent cross-links to stabilize collagen in the living animal, and they are

gradually replaced by more heat-stable trivalent cross-links, mainly composed of pyridinolines (Dubost et al., 2013). As the animal matures, collagen synthesis and turnover decrease, while the concentration of non-reducible trivalent cross-links increases, contributing to meat toughness (Purslow et al., 2012; Roy et al., 2021). Collagen structure consists of a triple helix made of three alpha (α) chains composed of polypeptides in the repeating sequence of Glycine–X–Y, where X and Y are often proline and hydroxyproline (Mansell & Bailey, 2004; Patino et al., 2002). If two α chains are linked by an inter- or intra- molecular crosslinks, a dimer is obtained, and it is referred to as the beta (β) component, while when three α chains are crosslinked a trimer is obtained, referred to as gamma (γ) component (Carmichael & Lawrie, 1967). Collagen type 1, the most abundant collagen type in the intramuscular connective tissue, consists of two α1 chains and one α2 (Henriksen & Karsdal, 2019; Light & Champion, 1984). However, more than 29 types of collagen are known and distributed on skins, bones, tendons, vascular systems, or intramuscular connective tissues that vary in sequence, structure, and function (D. Liu et al., 2015).

Collagen content and solubility, collagen network architecture, and especially the density and maturity of collagen cross-links play essential roles in meat texture (X. Li et al., 2021). Some studies have proposed the weakening of the collagen in meat during the aging period. Palka (2003) evaluated the collagen solubility of beef *Semitendinosus* aged for 5 and 12 days and found that aging for 12 days resulted in a two-fold increase in the quantity of soluble collagen in the raw muscle. Wang et al. (2022) evaluated the effects of ultrasound applications (0, 300, and 600 W for 20 min at the frequency of 20 kHz) on beef *Semitendinosus* muscle and found that collagen solubility increased for all frequencies in muscles aged for 8 days. The same group suggested that collagen structure was initially disrupted by the ultrasound treatment and was

further amplified by the aging time. However, there is still much controversy regarding the weakening of the collagen structure during aging as some studies were not able to find any modifications in collagen structure during aging (Kannan et al., 2006; Silva et al., 1999).

2.1.3. Elastin

As described by its name, elastin provides resilience and elasticity to tissues and organs (Kristensen & Karsdal, 2016). The elastin precursor is tropoelastin, primarily synthesized in the fibroblasts (Kristensen & Karsdal, 2016; Sandberg et al., 1981). When secreted out of the cell, tropoelastin monomers aggregates and forms cross-links, mainly desmosine and isodesmosine, to form mature elastic fibers (K. Wang et al., 2021).

Bendall (1967) measured the elastin content in 18 different choice cuts of meat and found that for majority of the muscles, elastin only corresponds to less than 0.2% of the dry muscle content after fat was removed, representing less than 5% of the total connective tissue (Bendall, 1967; Cross et al., 1973). Therefore, its contribution to meat texture is likely insignificant.

2.1.4. Reticulin

Reticular fibers are made of type III collagen and help support organs such as the liver, spleen, bone marrow and lymphatic organs (Stecco et al., 2015). Even with a similar composition to collagen fibers, it has been shown that reticular and collagen fibers differ in the composition of the matrix in which they are embedded, where reticular fibers contain a more significant amount of associated carbohydrates compared to collagen fibers (Montes et al., 1980).

2.2. Connective Tissue Cells

The most common cells in the connective tissue are the fibroblasts, but connective tissue may also contain macrophages, adipocytes, chondroblasts, and osteoblasts (Stecco et al., 2015). Fibroblasts are involved in tissue remodeling and wound healing processes, they secrete matrix

degradative enzymes, MMPs, and are also responsible for the maintenance of the ECM components, such as collagen, proteoglycans, growth factors, and cytokines (J. H. C. Wang et al., 2007). Macrophages are cells with an essential role in the immune system with decisive functions in homeostasis and defense (Martinez et al., 2008). Adipocytes form a specialized connective tissue when they become the predominant cell, the adipose tissue, which has the primary function of storing energy as fat (Stecco et al., 2015). Chondroblasts and osteoblasts are specialized cells that synthesize cartilage and bone, respectively (Bhagavan & Chung-Eun, 2015).

3. Contribution of connective tissue to meat tenderness and potential ways to mitigate the impact of connective tissue

Tenderness is not equal in different meat cuts (Belew et al., 2003), and this is due to muscles having different functions and compositions (Sullivan & Calkins, 2011). For example, *Semimembranosus*, a locomotive muscle, has been reported to be tougher than *Longissimus thoracis*, a postural muscle (Gajaweera et al., 2020; Rhee et al., 2004; Torrescano et al., 2003). Or, *Biceps femoris*, a locomotive muscle, was found to be tougher than *Psoas major*, a postural muscle (Carmack et al., 1995; Rhee et al., 2004; Sullivan & Calkins, 2011). The variations in tenderness among these meat cuts are mainly influenced by myofibrillar and connective tissue (Girard et al., 2012). The sarcomere contraction forming actomyosin bonds in the post-rigor provides a myofibrillar stiffness (Huff Lonergan et al., 2010), while the intramuscular connective tissue provides a background toughness in raw meat (T. Nishimura, 2010). Most consumers prefer tender cuts; however, tougher cuts can go through processes to increase their palatability

and values. Therefore, improving meat tenderness is the key in providing a better eating perception, where different approaches can be used to tenderize the meat.

3.1. Cooking Techniques

Thermal treatment is an important step that results in denaturation and dissociation of myofibrillar proteins, shrinkage of muscle fibers, aggregation and gel formation of sarcoplasmic proteins and solubilization of collagen, which affects meat texture (Chang et al., 2011).

Sous-vide cooking is a relatively new style of cooking that is increasing in popularity. This cooking technique consists of sealing the meat in a vacuum bag and cooking in a low temperature for a long time, and has shown to significantly improve tenderness (Naqvi et al., 2021). Uttaro et al. (2019) reported a decrease in shear force for the muscles *Supraspinatus* and *Rectus femoris* in beef cooked in sous-vide conditions for 4 hours immersed in water at 59 °C when compared to steaks cooked for 22 minutes at 70 °C. Collagen denaturation occurs between 52 and 63° C and leads to conformational changes of collagen, such as the breakdown of hydrogen bonds, shrinkage of collagen and collagen solubilization that leads to the formation of gelatin (Tornberg, 2005). However, in the presence of more heat-stable mature collagen-crosslinks, it takes longer to solubilize collagen (Tornberg, 2005). Sous-vide cooking is associated with the reduced shrinkage of fibers generated during cooking and also the increase of collagen solubility, thus, improving meat tenderness (Naqvi et al., 2021; Park et al., 2020).

3.2. Acid Marination

Marination in acidic solutions have been traditionally used in culinary technique as a way to improve tenderness of meat (Berge et al., 2001; Lewis & Purslow, 1991), particularly on connective tissue softening. Wang and Tang (2018) used *Semitendinosus* muscle marinated in 0.5% of malic acid for 3, 6, 12 and 24 hours and showed that shear force decreased after 6 hours

of acid marination. Chang et al. (2010) utilized the *Semitendinosus* to compare the effectiveness of marinade with organic acids for 24 hours and found that the mechanical strength of the connective tissue without marination was higher than those treated with 1.5% or lactic acid, 1.5% of acetic acid or 1.5% of citric acid. The decrease in the mechanical strength of collagen possibly occurs from the solubilization of collagen by the hydrolysis of peptide bonds and the breakage of collagen cross-links, which weakens the structure and allows it to be more readily soluble (Burke & Monahan, 2003; Chang et al., 2010).

3.3. Blade Tenderization

Blade tenderization is a popular mechanical process in Europe and North America that enhances consumer acceptability, which consists of a set of needles or blades that cut through muscle fibers and connective tissue (Jadeja, 2016). It significantly improves tenderness of less tender cuts of meat (Obuz et al., 2014). In normal and wooden breast chicken, blade tenderization were shown to decrease shear force values when compared to breasts that were not blade tenderized (Tasoniero et al., 2016). In *LL* steaks from cull cows, blade tenderization was also shown to decrease shear force values (Obuz et al., 2014).

3.4. Post-mortem aging

Postmortem aging of meat is a common practice in the meat industry that aims to improve the palatability of meat. Meat tenderness improvement is achieved (Marino et al., 2013; Matney et al., 2021; Nishimura et al., 2008) as a consequence of endogenous enzymatic protein degradation of myofibrillar components, mainly due to calpains (Lomiwes et al., 2014). Colle et al. (2015) found an increased in tenderness for *LL* of beef when aged for 14 days compared to unaged samples, and further improvement in *Longissimus* tenderness is achieved after 63 days of postmortem aging. In addition to the myofibrillar protein degradation, weakening of the

connective tissue during the aging period has also been suggested (Nishimura et al., 2008; Takahashi, 1996). Lewis et al. (1991) showed a decrease in the breaking strength of the perimysium connective tissue in raw meat of bovine *Semitendinosus* aged for 14 days. Also, Liu et al. (1995) demonstrated the weakening of IMCT on chicken *Semitendinosus* muscle aged for 12 hours using a scanning electron microscope. In pork, IMCT of *Semitendinosus* muscle was shown to weaken after 8 days of aging (Nishimura et al., 2008). Although the degradation of IMCT during aging is debatable, few researchers have suggested MMP as a contributor in collagen degradation during post-mortem aging of meat (Christensen & Purslow, 2016; Pambuka et al., 2007; Sylvestre et al., 2002).

4. MMPs and their potential impacts on the post-mortem meat tenderization process

In physiological conditions, tissue equilibrium exists between synthesis and degradation of connective tissue, provided by mesenchymal cells and MMPs (Herouy, 2001). MMPs are a family of zinc-dependent endopeptidases that have a crucial role on physiological processes, such as tissue remodeling and degradation of ECM proteins (Shimoda, 2019). These enzymes are synthesized in the inactive form as pro-MMPs and must be activated to perform their function. The activation depends on the balance between enzymes and the endogenous tissue inhibitors of metalloproteinases (TIMPs), which are the major physiological inhibitors for MMPs (Christensen & Purslow, 2016). Pro-MMPs can be activated *in vitro* by chemical agents, oxidized glutathione, SDS, reactive oxygens, low pH and heat treatment (Visse & Nagase, 2003). More than 20 MMPs are known in mammals and have similar structural characteristics but different substrate preferences (Christensen & Purslow, 2016). When active, MMPs have

different roles and can serve as collagenases (MMP-1, MMP-8, and MMP-13), stromelysins (MMP-3, MMP-10, and MMP-11), gelatinases (MMP-2 and MMP-9), membrane-type MMPs such as transmembrane (MMP-14, MMP-15, MMP-16, MMP-24), membrane-anchored (MMP-17, MMP-25), and matrilysins (MMP-7 and MMP-26) (Olejarz et al., 2020).

4.1. Collagenases and Gelatinases MMPs

Both collagenase MMPs-1, 8, and 13 and gelatinase MMPs – 2 and 9 are known to utilize collagen, gelatin, and ECM molecules laminin and aggrecan core proteins as substrates (Murphy & Nagase, 2009; Visse & Nagase, 2003). Xia et al. (2013) found that the addition of MMP-1 in intact and tightly packed young skin dermis resulted in fragmentation and alterations in the structure and organization of collagen fibrils similar to what is seen in aged skin dermis. Hästbacka et al. (2007) collected peritoneal fluid and blood serum from critically ill patients with secondary peritonitis and compared to blood serum of healthy patients. They found that serum levels of MMP-8 were higher in the ill patients than in the controls. This finding suggested collagenolytic activity from bacterial MMP-8 is responsible for the damage of peritoneum (consisting of collagen I, collagen IV, fibronectin, and laminin) in these patients. Finally, Neuhold et al. (2001) used transgenic mice with overexpression of MMP-13 to study the pathogenesis of osteoarthritis. In those mice, increased MMP-13 in the chondrocytes was in parallel with an excessive cleavage of type II collagen and an articular cartilage degeneration compared to wild-type mice.

Although MMP-2 and MMP-9 are categorized as gelatinase, they are important for the degradation of the basement membrane in processes of angiogenesis, and cancer invasion and metastasis (Seabra et al., 2014). Moreover, MMP-9 plays a major role in the degradation of ECM in physiology and pathophysiology processes that involve tissue remodeling (Yabluchanskiy et

al., 2013). Zeng et al. (1999) studied the breakdown of the basement membrane type IV collagen in colorectal cancer by MMP-2 and MMP-9 as an essential step for tumor invasion and metastasis and found that type IV collagen degradation correlates with a local increase in MMP-9 expression.

4.2. MMPs in meat

It has been discussed that MMPs may influence meat quality and play a role in muscle development, fibrogenesis and adipogenesis (Christensen & Purslow, 2016). Kubota et al. (2003) identified MMP-9 as a potential enzyme involved in the disintegration of the intramuscular connective tissue that induces the post-mortem tenderization of fish muscle during chilled storage. Sylvestre et al. (2002) reported a presence of an active MMP-2 in lamb *Longissimus dorsi* and *Semimembranosus* at 21 days of aging, suggesting its role on collagen degradation during the postmortem aging meat. Likewise, Veiseth-Kent et al. (2018) found an increase in MMP-2 activity at 13 days postmortem for beef *Biceps femoris*, *Infraspinatus*, *Longissimus lumborum* and *Psoas major* and also a degradation of the aggrecan, a crucial component of proteoglycans in the postmortem period. However, more studies need to be done to stablish the importance of this enzyme in the post-mortem period.

5. Conclusion

Meat tenderness can be negatively impacted by connective tissue abundance. However, strategies to weaken the connective tissue can be achieved by cooking methods, acid marination, blade tenderization and post-mortem aging. It is highly possible that connective tissue components may be degraded by MMPs during postmortem aging. However, few research has investigated the role of MMPs in meat quality, and there is still a gap in knowledge on which characteristics MMPs could be affecting and which class of MMPs could be causing the effects.

Hence, this research aims to identify which components of the IMCT are being degraded during the postmortem period and how MMPs participate in this process.

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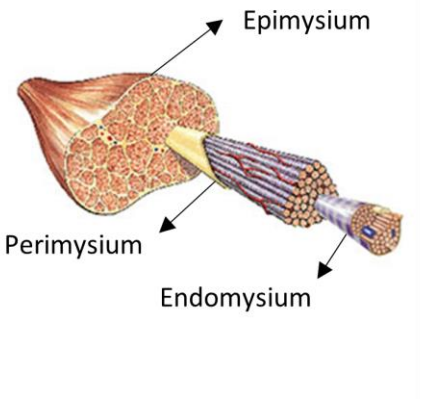

Figure 1.1. Muscle Structure. Adapted from Salvage et al. (2020).

Chapter 2 - Structural changes in the intramuscular connective tissue components during extended aging may improve beef tenderness

Abstract

The aim of this study was to characterize structural and property modifications of Intramuscular connective tissue (IMCT) during extended aging. *Longissimus lumborum* (*LL*), *Gluteus medius* (*GM*), and *Gastrocnemius* (*GT*) were collected from 10 USDA choice carcasses, fabricated and assigned to one of four aging periods: 3, 21, 42, or 63 days (n = 120). As expected, tenderness improved, and connective tissue texture softened after 21 days of postmortem aging ($P < 0.05$). In addition, transition temperature of collagen decreased ($P < 0.01$) after 42 days of postmortem aging. It is interesting to note the collagen structure was also altered where relative % of γ chain decreased after 42 days of postmortem aging ($P < 0.05$), and the α1 chain % increased at 63 days ($P < 0.01$). Finally, The *LL* and *GT* had a decrease in the 75 kDa aggrecan fragments from 3 to 21 to 42 days ($P < 0.05$). This study provided evidence that IMCT softens during postmortem aging due to the modifications of IMCT components.

Keywords: Trained Panel, Collagen, Aggrecan, Collagen Crosslinks, Differential Scanning Calorimeter

1. Introduction

It is well established that muscles are not created equal throughout a beef carcass (Sullivan & Calkins, 2011). Some muscles are inherently tougher than the others due to various attributes such as sarcomere length, myofibrillar protein integrity and connective tissue amount (Koohmaraie, 1994). Intramuscular connective tissue (IMCT) contributes to tensile stiffness of muscle fibers and provides the "background toughness" in beef (Nishimura, 2010). This is due to IMCT serving as the scaffold for muscle cell growth and development, and they also organize and support the neurons and capillaries for each muscle cell (Purslow, 2020). The IMCT amount, composition and structure vary greatly among muscles due to their inherent differences in function. And, "tougher" or "lower quality" cuts are usually associated with higher amounts of connective tissue (Torrescano et al., 2003).

Research has demonstrated that postmortem aging can improve beef tenderness resulting from the degradation of myofibrillar proteins mainly by the activity of calpains (Bhat et al., 2018; Carlson et al., 2017; Kim et al., 2010). It has been suspected that IMCT may also degrade during postmortem aging because in live animals there is a degradation and turnover of the extracellular matrix component of the connective tissue (Christensen & Purslow, 2016; Purslow et al., 2012). However, studies investigating the tenderization of IMCT during the aging period have shown contradictory results (Dubost et al., 2013; Listrat et al., 2020; Nishimura et al., 1998). Pierson and Fox (1976) have failed to find differences in collagen solubility during postmortem aging. On the other hand, Nishimura et al. (1997) reported that the mechanical strength of IMCT decreased during postmortem aging of beef. Finally, Takahashi (1996) reviewed several studies and concluded that the tenderization of the meat is not only from the

degradation of myofibrillar proteins, but also the result from the weakening of endomysium and perimysium.

Tenderness of beef cuts have been repeatedly cited as the most important determinant of consumer purchasing decision (Miller et al., 2001; Platter et al., 2005), and consumers are usually less willing to experiment with beef cuts other than the traditional middle meats due to their abundance in connective tissue. More strategies to manage tougher or lower quality beef cuts could be developed with a better understanding of the mechanisms that lead to the softening of IMCT during postmortem storage. Nevertheless, there is a considerable lack of knowledge regarding the mechanism that softens the connective tissue in meat. Therefore, this study aimed to characterize structural and property modifications of IMCT during extended aging up to 63 days postmortem of three different beef muscles.

2. Materials and Methods

2.1. Sample collection and fabrication

Subprimals from ten USDA high choice carcasses were purchased from a beef processing facility in Nebraska. Both sides of boneless beef striploin (IMPS 180), boneless top sirloin butt (IMPS 184), and heel (IMPS 171F) were collected at 2 days postmortem from each carcass. The subprimals were vacuum packaged and transported to Kansas State University meat laboratory to be fabricated in the next day at three days postmortem. Each striploin, top sirloin butt and heel were first fabricated to remove the accessory muscles not included in the study, leaving only the *Longissimus lumborum* (*LL*), *Gluteus medius* (*GM*), and *Gastrocnemius* (*GT*), respectively. Four 2.54 cm steaks and four 1.27 cm steaks were fabricated from the muscles from the left side of the carcasses, and four 2.54 cm steaks were fabricated from the muscles from the right side of the

carcasses. The muscles *LL* and *GM* were cut from anterior to posterior, and *GT* was cut from dorsal to ventral. Two 2.54 cm steaks (one on the left, and one on the right side) and one 1.27 steak from each subprimal were assigned to be aged at 2 ± 2°C in one of the four designated aging periods: 3, 21, 42 or 63 days. The 1.27cm steaks were designated for laboratory analysis, while the 2.54 cm steaks from the left side were designated for shear force analysis, and the 2.54 cm steaks from the right side were designated for trained panels. The shear force and trained panel samples were frozen at -20°C following their designated aging time. The laboratory analysis steaks were cut into cubes, frozen in liquid nitrogen, pulverized with commercial blenders (model 51BL32, Waring Commercial, Torring, CT, USA), and stored at -80 °C until further analysis.

2.2. Warner-Bratzler shear force (WBSF) and connective tissue shear force (CTSF)

The steaks were prepared following the AMSA Research Guidelines for Cookery and Evaluation (American Meat Science Association, 2016). Steaks were thawed at 2°± 2°C for 24 h prior to cooking. Each steak was grilled on a Cuisine Art Griddle Deluxe Clamshell (Cuisine Art, Stamford, CT) to an internal temperature of 71° C measured by a thermometer inserted into the geometric center of each steak (Thermapen MK4; Thermoworks, American Fork, UT). Cooked steaks were allowed to be cooled down at 2 ± 2°C for 24 hours. Six 1.27 cm diameter cores were taken parallel to the muscle fiber direction from each sample and sheared perpendicular to the muscle fiber orientation using a V-shaped WBSF blade coupled to an Instron Universal Testing System (Model5569; Instron Corporation, Norwood, MA) set at a speed of 250 mm/min and a load cell of 100 kg. On the shear diagram collected from the Bluehill Universal software (Instron Corporation) for each sample, the shear curve was divided into two halves from the starting to the ending point (Figure 2.1). The starting and end points for each shear curve were designated

as when the curve reached 1 kg of force. The peak on the first half of the shear curve was determined as the WBSF, while the CTSF was determined as the peak that occurred in the second half of the shear curve, as proposed and verified by Girard et al. (2012). The mean WBSF and CTSF values of the six cores were calculated for each sample.

2.3. Trained panels

Five training sessions over a 2-week period prior to the beginning of the panels were performed to calibrate the panelists as described in Chun et al. (2020). In each session, selected panelists consisted of meat science faculty, graduate students and staffs from the Kansas State University Department of Animal Sciences and Industry were trained to evaluate myofibrillar tenderness, connective tissue amount and overall tenderness of six different beef muscles (*Psoas major, Semitendinosus, GM,* and *LL* and *GT*) cooked at different temperatures ranging from 60 °C to 77 °C to calibrate the panelists' palate to the leader of the trained panel.

Twenty sensory panels were conducted with six samples per panel served in a random order to eight trained panelists. Each panelist was assigned to a booth containing a tablet, plastic fork, napkins, an expectorant cup, a cup of water, apple slices, and unsalted crackers. Steaks were prepared the same as described in shear force section, but was only allowed to be rested for three minutes in room temperature and cut into 1.3 cm x 1.3 cm x 2.54 cm cubes. One warm up sample consisted of two cubes were first served to the panelists followed by two cubes of each six different samples. Each steak was rated on a continuous line scale using a digital survey (Qualtrics Software, Provo, UT, USA) on electric tablets (Model 5709 HP Stream 7; Hewlett-Packard) for myofibrillar tenderness, connective tissue amount, and overall tenderness. Each scale was anchored at both ends and midpoint with the descriptive terms (0 = extremely tough/

none/ extremely tough; 50= neither tough or tender/ medium/ neither tough or tender; 100 = extremely tender/ extremely abundant/ extremely tender.

2.4. Sample preparation for collagen content and collagen crosslinks

Collagen was hydrolyzed according to the method described by Avery et al. (2009) with modifications. One hundred mg of pulverized meat sample was hydrolyzed in 2 mL of 6N hydrochloric acid (HCl) in a forced air oven (Isotemp 100L Oven FA; Fisher Scientific, Pittsburg, PA) at 115°C for 24 h. Following hydrolysis, samples were cooled to room temperature, and HCl was completed evaporated from the samples using a Vacuum Evaporation System (RapidVap; Labconco Corporation, Kansas City, MO, USA). The dried samples were rehydrated with 2.5 mL of ultrapure water, centrifuged at 2,000 × g for 20 minutes, and the supernatant was stored at -80° C until hydroxyproline and crosslink analysis.

2.5. Collagen content

Collagen concentration was determined by the hydroxyproline assay described by Bergman and Loxley (1963) with modifications. Two hundred fifty µL of the rehydrated samples were first diluted 1:80 using ultrapure water, followed by adding the Chloramine-T Oxidant Reagent (6 mM of chloramine-T hydrate in buffer solution; 140 mM citric acid monohydrate, 37.5 mM sodium hydroxide, 660 mM sodium acetate trihydrate, and 29% 1-proponal, pH 6.0) to each sample and incubated for 20 min at room temperature. Following the incubation, dimethylaminobenzaldehyde (DMBA) color reagent (60mM DMBA, dissolved in 21% perchloric acid, 65% two-proponal and 14% ultrapure water) was added to each sample, incubated in a water bath set at 60°C for 90 min and cooled in a cold-water bath for 3 min. A spectrophotometer equipped with a microplate reader (BioTek Eon; BioTek Instruments Inc., Winooski, VT) was used to determine hydroxyproline concentration at an absorbance of 588 nm.

An appropriate hydroxyproline standard curve and a conversion factor of 7.14 for hydroxyproline to collagen ratio was used to determine collagen content in each sample. Collagen content was displayed as mg/g of wet tissue.

2.6. Mature collagen crosslink measurement

The rehydrated sample were cleaned and analyzed for collagen crosslink analysis following methods described by Viguet-Carrin et al. (2009) with modifications. Four hundred μL of the rehydrated sample was combined with sample buffer (6:1 acetonitrile and acetic acid) and cleaned using a Bond Elut Cellulose cartridge (300 mg, 3 mL capacity, Agilent Technologies, Santa Clara, CA) through a HyperSepTM glass block vacuum manifold (Thermo Scientific, Waltham, MA). Each cellulose cartridge was first equilibrated with a wash buffer (8:1:1, acetonitrile, acetic acid, and ultrapure water), followed by loading the diluted samples and 1% heptafluorobutyric acid (HFBA) to elute the collagen crosslinks.

An ultra-high-pressure liquid chromatography system (Acquity UPLC H-Class; Waters Corporation, Milford, MA) equipped with a degasser, quaternary pump, sample manager and an Acquity UPLC Fluorescence Detector (Waters Corporation) was used to determine mature crosslinks pyridinoline (PYD) and deoxypyridinoline (DPD). A PYD/DPD standard (P/N 4101; Quidel Co., San Deigo, CA) was used to produce a standard curve to determine linearity range of assays and detection limits. The crosslinks were separated using an HSS T3 2.1 x 100 mm, 1.8 μm column (Waters Corporation), at a flow rate of 0.5 mL/min with a column temperature of 60°C. Solvent A (0.2% HFBA in ultrapure water) and solvent B (100% acetonitrile) were used as a gradient solution. After a 10 min isocratic step at 100% solvent A, PYD/DPD were eluted with 85% solvent A and 15 % solvent B for a total run time of 20 min for each sample. The PYD and DPD were measured for fluorescence at an excitation of 297 nm and emission of 395 nm.

Between each sample, 100% solvent B was used to rinse off residues of previous samples followed by 100% solvent A to equilibrate the column. Quantification of the PYD and DPD crosslinks were determined by a standard curve using the peak area ratio (crosslink area/standard area). The concentration of PYD and DPD were multiplied by the dilution factors to get final concentration in ppm. Mature collagen crosslinks density was expressed as mol/mol of collagen, and the chemical masses of 428.44, 412.44 and 300,000 g/mol were used for PYD, DPD and collagen, respectively.

2.7. Perimysial Peak Transitional Temperature Measurement

Perimysial fraction extraction was conducted following the method described by Light and Champion (1984) with modifications. Five hundred mg of pulverized muscle tissue was homogenized in 0.05 M CaCl2 using a bead homogenizer (Bead Blaster 24; Benchmark Scientific, Sayreville, NJ). The homogenate was filtered through a 1 mm standard testing sieve (VWR International, Radnor, PA), and the perimysial fraction collected from the screen and transferred to a microcentrifuge containing 1X PBS for no more than 24 hours at 4°C prior to the peak transitional temperature measurement.

Collagen denaturation temperature was assessed according to methods described by Vierck et al. (2018) with modifications. Prior to the measurement, the non-bound water of the perimysial fraction was removed by blotting the samples with filter paper. Five to 10 mg of hydrated samples was placed in an TZero Pan (TA instruments, New Castle, DE), and sealed with a TZERO hermetic lids (TA instruments). The sample pan and a reference pan were subjected to a temperature program set to heat up the sample at a 5°C/min increment from room temperature to 80°C using differential scanning calorimeter (DSC Q1000; TA instruments). The

data were analyzed using TA Universal Analysis software (TA instruments) to determine the peak transitional temperature (Figure 2.2).

2.8. Sarcoplasmic proteins extraction

Two hundred mg of pulverized samples were homogenized in extraction buffer (20 mM Tris-HCl, 125mM NaCl, 1% Triton X) using a bead homogenizer (Bead Blaster 24; Benchmark Scientific). The homogenized sample was centrifuged (Centrifuge 5810 R; Eppendorf, Hamburg, Germany) at 16,000 × g at 4 °C for 20 minutes, and the supernatant was collected and designated as the sarcoplasmic protein. The protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), and protein concentration was adjusted to 3,000 µg/ml.

2.9. Collagen Extraction and Gel Electrophoresis

Collagen was extracted following the methods described by Capella-Monsonís et al. (2018) with modifications. Three grams of pulverized meat was combined with 0.1 M NaOH and shaken overnight at 4o C. After shaking, the samples were centrifuged (Centrifuge 5810 R; Eppendorf) at 3,000 × g for 10 minutes and washed with ultrapure water 3 times. The pellet was mixed with 0.5M of acetic acid containing of 0.1% of pepsin from porcine gastric mucosa (J61679; Alfa Aesar, Haverhill, MA, USA) for 24 hours at 4o C. After incubation, the tubes were centrifuged (Centrifuge 5810 R; Eppendorf) at 10,000 × g for 10 minutes, and the collagen from the supernatant was precipitated by the addition of Tris base and NaCl at a concentration of 0.5 and 2.6 M, respectively. The samples were centrifuged again (Centrifuge 5810 R; Eppendorf) at 16,000 × g for 1 hour, and the pellet was redissolved in 0.5 M of acetic acid. The samples were desalted using Zeba Spin Desalting Columns (MWCO 40K; ThermoScientific) by centrifuging the samples in the column at 1,000 × g for 2 minutes. The desalted samples were concentrated to

~0.5 mL in volume using a vacuum evaporator (RapidVap; Labconco Corporation). The hydroxyproline assay as described in the collagen content section was used to determine the collagen concentration of each sample.

Collagen concentration of each sample was adjusted to 50 mg/mL using 0.5M of acetic acid. Samples were neutralized with 1 M NaOH and mixed with Laemmli non-reducing Sample Buffer (J63615; Alfa Aesar, Haverhill, MA) at a 10:3:1 ratio. Two μg of extracted collagen was loaded to each well of a Novex WedgeWell 6% Tris-Glycine Gel (Invitrogen, Carlsbad, CA) and collagen structure was separated by gel electrophoresis using a Mini Gel Tank (Invitrogen) at a constant voltage of 100 V for 160 minutes in tris-glycine SDS running buffer. Following the separation, gels were stained for 30 minutes using ProSignal Blue Protein Stain (Prometheus, Genesee Scientific, San Diego, CA) and washed for 5 minutes with water. Images were taken using an iBright Imaging System (FL1500, Thermo Fisher Scientific) analyzed using iBright Analysis Software (Thermo Fisher Scientific). Four bands were identified for the collagen structure (Figure 2.3) as Gamma (γ), Beta (β), Alpha 1 (α1) and Alpha 2 (α2) in each sample (Carmichael & Lawrie, 1967), and the relative percentage of each band in each sample was calculated by dividing the band intensity by the intensity of all bands in that specific lane.

2.10. Western Blot of Aggrecan

Extracted sarcoplasmic proteins were combined with Laemmli SDS sample buffer (Alfa Aesar) at 1:1 ratio. Five μL of Prometheus prestained protein ladder (Genesee Scientific, San Diego, CA), 30 μg of sarcoplasmic protein of a prepared reference (sarcoplasmic protein of a *Semitendinosus* muscle aged for 2 days), and 30 μg of sarcoplasmic protein from each sample were loaded into a 10% Tris-Glycine Gel (Invitrogen), and proteins were separated using a Mini Gel Tank (Invitrogen) in a tris-glycine SDS running buffer at a constant voltage of 180 V for 50

minutes. Following gel electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen) using iBlot™ 2 gel transfer device (Invitrogen). All blots were blocked for 90 minutes using OneBlockTM Western-FL Blocking Buffer (Genesee Scientific). Following blocking, the membrane was immersed in primary antibody - mouse anti-aggrecan (MBS442010, MyBioSource, San Diego, CA) at a 1:50 dilution in blocking buffer and incubated overnight at 4 °C. After the primary antibody incubation, the membrane was incubated with a secondary antibody - Goat anti-mouse IgG Alexa Fluor Plus 555 (A32727, Invitrogen) at 1:2,000 dilution in blocking buffer for 90 minutes. Membranes were scanned using the 555 channel in iBright Imaging System (FL1500, Thermo Fisher Scientific) and analyzed using iBright Analysis Software (Thermo Fisher Scientific). A band at 95 kDa and another at 75 kDa were quantified and compared to the reference sample (Figure 2.4). Aggrecan fragmentation was expressed as fold changes relative to bands of the reference sample on each membrane.

2.11. Statistical analysis

All data were analyzed as a split-plot using PROC GLIMMIX of SAS (version 9.4, Cary, NC). The model included the whole-plot factor of meat cut, the sub-plot factors of aging time and the cut × aging time interaction. Animal within meat cut was considered as the error term with individual animal used as the experimental unit. For all analysis, satterthwaite approximation was used to estimate the degrees of freedom. Differences among means was detected at the 5% level using the least significance difference.

3. Results and Discussion

3.1. Warner-Bratzler shear force and Connective tissue shear force

No interactions were detected between muscle x aging period ($P > 0.10$), but a main effect was found for muscle (Table 2.1) and aging period (Table 2.2) for WBSF ($P < 0.01$). *LL* had lower WBSF than *GM* and *GT* ($P < 0.01$), but *GM* and *GT* did not differ in WBSF from one another ($P > 0.10$). On the other hand, steaks at 3 days aging had greater WBSF than the other aging periods ($P < 0.01$), and it was interesting to note that the steaks did not become more tender with further aging beyond 21 days postmortem ($P > 0.10$). It was expected to find *GM* and *GT* to be tougher than *LL* as this has been demonstrated in many studies (Chun et al., 2020; Sullivan & Calkins, 2011; Torrescano et al., 2003), which muscles used for locomotion usually have a greater amount of connective tissue than muscles for support due to their needs to sustain greater forces (Belew et al., 2003; Lonergan et al., 2019). It was also expected to find a decrease in WBSF with postmortem aging. During the aging of meat, meat tenderness improvement is achieved as a consequence of protein degradation of myofibrillar components, mainly due to calpains (Huff- Lonergan et al., 2010; Lomiwes et al., 2014; Marino et al., 2013). Hammond et al. (2022) found a decrease in WBSF from 2 to 21 days for 8 different beef muscles. Moreover, Matney et al. (2021) found a decrease in shear force in beef *LL* and *Semitendinosus* aged from 7 to 28 to 112 days. Interestingly, Colle et al. (2015) found no difference in the shear force for the beef *GM* when aged from 2 to 63 days, but found a decrease in the shear force for *LL* with the extended aging from 2 to 14 and 21 to 63 days.

There was a muscle x aging interaction for CTSF ($P < 0.05$; Table 2.1). *LL* steaks at 3 days postmortem had higher CTSF than *LL* steaks from 42 and 63 days of postmortem aging ($P < 0.05$) and showed a tendency to be higher than *LL* from 21 days of aging ($P = 0.08$). *GM* showed no difference in CTSF among the different aging periods ($P > 0.10$). Three days aged *GT* steaks had a higher CTSF value than the *GT* steaks from the rest of the aging periods ($P < 0.05$).

No difference in CTSF among the muscles was found for 3 and 21 days ($P > 0.10$), but *LL* had lower CTSF values than *GM* and *GT* after 42 days of postmortem aging ($P < 0.05$). The CTSF represents the shear-force of the perimysium network and is affected by the amount of connective tissue and their solubility (Girard et al., 2012). The differences found at 42 days postmortem in this study indicated that *GM* and *GT* may have tougher connective tissue texture than *LL*. Moreover, the decrease in the CTSF for *LL* and *GT* as the aging periods progress demonstrated potential evidence for the weakening of the IMCT structures.

3.2. Trained panel

No muscle x aging interaction was found for myofibrillar tenderness, connective tissue amount or overall tenderness ($P > 0.10$). However, there was a muscle (Table 2.1) and an aging effect (Table 2.2) for all three characteristics ($P < 0.05$). As expected, steaks aged for only 3 days were rated to have lower myofibrillar tenderness ($P < 0.05$), more connective tissue amount ($P < 0.05$) and less tender for overall tenderness ($P < 0.01$) compared to steaks from the other aging periods, but no differences were found for these characteristics among the 21, 42 and 63 days aged steaks ($P > 0.10$). In addition, *LL* was rated with lower connective tissue amount than *GM* and *GT* ($P < 0.05$), which *GM* and *GT* did not differ from each other ($P > 0.10$). Finally, it was interesting to note that *LL* and *GT* did not differ in myofibrillar and overall tenderness rating ($P > 0.10$), which were both more tender than *GM* ($P < 0.01$).

The findings from this study are in agreement with Hammond et al. (2022) and Chun et al. (2020), who also found an increase in myofibrillar tenderness and overall tenderness and a decrease in the connective tissue amount after 21 days of aging for 11 different beef muscles. The initial increase in the myofibrillar tenderness was expected as a result of the degradation of myofibrillar proteins through enzymatic protein degradation (Lomiwes et al., 2014; Marino et al.,

2013; Matney et al., 2021; Nishimura et al., 2008). Phelps et al. (2016) documented calpains were active until 42 days of postmortem aging using beef *Semitendinosus* muscle aged from 7 to 70 days, and myofibrillar proteins Desmin and Troponin-T continuously degraded until day 70. It was peculiar to find *LL* with similar myofibrillar and overall tenderness as *GT* in this study, which disagreed with Chun et al. (2020) who found *GT* with tougher myofibrillar component than *LL*. Although *GT* had a greater connective tissue amount detected by the panelists, the connective tissue did not seem to affect its overall tenderness in this study.

Lewis et al. (1991) showed that aging bovine *Semitendinosus* muscle for 14 days decreased the breaking strength of perimysium measured using a tensile strength test. Additionally, Nishimura et al. (1998) showed a decrease in shear-force values for the isolated endomysium and perimysium of beef *Semitendinosus* muscle after 14 days of aging. Finally, Liu et al. (1995), demonstrated a structural weakening of endomysium and perimysium in chicken *Semitendinosus* after 12 hours of postmortem aging using scanning electron microscopy. The decrease in connective tissue amount detected by trained panelists agreed with the results found for CTSF from this study and reinforced that there is a possibility that connective tissue structures were weakened with the extended aging periods.

3.3. Total Collagen Content and Collagen Crosslinks Density

There was no muscle x aging period interaction or main effect for aging ($P > 0.10$) found for total collagen content, but a main effect for muscle was detected ($P < 0.01$; Table 2.3), which *LL* had lower total collagen content compared to *GM* and *GT* ($P < 0.01$).

It was expected that muscles of locomotion had more collagen content than muscles of support. Chun et al. (2020), Torrescano et al. (2003) and Stolowski et al. (2006) all reported that locomotive muscles like *GT* and *Semitendinosus* and transitional muscles like Tensor fasciae

latae and Vastus lateralis in beef all had greater collagen content than *LL*. As mentioned earlier, our results did not find any alterations in total collagen content due to aging. However, Chun et al. (2020) showed a decrease in total collagen content for the *LL*, *Tensor fascia latae* and *GT* when aged from after 21 days of postmortem aging. Similarly, another study found collagen content decreased from 1 to 14 days of postmortem aging in lamb *LL* (Starkey et al., 2015). On the other hand, Kolczak et al. (2008) also failed to find differences in collagen content in the muscles *Semitendinosus* and *Psoas major* from beef carcasses aged from 1 to 12 days. The inconsistent results among studies are likely due to differences in water holding capacity, resulting in different level of purge loss during extended aging. Perhaps, purge loss may alter the moisture and soluble collagen content of the samples, which masked the potential alteration of total collagen content from the aging process.

There was no muscle x aging interaction nor main effect for muscle found for the mature collagen crosslink PYD density ($P > 0.10$). However, a main effect for aging period was found ($P < 0.05$; table 2.2). At 3 days aging, PYD density was lower than samples from 42 and 63 days of postmortem aging ($P < 0.05$) and did not differ from samples from 21 days of postmortem aging ($P > 0.10$).

Wu et al. (2021) also evaluated 6 different beef shank cuts and demonstrated that as PYD density increases, the overall solubility of collagen decreases, which results in a tougher connective tissue texture in cooked meat products. Chun et al. (2020) and Dubost et al. (2013) showed locomotive muscles like *GT*, *Semimembranosus* and *Biceps femoris* have greater PYD density than supportive muscles like *Longissimus dorsi*. However, PYD density was similar for the different muscles in this study. The lack of differences in collagen crosslink density among the muscles may be attributed to animal age and the lack of physical exercise. All the carcasses

used in this study were from feedlot cattle graded as A maturity, which are from young beef cattle under 30 months of age. When collagen crosslinks are initially formed in the young animal, they are in the form of divalent collagen crosslinks, which are reducible and heat-labile (Gaar et al., 2020). As the animal matures, the divalent collagen crosslinks form into more stable trivalent crosslinks that contributes to cooked meat toughness (Purslow et al., 2012; Roy et al., 2021). Furthermore, all the cattle used in this study finished in the feedlot system. Feeding a high-energy diet are known to accelerate collagen turnover, thus, preventing the maturation of divalent crosslinks into trivalent crosslinks (Wu et al., 1981).

Hammond et al. (2022) used A maturity beef carcasses collected from the same packing plant in her study and also found no difference in PYD density between *GM* and *Longissimus thoracis*. It is also interesting to note that we found *GT* with similar overall tenderness as *LL* in our trained panels. This finding was peculiar because our results showed that *GT* had greater connective tissue amount and total collagen content than *LL*. However, this can be explained that as myofibrillar tenderness score and PYD density were similar between *LL* and *GT*, they ended with similar muscle fiber and connective tissue texture, thus, resulted in similar overall tenderness. Finally, it was unexpected to find PYD density increased with postmortem aging of these samples. The PYD density was calculated as the molarity of PYD per molarity of collagen and an increase in the PYD density could mean that PYD crosslinks were staying in the beef while there was a decrease in collagen concentration likely due to a purge loss during aging.

There was a muscle x aging interaction for DPD density ($P < 0.05$; Table 2.3), where *GM* had the least DPD density at 42 days postmortem aging compared to the other aging periods within the same muscle ($P < 0.05$). Although DPD density shows a muscle x aging interaction,

DPD is found in very low concentrations in meat. Thus, it is likely that it does not have a significant influence on connective tissue texture.

3.4. Transition temperature of connective tissue

There was no muscle x aging period interaction ($P > 0.10$) or main effect for muscle ($P > 0.10$) for the transition temperature of connective tissue, but there was a main effect for aging ($P < 0.01$; Table 2.2). The transition temperature did not differ from 3 to 21 days ($P > 0.10$), but decreased after 42 days of postmortem aging ($P < 0.01$). Smith & Judge (1991) conducted a study using beef *Semimembranosus* muscle from different USDA maturities and found a positive correlation of transition temperature of collagen with PYD density and concluded that muscles with higher PYD density had higher thermal denaturation temperature. Moreover, Roy et al. (2021) demonstrated that the *GM* muscles of mature cattle had higher PYD density and also higher transition temperature than calves, which were shown to have lower PYD density. In this study, the decreases in connective tissue transition temperature from samples at 42 days of postmortem aging should be caused by changes in the connective tissue structure and the degree of collagen crosslinks. However, we did not find the expected alterations in mature collagen crosslinks using the traditional acid hydrolysis and HPLC methodology. This could be attributed to the use of HCl in this study to hydrolyze all collagen fibers into amino acids and crosslinks regardless of their degradation status (Bergman & Loxley, 1963). Therefore, it is important for us to recognize the traditional volume-based crosslink density measurement is likely not a good method to determine alteration in collagen structure.

3.5. Collagen Structure

There was no muscle x aging interaction for the γ chain relative % ($P > 0.10$), but there was an aging ($P < 0.05$; Table 2.2) and a muscle effect ($P < 0.01$; Table 2.3). *LL* had greater γ

chain % compared to *GM* and *GT* ($P < 0.01$), but *GM* and *GT* γ chain % were not different from one another ($P > 0.10$). Also, γ chain % was similar from 3 to 42 days post-mortem ($P > 0.10$), but it decreased after 42 days of post-mortem aging ($P < 0.05$). There was also no muscle x aging interaction for the β chain relative % ($P > 0.10$), but a main effect for muscle ($P < 0.01$; Table 2.3) were found. The *LL* had lower β chain % than *GM* and *GT* ($P < 0.01$), which *GM* and *GT* were similar in β % ($P > 0.10$). Moreover, a trend for the aging effect was noted for the β chain relative %, which β chain % tended to decrease from 3 to 21 days of aging ($P = 0.08$). There was no muscle x aging interaction for the α1 relative % ($P > 0.10$), but and an aging ($P < 0.01$; Table 2.2) and a muscle effect ($P < 0.01$; Table 2.3) were found. *GM* and *GT* were similar in α1 chain % ($P > 0.10$) and had greater α1 chain % than *LL* ($P < 0.01$). There was an increase for α1 chain % from the samples at 63 days postmortem aging when compared to those from 3 and 21 days postmortem ($P < 0.01$). However, α1 chain % from samples at 42 days were similar to those from all other aging periods ($P > 0.10$). Finally, a muscle x aging interaction was found for α2 chain relative % ($P < 0.05$; Table 2.3). *LL* and *GM* had similar α2 chain % in all aging periods ($P > 0.10$), while *GT* had an increase in α2 chain % from 3 to 21 days ($P < 0.01$), followed by a decrease from 21 to 42 days ($P < 0.05$).

Collagen fibers consists of a triple helix structure made of three polypeptide alpha (α) chains. When two α chains are linked by an inter- or intra- molecular crosslinks, a dimer is obtained, and it is referred to as the beta (β) chain. On the other hand, when three α chains are crosslinked, a trimer is obtained, which is referred to as gamma (γ) chain (Carmichael & Lawrie, 1967). The data found in this study provided strong evidence on the alteration of collagen structure throughout the extended aging periods. Both γ and β chains relative % decreased throughout the aging periods, suggesting the weakening of the collagen structure by the

breakdown of the crosslinks on those complex structures into polypeptide chains. This degradation of collagen structure was further confirmed with the increase of α1 % at 63 days. On the other hand, the α2 % of *GT* sample collagen had a fluctuating pattern, which could be attributed to continuous breakdown of α2 into peptide chain fragments.

The breakdown of the collagen structure was also reported by Akkus et al. (2005), who showed a decrease in β, α1 and α2 chains in human femurs subjected to γ-irradiation. Moreover, Burton et al. (2014) utilized γ-irradiation on bovine tibiae and demonstrated that irradiated bones had a high variability in the γ, a complete lack of β, and less dense α chains, with the presence of lower molecular weights fragments recognized as peptide chain fragments. Additionally, irradiated samples was found to have a decrease in transition temperature of collagen (Burton et al., 2014), which agreed with this study that also showed a relationship between degradation of the collagen structure and collagen heat stability.

The modifications of the collagen structure during postmortem aging found in this study suggested that native collagenases were active in postmortem muscles. Matrix metalloproteinases (MMPs) and cathepsins are known to contribute to collagen turnover in the live animal (Holmbeck & Birkedal-Hansen, 2013), thus, they could potentially be influencing on the collagen breakdown seen in this study.

3.6. Aggrecan fragmentation

There was a muscle x aging interaction for the 95 and 75 kDa aggrecan fragments ($P < 0.05$; Table 2.3). For the 95 kDa aggrecan fragment band, both *LL* and *GT* at 21 days had the greatest band intensity compared to those from the rest of the aging periods in *LL* and *GT* ($P < 0.01$). However, *GM* did not show any change in the 95 kDa aggrecan fragment intensity among the aging periods ($P > 0.10$). For the 75 kDa aggrecan fragment band, both *LL* and *GT* had a

decrease in the aggrecan fragment intensity from 3 to 21 to 42 days ($P < 0.05$), but remained stable after 42 days of postmortem aging ($P > 0.10$). Similar to the *GM* aggrecan fragment band at 95 kDa, the 75 kDa aggrecan fragment band for *GM* did not show any alteration in intensity among the aging periods ($P > 0.10$). It was interesting to note that all muscles had similar 95 kDa aggrecan fragment band intensity for aging periods at 3, 42 and 63 days ($P > 0.05$). However, *LL* had greater 95 kDa aggrecan fragment intensity than those from *GM* and *GT* at 21 days postmortem ($P < 0.05$), which *GM* and *GT* had similar 95 kDa aggrecan fragment intensity ($P > 0.05$). Finally, *LL* and *GT* had greater aggrecan fragment intensity than those from *GM* for both 3 and 21 days aging periods ($P < 0.01$). However, all 3 muscles had similar of aggrecan fragment intensity after 21 days of aging ($P > 0.10$).

Aggrecan is a large aggregating proteoglycan found in several tissues but mainly in the articular cartilage, and it has a molecular weight of approximately 2,500 kDa (Vertel & Ratcliffe, 2000; Yamakoshi, 2014). Aggrecan consists of a interglobular domain that separates two globular regions, and the interglobular domain is susceptible to cleavage by most proteinases, including MMPs and aggrecanases (Sivan et al., 2014). The antibody used in this study was designed to recognize the N-terminal neoepitope sequence generated by the cleavage of aggrecan in the interglobular domain (Hughes et al., 1995).

Fosang et al. (1993) demonstrated proteolytic cleavage of aggrecan by extracting aggrecan fragments containing two globular regions (G1 and G2) from human joint and pig larynges. Aggrecan was digested in recombinant human fibroblast collagenase in vitro, and the aggrecan cleavage in between those domains after the digestion was reported (Fosang et al., 1993). Moreover, Veiseth-Kent et al. (2018) evaluated the breakdown of aggrecan in 4 different bovine muscles aged from 2 to 13 days and found an increase in two aggrecan fragments (31 and

46 kDa) for *LL* when aged for 13 days. In addition, Eggen et al. (1998) evaluated decorin, another proteoglycan component in meat and found that decorin is also degraded during beef *Semitendinosus* muscle aging. It is well established that Aggrecanases and MMPs are the main enzyme groups responsible for aggrecan turnover (Nagase & Kashiwagi, 2003), and it is possible that the collagenase MMPs can be responsible for the remodeling of both collagen structure and aggrecan found in this study.

4. Conclusion

This study provided evidence that IMCT softens during postmortem aging due to the modifications of IMCT components such as proteoglycans and the collagen structure. In addition, IMCT structural components are likely weakened by native collagenases during postmortem aging. However, further study is needed to better characterize the enzymes responsible for the IMCT degradation during the postmortem aging of beef. With better understanding in the enzymes responsible for the IMCT degradation, industries can better utilize this knowledge for the management of tougher beef cuts.

5. References

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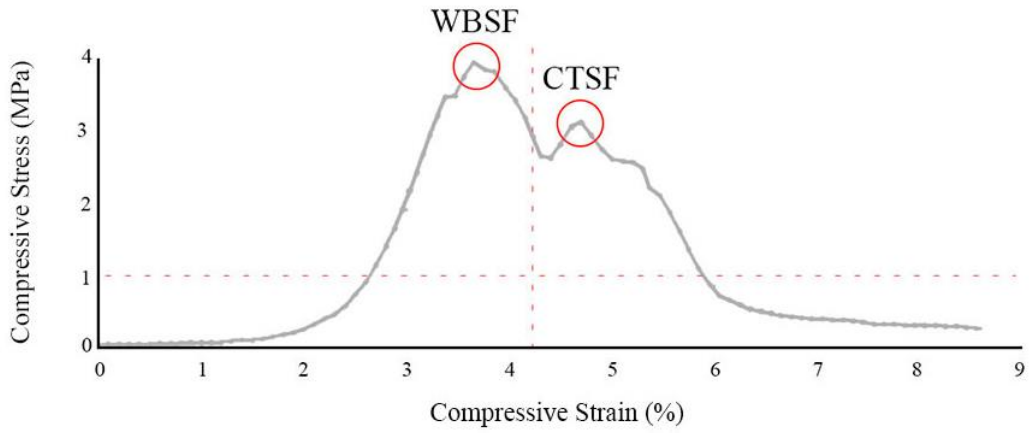

Figure 2.1. Representative image of Warner- Bratzler shear force (WBSF) and Connective tissue shear force (CTSF) peak identification from the shear test.

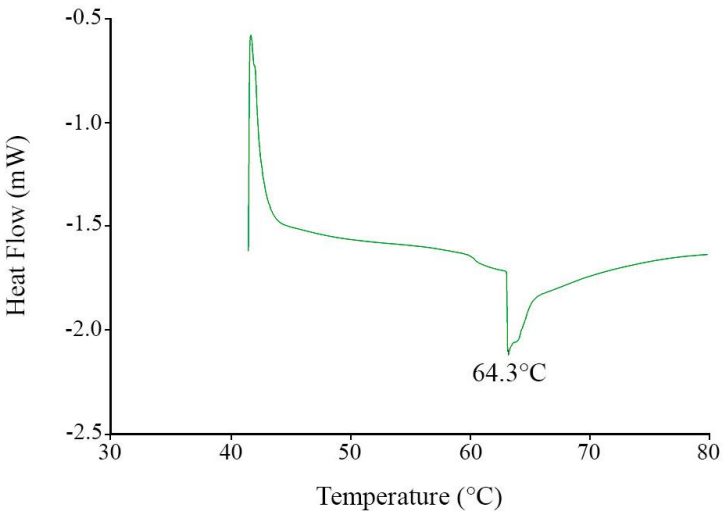

Figure 2.2. Representative perimysial peak transition temperature curve from the differential scanning calorimeter.

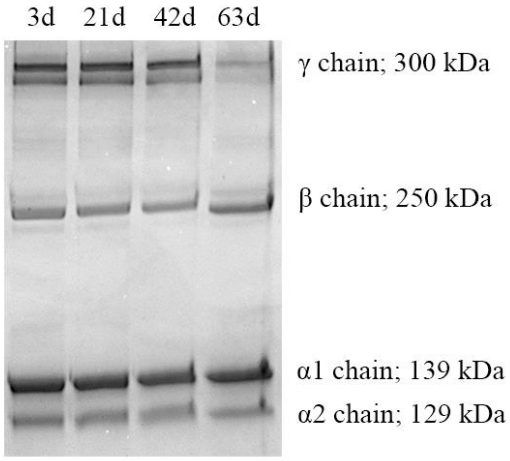

Figure 2.3. Representative image of the collagen structure represented by the γ, β, α1 and α2 chains at 3, 21, 42 and 63 days for the muscle *Longissimus lumborum* muscle.

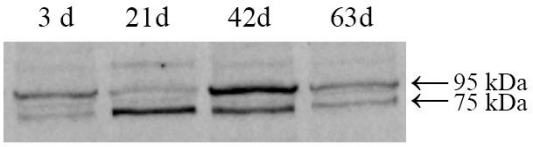

Figure 2.4. Representative image of Western Blot indicating the 95 kDa and 75 kDa aggrecan fragments in the *Gastrocnemius* muscle.

Table 2.1. Warner-Bratzler shear force (WBSF), connective tissue shear force (CTSF), myofibrillar tenderness, connective tissue amount and overall tenderness evaluated by trained panelists of three beef muscles aged for 3, 21, 42 and 63 days (n = 120).

Items	Muscle				
	Longissimus lumborum	**Gluteus Medius**	**Gastrocnemius**	**SEM[1]**	**P-value**
WBSF, kgf	3.41^{b}	4.39^{a}	4.29^{a}	0.14	< 0.01
CTSF, kgf					
3 days	3.36^{Aa}	3.48^{Aa}	3.83^{Aa}	0.18	< 0.05
21 days	2.97^{ABa}	3.26^{Aa}	3.06^{Ba}		
42 days	2.7^{Bb}	3.7^{Aa}	3.26^{Ba}		
63 days	2.5^{Bb}	3.35^{Aa}	3.29^{Ba}		
Myofibrillar tenderness[2]	64.19^{a}	55.60^{b}	64.00^{a}	0.99	< 0.01
Connective tissue amount[3]	12.72^{b}	16.68^{a}	17.93^{a}	1.24	< 0.01
Overall tenderness[2]	60.28^{a}	50.28^{b}	57.94^{a}	1.26	< 0.01

$^{A-B}$ Within a column, means without a common superscript differ at $P < 0.05$.
$^{a-b}$ Within a row, means without a common superscript differ at $P < 0.05$.
[1] Standard error of the mean.
[2] Sensory scores: 0 = extremely tough; 50 = neither tough nor tender; 100 = extremely tender.
[3] Sensory scores: 0 = none; 50 = medium; 100 = abundant.

Table 2.2. Main effect of aging time for Warner-Bratzler shear force (WBSF), myofibrillar tenderness, connective tissue amount and overall tenderness evaluated by trained panelists, transition temperature of collagen, pyridinoline (PYD) density, and structural components of collagen (γ, β and α1) of three beef muscles aged for 3, 21, 42 and 63 days (n = 120).

Items	Aging					
	3 days	21 days	42 days	63 days	SEM[1]	P-value
WBSF, kgf	4.68[a]	3.93[b]	3.89[b]	3.64[b]	0.13	< 0.01
Myofibrillar tenderness[2]	57.68[b]	61.37[a]	62.41[a]	63.64[a]	1.11	< 0.01
Connective tissue amount[3]	19.44[a]	14.01[b]	13.87[b]	14.34[b]	1.69	< 0.05
Overall tenderness[2]	50.69[b]	56.12[a]	58.45[a]	59.43[a]	1.52	< 0.01
PYD density, mol/mol of collagen	0.13[b]	0.14[ab]	0.16[a]	0.16[a]	0.01	< 0.05
Transition temperature of collagen, °C	65.41[a]	65.51[a]	64.84[b]	64.70[b]	0.20	< 0.01
γ chain relative %	25.03[a]	27.57[a]	24.78[a]	18.48[b]	3.14	0.04
β chain relative %	26.84	22.29	24.02	25.81	1.84	0.08
α1 chain relative %	34.10[b]	34.56[b]	37.40[ab]	40.84[a]	1.80	<0.01

[a-b] Means without a common superscript differ at $P < 0.05$.
[1] Standard error of the mean.
[2] Sensory scores: 0 = extremely tough; 50 = neither tough nor tender; 100 = extremely tender.
[3] Sensory scores: 0 = none; 50 = medium; 100 = abundant.

Table 2.3. Total collagen content, pyridinoline (PYD) density, deoxypyridinoline (DPD) density, aggrecan fragmentation, and collagen structural components (γ, β, α1, and α2 chains) of three beef muscles aged for 3, 21, 42 and 63 days (n = 120).

Items	Muscle				
	Longissimus lumborum	Gluteus Medius	Gastrocnemius	SEM[1]	P-value
Total collagen, mg/g wet tissue	5.01^{b}	7.96^{a}	9.07^{a}	0.82	< 0.01
DPD density, mol/mol of collagen					
3 days	0.026^{Aa}	0.027^{Aa}	0.018^{Aa}	0.005	< 0.05
21 days	0.036^{Aa}	0.020^{ABb}	0.013^{Ab}		
42 days	0.036^{Aa}	0.011^{Bb}	0.014^{Ab}		
63 days	0.028^{Aa}	0.020^{Aab}	0.009^{Ab}		
95 kDa Aggrecan fragment, fold change					
3 days	0.43^{Ba}	0.21^{Aa}	0.14^{Ba}	0.10	< 0.05
21 days	1.09^{Aa}	0.35^{Ab}	0.62^{Ab}		
42 days	0.60^{Ba}	0.39^{Aa}	0.28^{Ba}		
63 days	0.50^{Ba}	0.40^{Aa}	0.28^{Ba}		
75 kDa Aggrecan fragment, fold change					
3 days	0.77^{Aa}	0.09^{Ab}	0.70^{Aa}	0.10	< 0.01
21 days	0.50^{Ba}	0.02^{Ab}	0.52^{ABa}		
42 days	0.17^{Ca}	0.04^{Aa}	0.29^{Ba}		
63 days	0.06^{Ca}	0.04^{Aa}	0.22^{Ba}		
γ chain relative %	32.12^{a}	19.75^{b}	20.03^{b}	2.72	< 0.01
β chain relative %	20.98^{b}	27.48^{a}	25.76^{a}	1.71	< 0.01
α1 chain relative %	33.74^{b}	37.95^{a}	38.50^{a}	1.69	< 0.01
α2 chain relative %					
3 days	13.04^{Aa}	16.12^{Aa}	12.97^{Ca}	1.28	< 0.05
21 days	12.95^{Ab}	15.04^{Ab}	18.78^{Aa}		
42 days	14.02^{Aa}	12.94^{Aa}	14.41^{BCa}		
63 days	12.80^{Ab}	15.20^{Aab}	16.74^{ABa}		

$^{A-C}$ Within a column, means without a common superscript differ at $P < 0.05$.
$^{a-b}$ Within a row, means without a common superscript differ at $P < 0.05$.
[1] Standard error of the mean

Chapter 3 - Matrix Metalloproteinase- 9 may contribute to collagen structure modification during postmortem aging of beef

Abstract

Matrix metalloproteinases (MMPs) are endopeptidases responsible for remodeling the extracellular matrix of intramuscular connective tissue in live animals. However, the effect of MMPs in beef is not known. Therefore, 3 different experiments were performed to: 1) Characterize MMP activity during postmortem aging of beef; 2) determine if the native beef MMP can contribute to connective tissue degradation in a simulated standard industry postmortem aging condition and 3) to explore approaches to improve the native beef collagenase activity. In experiment 1, the muscles *Longissimus lumborum* (*LL*), *Gluteus medius* (*GM*) and *Gastrocnemius* (*GT*) were aged for 3, 21, 42 and 63 days. MMP-9 was shown to be the active collagenase during aging of meat, where *GT* had higher MMP-9 activity than *LL* and *GM* ($P < 0.01$), and the highest activity was detected at 3 days of postmortem aging ($P < 0.05$). In experiment 2 and 3, the muscles *LL*, *GM* and *GT* aged for 3 days were used as a model system. MMP-9 was not detected prior to 42 days of storage in the cooler, where the activity was higher for the 63 than 42 days of aging ($P < 0.05$). Moreover, supplementation with 20 µM of $ZnCl_2$ showed the highest MMP-9 activity when compared to other levels of supplementation ($P < 0.01$). Further research still needs to be done to better understand MMP-9 mechanism during postmortem aging of meat. With a better understanding of MMP-9 in the aging process, the beef industry can provide better connective tissue management strategies for lower-quality beef cuts.

Keywords: MMP-9, Zymography, Zinc, Collagenase, Western Blot.

1. Introduction:

The mechanism in which meat tenderness is improved during postmortem aging resulted from the degradation of myofibrillar proteins by calpains is well researched (Huff-Lonergan et al., 2010; Kemp & Parr, 2012), but enzymes that could potentially contribute to intramuscular connective tissue (IMCT) softening are seldom studied (Alderton et al., 2004; Kubota et al., 2003). In live animals, the extracellular matrix of IMCT is degraded and remodeled by collagenases, gelatinases, stromelysins, matrilysins or membrane-type matrix metalloproteinases (MMPs), which are a group of calcium and zinc-dependent endopeptidases (Kar et al., 2010; Purslow, 2014). Some studies suggested that these MMPs are active during aging of meat and are potential candidates to degrade IMCT components, such as proteoglycans and collagen (Pambuka et al., 2007; Sylvestre et al., 2002; Veiseth-Kent et al., 2018). However, there is no concrete evidence of MMPs participating in the postmortem aging of meat under regular aging conditions.

After the slaughter of the animal, there is a cessation of mitochondrial respiration that shifts the muscle metabolism to anaerobic, that combined with external cooling result in the drop of pH of meat to 5.6-5.7 and a core temperature below 4°C (Ertbjerg & Puolanne, 2017). At the same time, there is an increase in free calcium in the sarcoplasm prevenient from the calcium leakage at the sarcoplasm reticulum (Ertbjerg & Puolanne, 2017). Those modifications affect the muscle environment and the activity of several enzymes (Ashmore et al., 1972; Dransfield, 1994). Pambuka et al. (2007) evaluated the effect of temperature and pH on the activity of ostrich MMPs and found an optimal activity of 45°C and pH around 7-8. Similarly, Wang et al. (2014) evaluated MMP-2 activity in common carp and found that MMP-2 achieves optimum

activity at 40°C and pH of 8. Both studies found that MMPs decreased in activity at lower temperatures and pH.

The role of MMPs in the degradation of IMCT components in the postmortem aging of meat needs to be better understood. This way, industries can better utilize this knowledge for the management of tougher beef cuts. Therefore, the objectives of this study were to 1) characterize MMP activity during postmortem aging of beef; 2) determine if the native beef MMP can contribute to connective tissue degradation in a simulated standard industry postmortem aging condition and 3) to explore approaches to improve the native beef collagenase activity.

2. Materials and Methods

2.1. Sample Collection and Preparation

The sample collection, fabrication, and homogenization process were as described in Chapter 2. Three separate experiments with each meant to address one specific objective were conducted.

2.2. Experiment 1: Characterize MMP activity during postmortem aging of beef

2.2.1. Determining collagenase activity by collagen zymography in 4 different aging periods

In experiment 1, sarcoplasmic proteins were extracted and adjusted to 3 mg/mL from *Longissimus lumborum* (*LL*), *Gluteus medius* (*GM*), and *Gastrocnemius* (*GT*) from 10 carcasses aged for 3, 21, 42 or 63 days as described in Chapter 2. Ten % polyacrylamide gels containing collagen type 1 (CellAdhere; Stemcell Technologies, Vancouver, Canada) were casted following the procedures described by Tajhya et al. (2017) with modifications. A resolving gel was prepared (10% acrylamide: bis-acrylamide = 37.5:1, w/w, containing 0.5 mg/mL of collagen)

and polymerized with 0.072% ammonium persulfate (APS) and 0.11% tetramethylethylenediamine (TEMED). A stacking gel (5% acrylamide: bis-acrylamide = 37.5:1, w/w, containing no collagen) was polymerized with 0.15% APS and 0.3% TEMED.

The collagen zymography procedure followed the method described by Zhao et al. (2016) with modifications. Sarcoplasmic proteins were combined with a Tris-Glycine SDS sample buffer (Novex Life Technologies, Carlsbad, CA) at 1:1 ratio. Five μL of Prometheus prestained protein (Genesee Scientific, San Diego, CA), 30 μg of sarcoplasmic protein of a prepared reference (*Semitendinosus* muscle aged for 2 days) and 30 μg of sarcoplasmic protein from each sample were loaded into the wells of each gel. Proteins were separated using a Bio-Rad Mini-PROTEAN Tetra System electrophoresis unit (Bio-Rad, Hercules, CA) in ice-cold 1X SDS running buffer (Bioland Scientific, Paramount, CA). The system was run at a constant voltage of 125 V at 4°C for 3 hours.

Following electrophoresis, the gels were incubated for 1 hour in 1X zymogram renaturing buffer (Bio-Rad) at room temperature with one change of buffer at 30 minutes. Gels were incubated in 1X zymogram developing buffer (Bio-Rad) for 30 minutes at room temperature. Solution was discarded, and the developing buffer was replaced to be incubated at 37°C in a forced air general incubator (Symphony, VWR, Radnor, PA) for 36 h. Following the incubation, the gels were rinsed with ultrapure water for 10 minutes and stained for 30 minutes in a Coomassie blue stain (2.3 mM Coomassie blue G250, 40% methanol, 10% glacial acetic acid, and 50% ultrapure water) at room temperature. Finally, the gels were destained using a destaining solution (30% methanol, 10% glacial acetic acid, and 60% ultrapure water) at room temperature for 75 minutes until white bands became visible.

Images were taken using an iBright Imaging System (FL1500, Thermo Fisher Scientific, Waltham, MA) and analyzed using iBright Analysis Software (Thermo Fisher Scientific). A white band representing collagenase activity was detected on the collagen zymography gels at 72 kDa for each well (Figure 3.1 and 3.2). Collagenase activity was expressed as fold changes relative to the reference sample on every gel.

2.2.2. Western Blot for MMP identification

Five μL of Prometheus prestained protein (Genesee Scientific) and 30 μg of sarcoplasmic protein from the *GT* muscle were loaded into 10% Tris-Glycine Gels (Invitrogen, Carlsbad, CA), and proteins were separated in 1X tris-glycine SDS running buffer using a Mini Gel Tank (Invitrogen) at a constant voltage of 180 V for 50 minutes. Following gel electrophoresis, the proteins were transferred to iBlot 2 PVDF membranes (Invitrogen) using iBlot 2 gel transfer device (Invitrogen). All blots were blocked for 90 minutes using OneBlock Western-FL Blocking Buffer (Genesee Scientific).

Following blocking, each membrane was incubated with a different set of primary antibody: 1) Mouse monoclonal MMP-1 (H18G8-s; DHSB, Iowa City, IA); 2) mouse monoclonal MMP-2 (MAB3308, Sigma-Aldrich, San Luis, MO); 3) mouse monoclonal MMP-8 (MAB9081; R&D Systems, Minneapolis, MN); 4) rabbit polyclonal MMP-9 (PA5-27191, Invitrogen); 5) mouse monoclonal MMP-13 (MAB 511; R&D Systems) in various dilution ranging from 1:2,000 to 1:50 (determined according to the manufacturers' recommendations) overnight at 4 °C. After the primary antibody incubation, membranes were washed, and the membranes were incubated in the corresponding secondary antibodies: anti-mouse Alexa Fluor Plus 488 (A32723, Invitrogen) was used for membranes incubated in anti-MMP-1, MMP-2, MMP-8 and MMP-13; anti-rabbit Alexa Fluor Plus 647 (A32733, Invitrogen) was used for

membranes incubated in MMP-9 at 1:2,000 dilution and incubated for 90 minutes in room temperature. Following washing, the membrane was scanned using the 488 and 647 channels in the iBright Imaging System (FL1500, Thermo Fisher Scientific) and analyzed using iBright Imaging System (FL1500, Thermo Fisher Scientific) to identify the MW of the corresponding detected MMPs.

2.3. Experiment 2: Determine if the native beef MMP can contribute to connective tissue degradation in a simulated standard industry postmortem aging condition

In experiment 2, sarcoplasmic proteins were extracted and adjusted to 3 mg/mL from *LL*, *GM*, and *GT* from 10 carcass aged for 3 days, and 10 % polyacrylamide gels containing 0.5 mg/mL of collagen type 1 (CellAdhere; Stemcell Technologies) were casted and ran in the same gel electrophoresis conditions as described previously in experiment 1. The sarcoplasmic protein of a reference sample and all muscles from each of the 10 carcasses were loaded into the wells of each gel. Following electrophoresis, the gels were incubated for 1 hour in 1X zymogram renaturing buffer (Bio-Rad) at room temperature with one change of buffer at 30 minutes. Followed by two incubations in 1X zymogram developing buffer (Bio-Rad) for 30 minutes at room temperature. The gels were assigned for 1 of 4 storage periods: 1) 3 d; 2) 21 d; 3) 42 d and 4) 63 d in 1X zymogram developing buffer (Bio-Rad) with the addition of 20 µM of $ZnCl_2$ and pH adjusted to 5.6 with 1M HCl at 4 °C to mimic industry beef aging condition. The developing buffer was drained and replaced every day to avoid microbial spoilage. After the respective storage periods, gels were stained and imaged as described in Experiment 1. Collagenase activity was expressed as fold changes relative to the reference sample on the 42 days gels for each carcass and also expressed as band intensity without being adjusted to the reference sample.

Representative images of collagenase activity from collagen zymography stored for 42 and 63 days are shown in Figure 3.3.

2.4. Experiment 3: Explore possible enhancement of the native beef collagenase activity by adjusting ZnCl2 concentration

In experiment 3, sarcoplasmic proteins were extracted and adjusted to 3 mg/mL from *LL*, *GM*, and *GT* from 10 carcass aged for 3 days, and 10 % polyacrylamide gels containing 0.5 mg/mL of collagen type 1 (CellAdhere; Stemcell Technologies) were casted and ran in the same gel electrophoresis condition as described previously in experiment 1. The sarcoplasmic protein of a reference sample and all muscles from each of the 10 carcasses were loaded into the wells of each gel. Following electrophoresis, the gels were incubated for 1 hour in 1X zymogram renaturing buffer (Bio-Rad) at room temperature with one change of buffer at 30 minutes. The renatured gels were incubated in 1 of 4 zymogram developing buffers: 1) Ca Only (1X developing buffer (Bio-Rad) containing 50 mM Tris-Cl, 5 mM $CaCl_2$, 200 mM NaCl, 0.02% Brij 35, pH 7.5); 2) Zinc 20 (1X developing buffer with the addition of 20 µM of $ZnCl_2$); 3) Zinc 50 (1X developing buffer with the addition of 50 µM of $ZnCl_2$); 4) Zinc 100 (1X developing buffer with the addition of 100 µM of $ZnCl_2$) for 36 h at 37 °C. Finally, gels were stained, destained and imaged as described in Experiment 1. Bands were quantified and calculated as fold changes relative to the reference sample on the Ca only gels for each carcass and also expressed as band intensity without being adjusted to the reference sample. Representative images of collagenase activity from collagen zymography in the different treatments are shown in Figure 3.4.

2.5. Statistical analysis

All data were analyzed as a split-plot using the using PROC GLIMMIX procedure of SAS (version 9.4, Cary, NC). For experiment 1, the model included the whole-plot factor of meat

cut, the sub-plot factors of aging time and the meat cut × aging time interaction. For experiment 2, the model included the whole-plot factor of gel storage time, the sub-plot factors of meat cut, and the storage time x meat cut interaction. For experiment 3, the model included the whole-plot factor of types of developing buffer, the sub-plot factors of meat cut, and the types of developing buffer x meat cut interaction. Satterthwaite approximation was used to estimate the degrees of freedom. The main effects of muscle and aging time were tested, and each animal was considered the experimental unit and each gel was considered the error term. For all experiments, differences among means were detected at the 5% level using the least significant difference.

3. Results and Discussion:

3.1. Experiment 1: Collagenase activity and identification

No muscle x aging interaction was found for the collagenase activity ($P > 0.05$); however, there was a muscle ($P < 0.01$) and an aging effect ($P < 0.01$; Table 3.1). The *GT* had higher collagenase activity than *LL* and *GM* ($P < 0.01$), which *LL* and *GM* were not different in collagenase activity from one another ($P > 0.10$). The highest collagenase activity was detected from steaks aged for 3 days, and the activity decreased after 21 days of postmortem aging and further decreased after 42 days of postmortem aging ($P< 0.01$). The collagenase activity remained stable from 42 to 63 days ($P > 0.10$). The immunoblotting procedure identified the 72 kDa collagenase detected in the collagen zymography as MMP-9 (Figure 3.4), and no other collagenase/gelatinase MMPs (MMP-1, MMP-2 MMP-8 and MMP-13) were detected.

MMP-9 belongs to the gelatinase group of MMPs together with MMP-2, and plays a major role in the degradation of ECM in pathophysiology processes that involve tissue remodeling (Olejarz et al., 2020; Yabluchanskiy et al., 2013). Active MMP-9 was also

immunologically identified by Kubota et al. (2003) in olive flounder (*Paralichthys olivaceus*) muscle at a molecular weight of approximately 78 kDa. Moreover, the same authors conducted gelatin zymography and found that MMP-9 was capable of degrading gelatin with 6 hours of postmortem storage at 4 °C and suggested that MMP-9 may be involved in the disintegration of IMCT that leads to tenderization of fish muscle during chilled storage (Kubota et al., 2003). In our study, it was interesting to note that MMP-9 decreased in activity throughout the extended aging periods; yet, we were able to detect MMP-9 activity in all 4 aging periods investigated. During the aging of meat, other proteases such as the calpains, also gets slowly inactivated throughout the aging periods. Colle & Doumit (2017) investigated calpain activity in beef *LL* and *Semimembranosus* using casein zymography and found that Calpain-1 was completely inactivated after 14 days of aging for both muscles, while Calpain-2 decreased in activity by 57% and 70% for *LL* and *Semimembranosus* after 42 days of postmortem aging, respectively. Protease activities are meticulously regulated by inhibitors; for example, calpastatin inhibits calpains activation (Sazili et al., 2004) and MMPs are regulated by endogenous tissue inhibitor of metalloproteinases (TIMPs) (Matrix et al., 2020). Therefore, it is possible the MMP-9 activity was slowly being inactivated by TIMPs during the postmortem aging in our study. Furthermore, many proteases like calpains and MMPs require autolysis for their activation (Kubben et al., 2007; Li et al., 2004). Therefore, another possibility is that the autolytic degradation of MMP-9 occurred upon its initial activation, resulting in a reduction of MMP-9 activity from d 21 of postmortem aging and beyond. Different from calpains, Ray et al., (2003) demonstrated a protective effect of MMP-9 activity from autolysis in the presence of fetuin-A in blood serum of mice. Perhaps, such protective effect allowed the MMP-9 to extend its activity in extended aging period beyond 63 d as found in our study.

In addition, Sylvestre et al. (2002) conducted gelatin zymography and found active MMP in lamb muscles. However, no difference in MMP activity was found between *Longissimus dorsi* and *Semimembranosus* nor between 0 to 21 days of postmortem aging. Finally, Veiseth-Kent et al. (2018) also utilized gelatin zymography and showed that *LL* had higher MMP activity than Infraspinatus in bovine. Both Sylvestre et al. (2002) and Veiseth-Kent et al. (2018) suggested that the active MMP was MMP-2, which we did not find MMP-2 in our study using immunoblotting technique. It is important to point out that neither of the previously mentioned studies performed any identification assay to identity the detected MMP, and they speculated the MMP identity based on the molecular weight of the MMP-2. Molecular weight should not be a tool for determining the identity of proteins, once several conditions can affect the position of the protein in the gel, such as running conditions, the presence of residues that can affect protein mobility and the similarity of molecular masses of many polypeptides (Griffith, 1972). On the other hand, immunoblotting allows for specific antibody-antigen interaction, allowing the targeted protein to be identified even in picogram level (Ghosh et al., 2014).

A higher activity for MMP-9 was shown in the *GT* compared to the *LL* and *GM*, exhibiting higher potential to degrade collagen and increase tenderness in meat during postmortem aging. Michelin et al. (2009) analyzed MMP-2 and MMP-9 activity in adult pacu fish (*Piaractus mesopotamicus*) muscles, and they found that MMP-2 was more active in the presence of muscles considered predominantly red than in muscles considered predominantly white. Based on muscle fiber typing work from other studies, *GM* and *LL* are considered to be white muscles, with higher relative % of type II fibers, while *GT* is considered a red muscle, which consists of much higher relative % of type I fiber (Kirchofer et al., 2002; Ustunel & Demir, 1997). Therefore, the higher MMP-9 activity in the *GT* muscle could be attributed to the

oxidative characteristic of this muscle. Unfortunately, the exact mechanism that regulates MMP-9 expression and activation in the postmortem period is still unknown.

3.2. Experiment 2: Determining the Activity of Native Beef Collagenase in Simulated Standard Industry Postmortem Aging Condition

No MMP-9 bands were detected prior to 42 days of storage in the cooler. No muscle x aging interaction or muscle effect was found for MMP-9 activity in a simulated standard industry postmortem aging condition ($P > 0.10$). However, an aging effect was found ($P < 0.05$). The MMP-9 activity was higher for the 63 days samples in comparison to the samples aged for 42 days ($P < 0.05$).

Contrary to what was found in experiment 1, MMP-9 activity increased from 42 days to 63 days of postmortem aging under standard industry aging condition (refrigerated), and this can be easily explained. In experiment 1, the enzymes were tested in a favorable environment for activity. Hence it was expressed as maximum MMP-9 activity at a given aging period. On the other hand, the simulated industry cooler condition and lower pH was not favorable for MMP-9 activity. Therefore, the MMP-9 activity was expressed as in situ, which it took longer (42 days) for MMP-9 to demonstrate recognizable collagen hydrolysis in collagen zymography.

Like many other native proteolytic enzymes, MMPs are sensitive to temperature and pH changes (Decaneto et al., 2015; Fasciglione et al., 2000). Kubota et al. (2003) investigated MMP-9 activity in olive flounder (Paralichthys olivaceus) muscle at 37°C, 16°C and 4°C utilizing gelatin zymography, and they found that there was almost no MMP-9 activity at 4°C for 12 hours. Similarly, the present study could not identify MMP-9 activity at 4°C when incubating up to 3 or 21 days. In addition, Li et al. (2015) found that MMP-9 activity in human trabecular meshwork cells decreased by 40% with just slight changes in temperature from 37 to 33 oC.

Furthermore, Yan et al. (2018) showed that MMP-2 activity from sea cucumber decreased by 50 % when temperature dropped from 40 to 30 oC. The same authors also found that the MMP-2 activity was optimized at a pH of 8.5 and decreased by 75% when pH was adjusted to 7 (Yan et al., 2018). Finally, Zhang et al. (2016) also showed that purified MMP-13 from sika deer antler reduced 50% in activity when temperature dropped from 37 to 25 °C and such activity decreased 80% when pH changed from 7 to 5.

Putting this knowledge from both experiment 1 and 2 together, the findings showed MMP-9 activity may be reduced when exposed to cold temperatures and lower pH, but not terminated, and its activity persists even after 63 days of refrigerated storage, which confirmed the potential of MMP-9 for IMCT degradation in beef in cold storage.

3.3. Experiment 3: Determining the Ideal Zinc Concentration to Improve Native Beef Collagenase Activity

There was no treatment x muscle ($P > 0.10$) or muscle effect ($P > 0.10$), but a treatment effect was identified ($P < 0.01$). All zinc supplementation treatments significantly increased MMP-9 activity when compared to the Ca only treatment ($P < 0.01$). Surprisingly, the Zinc 20 treatment exhibited the highest MMP-9 activity compared to the Zinc 50 and Zinc 100 treatments ($P < 0.01$), while no difference was found between Zinc 50 and Zinc 100 treatments ($P > 0.10$).

Zinc acts as a cofactor in regulating MMP activity and expression (Nosrati et al., 2019). Structurally, the catalytic domain of MMP-9 contains one zinc binding site close to the C-terminal, suggesting MMP-9 activity is likely driven by the concentration of zinc ions (Nagase & Woessner, 1999). Isaksen & Fagerhol (2001) studied calprotectin's effect to suppress the gelatinolytic activity of MMP-9 and found that the suppression of MMP-9 activity by

calprotectin can be overcome with the addition of 100 μM of zinc. In addition, Zong et al. (2017) supplemented zinc (0.2 μg/kg/day) to pregnant rats and found and increase in MMP-2 and MMP-9 expression in the placental tissues. These studies supported our findings that increasing zinc concentrations can stimulate MMP-9 activity. However, it is interesting to point out that our study also found that low level inclusion of zinc increased more MMP-9 activity than at high level inclusion. This finding suggested that zinc ion in excessive concentration may suppress MMP-9 activity. Yan et al. (2016) administered extreme high levels of zinc (3 mg/kg/day) via intraperitoneal injection in rats, and the injection decreased expression of MMP-9 by 70% in rats aorta compared to rats that received placebo or $CaCl_2$ only injections. However, the mechanism in which zinc stimulates or inhibits MMPs are still unclear and needs to be better comprehended (Nosrati et al., 2019).

Finally, Schulte et al. (2021) observed the effect of supranutritional zinc supplementation (120 mg/kg of dry matter) in steers and found that steaks from steers supplementation with zinc had a tendency to increase tenderness, but with no differences in myofibrillar protein degradation. Perhaps, the zinc supplementation could have increased MMP-9 activity, leading to a higher collagenolytic activity and a decrease in the background toughness for the steaks from those animals. The findings from experiment 3 demonstrated a potential route to stimulate MMP-9 activity in livestock and reducing background toughness in the meat from those animals.

4. Conclusion

Collagenase activity was found during aging of beef muscles and the collagenase was identified as MMP-9. When simulated under the refrigerated aging conditions, MMP-9 activity was reduced but still shown to be active after 42 days of postmortem aging. Moreover, low

inclusions of zinc were effective in improving MMP-9 activity. Future studies should continue to investigate the effect of zinc supplementation on beef cattle performance, beef quality, and MMP-9 activity during postmortem aging of meat. With a better understanding of MMP-9 in the aging process, the beef industry can provide better connective tissue management strategies for lower quality beef cuts.

5. References

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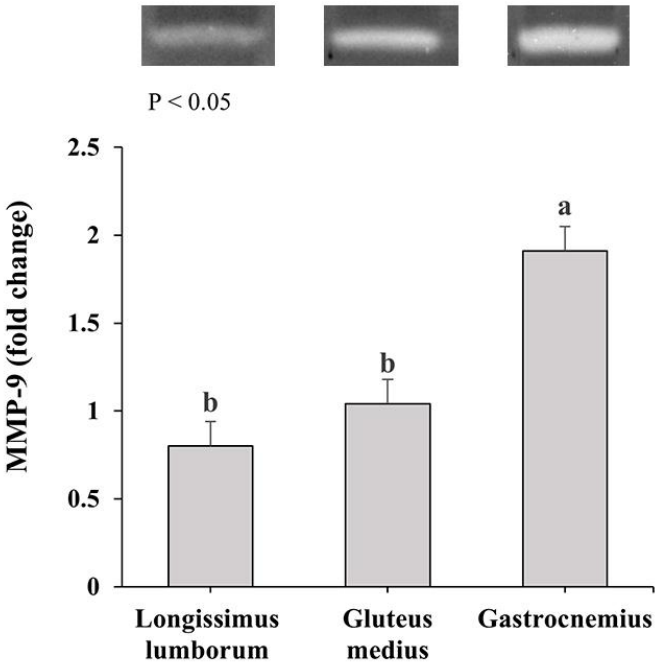

Figure 3.1. Representative collagen zymography showing MMP-9 activity at 72 kDa (top) and MMP-9 activity fold changes relative to a reference sample (bottom) in the muscles *Longissimus lumborum*, *Gluteus medius* and *Gastrocnemius*. Data are least-squares means ± SE. Different superscripts indicates means differ significantly ($P < 0.05$).

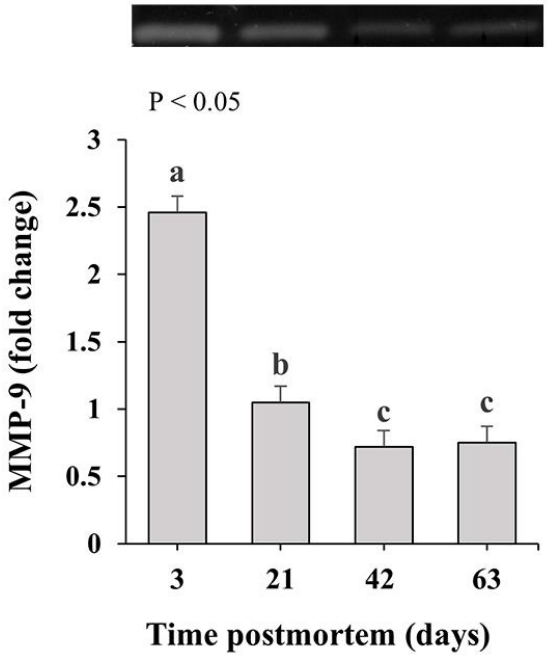

Figure 3.2. Representative collagen zymography showing MMP-9 activity at 72 kDa (top) and MMP-9 activity fold changes relative to a reference sample at 3, 21, 42 and 63 days of postmortem aging. Data are least-squares means ± SE. Different superscripts indicates means differ significantly ($P < 0.05$).

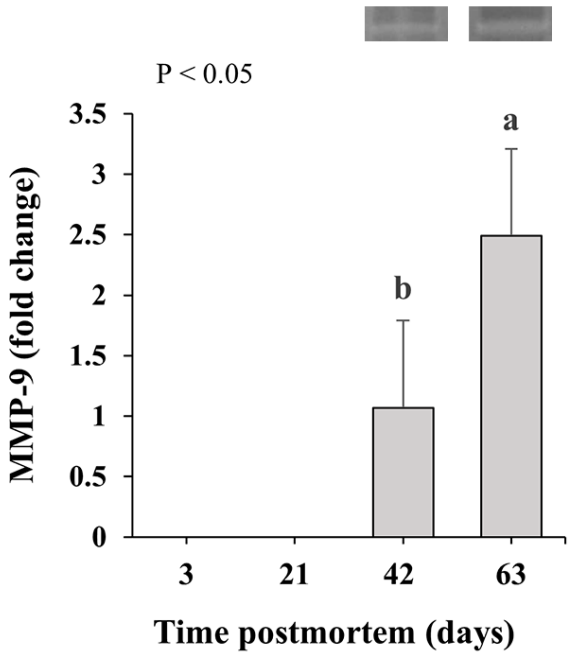

Figure 3.3. Representative collagen zymography showing MMP-9 activity at 72 kDa (top) and MMP-9 activity fold changes relative to a reference sample from the 42 days treatment (bottom). The collagen gels were subjected to simulated industry standard aging (4 oC) for 3, 21, 42 and 63 days. No activity was detected for 3 and 21 days. Data are least-squares means ± SE. Different superscripts indicates means differ significantly ($P < 0.05$).

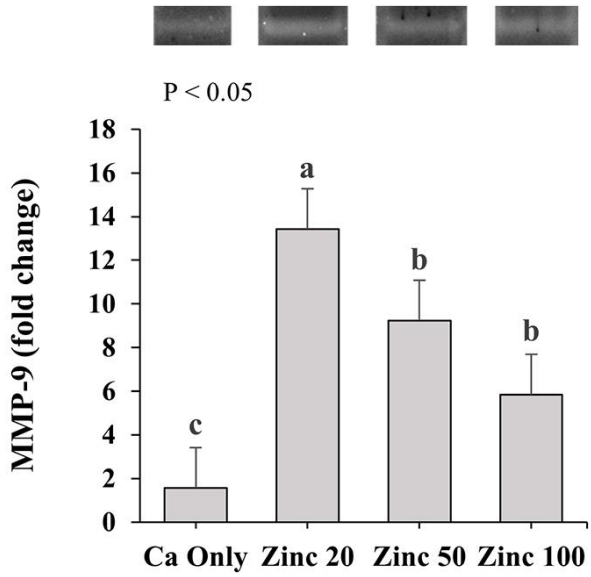

Figure 3.4. Representative collagen zymography showing MMP-9 activity at 72 kDa (top) and MMP-9 activity fold changes relative to a reference sample from the Ca only treatment (bottom). The collagen gels were subjected to different inclusions of calcium and zinc: Ca Only (5 mM CaCl2), Zinc 20 (5 mM CaCl2 + 20 µM of ZnCl2), Zinc 50 (5 mM CaCl2 + 50 µM of ZnCl2) and Zinc 100 (5 mM CaCl2 + 100 µM of ZnCl2). Data are least-squares means ± SE. Different superscripts indicates means differ significantly ($P < 0.05$).

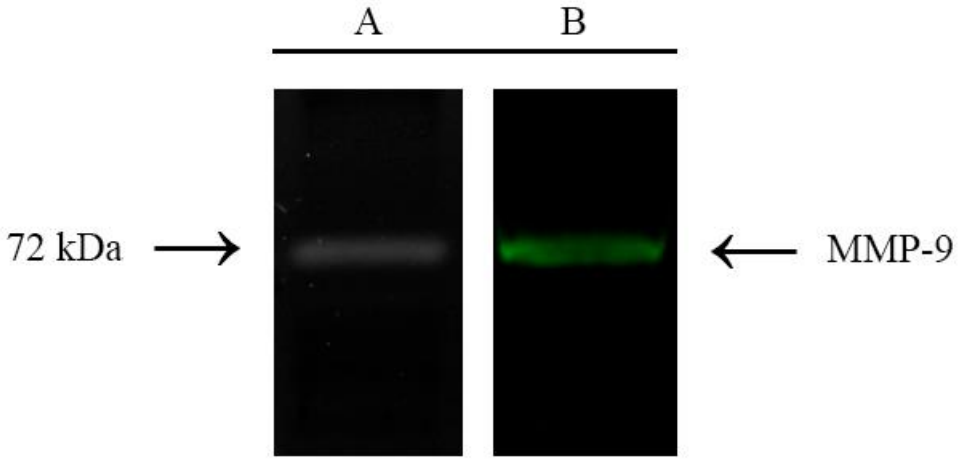

Figure 3.5. Representative image of a collagen zymography gel (A) and a Western Blot (B) indicating activity of MMP-9 at 72 kDa in the Gastrocnemius muscle.