An investigation to understand the contribution of collagen characteristics to Asian consumers' eating preference of six different beef shank cuts

by

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Abstract

The objective of this study was to evaluate factors affecting Asian consumers' visual and eating preferences as well as understanding the effect of mature collagen crosslink densities on cooked beef tenderness and connective tissue texture of six different beef shank cuts. Six beef shank muscles, three from the forequarter [biceps brachii (BB); deep digital flexor - foreshank (DDF-F); extensor carpi radialis (ECR)], and three from the hindquarter [flexor digitorum superficialis (FDS), deep digital flexor - hindshank (DDF-H); a combination of long digital extensor, medial digital extensor and peroneus tertius (LMP)] were collected from 12 USDA Low Choice beef carcasses. Shanks from the left sides were designated for cooked treatment (stewed in water for 90 minutes at 93°C) and used for Asian consumer panels, Warner-Bratzler shear force (WBSF) and cooked collagen content and characteristics. The right sides were designated for raw treatment and used for visual panels, objective color measurement, proximate analysis and raw collagen content and characteristics. In addition, soluble and insoluble collagen percentages were calculated. For Asian consumer panels, BB, FDS, and LMP all received higher ratings for tenderness, juiciness, and sensory overall liking, followed by ECR and DDF-H, with DDF-F received the lowest score ($P < 0.01$). For portion size preference and visual overall liking, BB and ECR received the highest rating, followed by DDF-H, DDF-F and LMP, with FDS received the lowest rating ($P < 0.01$). For connective tissue characteristics and mature collagen crosslink densities, DDF-F had the toughest connective tissue texture, greatest shear force value, most cooked collagen content, greatest insoluble collagen percentage as well as greatest raw and cooked pyridinoline (PYD) densities among all the beef shank cuts ($P < 0.05$). It was interesting to note that DDF-F, FDS, and LMP all started with similar raw collagen content ($P > 0.10$), but DDF-F ended with greater cooked collagen content than the others.

Cooking only decreased PYD density for DDF-F ($P < 0.05$), and PYD density for the rest of the beef shank cuts was not affected by cooking ($P > 0.05$). Furthermore, a correlation analysis was conducted in order to understand the contribution of collagen characteristics to cooked beef tenderness. Cooked collagen content, insoluble collagen percentage as well as raw PYD densities had positive correlations with connective tissue texture ($r = 0.550$, 0.498 and 0.560 respectively; $P < 0.01$) and WBSF ($r = 0.615$, 0.392 and 0.730, respectively; $P < 0.05$). This study showed that tenderness and juiciness directly affected Asian consumers' eating preference, while shank size affected their visual preference for beef shank cuts. Moreover, our results demonstrated that cooked (insoluble) collagen contributed to the background toughness, and PYD is a heat stable collagen crosslink that may require extensive heat treatment to degrade and allow for the solubilization of collagen. In addition, raw PYD density may be a great indicator for cooked beef connective tissue texture and ultimately, tenderness in beef cuts with high concentration of connective tissue prepared with moist heat cookery.

Table of Contents

List of Figures

List of Tables

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Dedication

I would like to dedicate this thesis to my parents Baimei and Rubin, as well as my husband Kanin. I could not have done this without their unconditional love and support.

Chapter 1 - Review of Literature

Introduction

Connective tissue sheaths serve as scaffold to allow the growth and development of muscle tissue (Nishimura et al., 2002). Additionally, the strength and elasticity of connective tissue sheath allows for the transmission of the forces generated by muscle contraction between muscles and bones during movement (Nishimura et al., 2002). Due to the inherent nature of connective tissue toughness, the connective tissues are known to provide the "background toughness" in meat tenderness evaluation (Hill, 1966). Therefore, meat tends to be less tender when there is high amount of connective tissue presented, which would also affect the overall eating experience from consumers.

The major component of connective tissue is collagen. Within the connective tissue, collagen fibers are stabilized by both divalent and trivalent crosslinks which are also referred to as the immature and mature crosslinks: the heat-labile immature crosslinks are mainly existed in younger animals, and transform into heat-stable mature crosslinks as animal age (Roy et al., 2015b). The replacement of immature crosslink with mature crosslink can decrease collagen solubility which resulted in tougher meat (Nishimura, 2010). Pyridinoline (PYD) and deoxypyridinoline (DPD) are some of the key mature crosslinks found in collagen, and Weston et al. (2002) reported that the density of mature crosslinks in collagen is considered to be one of the key factors in collagen-related toughness. Both Bosselmann et al. (1995) and Smith and Judge (1991) measured the collagen solubility and PYD density in bovine muscles and found an increase in PYD density as the collagen solubility decreased. This negative relationship between mature collagen crosslink density and collagen solubility may further transcribe into a similar relationship between mature collagen crosslink density and meat tenderness. In addition, Lepetit

(2007) further speculated that collagen crosslinks may be biomarkers for meat toughness. As the connective tissue plays such an important role in meat tenderness, it is necessary to conduct a detailed review to further understand its characteristics. Therefore, this literature review aims to describe the structure and component of connective tissue, collagen, and collagen crosslink, as well as how the modification of those components affect collagen solubility and meat tenderness.

Connective tissue

Connective tissue structure and component

The connective tissue consists of three components: cells, ground substances, and protein fibers (Betts et al., 2014). In general, the connective tissue cells such as fibroblast are scattered in a matrix, and the matrix consists of ground substances that are crisscrossed by protein fibers such as collagen, elastin, and reticulin, which were produced by the connective tissue cells. The ground substances are usually fluid or gel-like, and it determines the permeability of the connective tissue layer (Betts et al., 2014). The composition, quantity and structure of the three connective tissue components are dependent on the function of the specific tissues. For example, the connective tissue sheath endomysium provides structural support by surrounding the tiny muscle cells, while the tendon provides the connection between whole muscles and bones (Betts et al., 2014). Therefore, one cannot assume connective tissue components are similar across the board.

The skeletal muscle contains three main structural components, which includes the endomysium, perimysium, and epimysium (Purslow, 2005). Endomysium is the layer of connective tissue that surrounds the muscle fiber. Perimysium is the connective tissue layer that surrounds the muscle bundles, and epimysium is the outer layer of connective tissue that surrounds the whole muscle (McCormick, 1999). The amount of perimysium varies greatly from

one muscle to another, while the amount of endomysium stays constant (Nakamura et al., 2003; Lepetit, 2008). Among these three structural components of connective tissue in muscles, perimysium is considered the most important in meat tenderness and texture because it represents about 90% of total connective tissues within muscles (McCormick, 1999), while epimysium is not considered to be a major factor because it can be easily removed through the trimming of meat exterior (McCormick, 1999). The perimysium is categorized into primary and secondary perimysium. The primary perimysium is thinner and surrounds a group of muscle fibers to form into primary muscle fiber bundles, while the secondary perimysium is thicker and surrounds a group of primary muscle-fiber bundles and form into secondary muscle fiber bundles (Purslow, 2005; Nishimura et al., 2009). Both of the perimysial layers form a network structure that extends across the cross-section of the muscles.

Perimysium characteristics

Perimysium thickness will vary in animals with different age, weight and muscles (Purslow, 2010). Żochowska et al. (2005) investigated the perimysium thickness of biceps femoris (BF), semimembranosus (SM), and quadriceps femoris (QF) from wild boars in young (6 months) and mature (3 years) stages with average carcass weight of 20 and 60 kg, respectively. They found that BF had the thickest perimysium among all muscles, and young pigs had thinner perimysium than the mature animals. Similarly, Fang et al. (1999) found that as the pigs grew, the secondary perimysium became thicker, and the shear force value of raw porcine semitendinosus (ST) muscle increased from 0.24 kg at 1 month of age to 0.31 kg at 6 month of age. The same study also reported that the secondary perimysium thickness was positively correlated with shear force (r = 0.98) and concluded that perimysium thickness was closely related to pork toughness. On the other hand, Brooks and Savell (2004) reported that

perimysium thickness in bovine ST muscle was 2.4 times thicker than psoas major (PM) from the same animal. Finally, Liu et al. (1996) evaluated the thickness of perimysium of various chicken muscles and also observed a strong positive correlation (r = 0.95) between the shear force value and perimysium thickness.

Collagen

Collagen structure

The connective tissue contains different fibrous proteins, such as collagen, elastin, and reticulin (Betts et al., 2014). Among these three fibrous proteins, collagen is the most abundant, and it is the main structural component of the connective tissues (~55-95% of the connective tissue on a dry matter basis; (Tornberg, 2005). There are three major amino acids can be found in collagen: glycine, proline, and hydroxyproline. On a molar percentage basis, collagen contains about 33% glycine, 23% proline plus hydroxyproline, and 43% of other amino acids (Li and Wu, 2018). The repeating amino acid sequences of mainly glycine, proline and hydroxyproline form into a polypeptide chain, and three polypeptide chains are connected by hydrogen bonds to form a superhelix/triple helix collagen structure (Figure 1.1), also known as a collagen fibril (McCormick, 1999). The staggered collagen fibrils are packed side-by-side in parallel bundles to form into collagen fibers (Lodish et al., 2000). It is important to note that among all the amino acids, hydroxyproline is a unique amino acid in collagen, and it helps to stabilize the collagen structure (Stoilov et al., 2018). Therefore, hydroxyproline content is typically used as a marker to quantify the amount of collagen in muscles.

There are different types of collagen fiber that can be found in muscle tissues, and the more common ones are type I, II, III, and V (Nishimura et al., 1997; Listrat et al., 1999). Type I is mostly found in tendon, bone, and ligaments, type II is mostly found in cartilage, type III is

mostly found in skin and muscle, and type V is mostly found in fetal tissue (Lodish et al., 2000). Bailey et al. (1979) documented that type I and II were both found in the perimysium, while types III and V collagen were found in the endomysium. Among all of the collagen types, types I and III collagen are the predominant collagen found in skeletal muscles (Nishimura, 2010).

Collagen content variation among muscles

Collagen content varies from muscles to muscles. Locomotion muscles tend to have higher collagen content than supportive muscles. Wheeler et al. (2000) reported that the total collagen content in BF (7.1 mg/g muscle tissue) was higher in comparison to longissimus lumborum (4.1 mg/g muscle tissue) in pigs. Rhee et al. (2004) reported that the supportive muscle such as PM, gluteus medius (GM), and longissimus lumborum had lower collagen concentration (2.7 to 4.5 mg/g muscle tissue) in comparison to the locomotive muscles such as triceps brachii, SM, and ST (5.9 to 9.0 mg/g muscle tissue) in beef. Moreover, Vognarova et al. (1968) determined the collagen and elastin content in different cuts of veal and beef, and they concluded that the amount of connective tissue in meat was dependent upon the anatomical location and the physiological function of the muscles. In general, consumers prefer more tender meat, and the collagen content in meat becomes a factor that can influence the meat tenderness. reported that there was a strong positive relationship ($r^2 = 0.95$) between the value of meat and collagen content which indicated that increased collagen content decreased the eating quality of meat.

Heat treatment and collagen solubility

When heat is applied and reached to the denaturation temperature for collagen, this protein will go through conformation changes such as contraction and gelatinization (Tornberg, 2005). Martens et al. (1982) reported that initial collagen denaturation occurred at temperatures

between 53 and 63°C, and they speculated the denaturation was due to the breakage of hydrogen bonds, which resulted in the contraction of the collagen molecules. Palka (1999) evaluated the collagen solubility of ST muscle in beef with different endpoint temperature (50, 60, 70, 80, 90, 100, and 121°C) and confirmed that initial collagen denaturation in the perimysium occurred at 60°C, and gelatinization was observed when the temperature reached to 80°C. Ismail-Fitry et al. (2011) also used bovine ST muscle to construct restructured meat with application of different cooking temperatures (60, 70, and 80°C). They found that collagen gelatinized at 80°C, and the gelatinization actually contributed to binding of the restructured meat product.

In addition to evaluating the collagen exclusively, Li et al. (2008) utilized water-bath heating to cook bovine ST muscle and observed the change of perimysial and endomysial microstructure during heating. They found that the secondary perimysium remained intact from 40 to 60°C, and it started to dissolve as the temperature increased to 65°C. At an internal temperature of 75°C, perimysium became thinner due to the tearing of the perimysial layer. On the other hand, the endomysium were partially melted at 55°C and completely melted by 65°C (Li et al., 2008). Chang et al. (2011) also utilized water bath heating to cook bovine ST muscle to different endpoint temperature (40, 50, 60, 70, 80, and 90°C), and they observed similar results which showed the structure of endomysium started to dissolve by 50°C.

The utilization of different cooking time and temperature can influence collagen solubility and improve the overall eating quality. Voller-Reasonover et al. (1997) measured the texture and collagen solubility of poultry with three processing temperatures and exposure time (26 min at 115.6°C, 12 min at 121.1°C, and 6 min at 126.7°C) and reported that the longer exposure to moist heat allowed the meat to have greater collagen solubilization as well as lower hardness values which might improve tenderness in chicken. Thompson (2002) concluded that

grilling was appropriate for low connective tissue cuts, while roasting (exposure to lower temperature in dry heat for longer period of time) was more ideal for high connective tissue cuts because the water in meat will provide moisture and aid the collagen breakdown. Finally, slow cooking with moist heat cookery is the single most effective known method to soften the connective tissue and reduce connective tissue toughness (Calkins and Sullivan, 2007).

Animal age and collagen solubility

As the animal gets older, the collagen solubility will decrease. Young and Braggins (1993) measured the solubility of collagen of lamb SM and GM muscles from 4 months to 5 years of age and reported that the collagen solubility for both muscles declined with age increment. However, the collagen concentration remained the same.

Collagen solubility not only decreases as the animal aged, but it can also vary in different muscles from the same animal. Archile-Contreras et al. (2010) investigated the collagen content in bovine LD and ST muscles, and reported that the percentage of total collagen that was heat soluble was lower for ST than the LD muscle. Herring et al. (1967) compared the collagen solubility between bovine LD and SM muscles and reported LD has greater soluble collagen than SM, and the collagen solubility decreased as the cattle increase in maturity for both muscles. Additionally, Herring et al. (1967) showed that collagen solubility was positively correlated with tenderness in both muscles (r = 0.77 and 0.81, respectively) evaluated by trained panelists. These results demonstrated that locomotion muscles tend to have less soluble collagen concentration than the supportive muscles, and this may partially explain tenderness differences of different muscle location.

Effect of collagen content in meat tenderness

There are two components that determine the ultimate tenderness of muscle: the myofibrillar component which relates to the ultrastructure of muscle fiber, and stromal component which is related to the content, composition, and structure of connective tissue (Jeremiah et al., 2003a). The collagen fibers possess an extremely high tensile strength, and the physical properties change as the animals become older (Shimokomaki et al., 1972). Although connective tissue provides the background toughness to the meat, the collagen content may not be a factor that influence the overall tenderness. Crouse et al. (1985) evaluated the collagen content and palatability of bovine LD and SM muscles and reported that the total collagen and tenderness were not strongly correlated, and the correlation of total collagen with sensory panelists' perception of connective tissue was very low (r = -0.10). In addition, DeVol et al. (1988) measured WBSF, collagen content, and conducted sensory panel in porcine longissimus muscles with tenderness and connective tissue amount were evaluated by experienced panelists. They observed that the collagen content was not related to tenderness from sensory panel (r=-0.01) or WBSF values (r = -0.08), but the connective tissue amount was highly correlated with sensory tenderness rating (r = 0.91). Additionally, Cross et al. (1973) investigated the effects of intramuscular collagen and elastin on bovine muscle tenderness and reported that there were no significant correlations between total collagen content and amount of connective tissue rating, as well as no significant correlations between elastin content and amount of connective tissue. On the other hand, Torrescano et al. (2003) measured the WBSF values of raw samples from 14 different bovine muscles and reported that there was a positive correlation between total collagen content and WBSF (r = 0.72). The use of raw samples for tenderness measurement instead of cooked meat may be the reason for the strong positive correlation because the collagen in raw

meat had not been denatured and solubilized. Listrat et al. (2016) concluded in their review that the shear force of raw meat was highly correlated with its collagen content, while the level of correlation between cooked collagen content, solubility, or crosslink density and meat shear force may vary for different muscles and cooking conditions.

Collagen crosslink

Collagen crosslink formation

There are two types of collagen crosslinks: the intramolecular crosslinks and the intermolecular crosslinks. Within each collagen fiber, there are four crosslinking sites, where two are found toward the amino-terminal end, while the other two are found toward the carboxyl-terminal end. Of the two crosslinking sites at each terminal, one site can be found in the telopeptide region and one in the helical region of each terminal (Weston et al., 2002). The crosslink sites at the helical region are originated from the hydroxylysine residues, and the crosslink sites at the telopeptide region is originated from lysine residues. (Weston et al., 2002).

The crosslink formation begins with initiation by lysyl oxidase from lysine or hydroxylysine residues of the telopeptide region of the collagen molecule, and these residues will convert into either allysines or hydroxyallysine, respectively (Bailey, 1972; Lawrie, 1980; Bailey, 1989; McCormick, 1994). Within these two pathway of crosslink formation, the allysine produces aldimine crosslinks formed from lysine aldehydes, and it is easier to break down due to the initially-formed aldimines can be separated at low pH, which allowing the collagen monomers to be solubilized in acid (McCormick, 1999; Eyre and Wu, 2005). On the other hand, the hydroxyallysine results in ketoamine crosslinks arising from hydroxylysine aldehyde, and it is harder to analyze because the collagen is insoluble due to the stability of initial crosslinks and their maturation products (McCormick, 1999; Eyre and Wu, 2005).Finally, the allysine or the

hydroxyallysine react with a lysine, hydroxylysine, or histidine residue in the triple helix to form intramolecular or intermolecular crosslinks.

The intramolecular or immature crosslink can only link two collagen molecules together and makes it unlikely to contribute to the overall tensile strength of the fiber (Lawrie, 1980). Due to its nature to only connect two collagen molecules together, the immature crosslinks are also known as divalent bonds. The collagen molecule are aligned in a quarter-stagger formation, and the immature crosslinks have been assumed to condense with lysine and hydroxylysine in neighboring α-chains to form into intermolecular or mature crosslinks that can link one collagen molecule to the others (McCormick, 1999; Eyre and Wu, 2005; Bruce and Roy, 2019). It is important to note that the mature crosslink not only link the individual quarter-staggered collagen molecules, but it can also link a neighboring collagen fiber transversely (Bailey, 1989). The ability of mature crosslink to link to an extra neighboring collagen fiber on top of the two collagen molecules give it the name: trivalent bond (Lawrie, 1980).

The presence of mature crosslink is crucial to the mechanical stability of the collagen fiber as they provide the structure and strength for collagen's intended function (Bailey, 1989; Weston et al., 2002; Maynes, 2012). There are two main types of mature crosslinks: the first type is the result of enzymatic reaction (Bailey et al., 1998; Eyre and Wu, 2005; Depalle et al., 2015), while the second type comes from advanced glycation end-products (Bailey et al., 1998; Bailey, 2001; Depalle et al., 2015). The enzymatic reaction is related to initiation of lysyl oxidase into different aldehyde and converted to allysine and hydroxyallysine as described previously. The second type of mature crosslink is from the advanced glycation end-products. The enzymatic pathway are formed at specific terminal domains, while the advanced glycation end-products can form at different locations of the collagen molecule. However, the factors and pathways that lead

to formation of these glycation end-products are not fully characterized (Bailey, 2001; Viguet-Carrin et al., 2006).

The role of pyridinoline in thermal stability, animal maturity, and muscle location

In meat, the presence of mature crosslinks is a key factor in collagen-related toughness (Weston et al., 2002). The collagen fibers build up a three-dimensional network of mature crosslinks, which can significantly increase the stability of the collagen (Bailey, 1989). The PYD and its minor analog, DPD are two mature crosslinks of collagen that are derived from the enzymatic pathway (Burr, 2019). Both PYD and DPD are naturally fluorescence, and PYD has been used as a gold standard for measuring collagen crosslink in skeletal muscles (Eyre et al., 1984). PYD contains a pyridine, 6-sided ring structure (Bruce and Roy, 2019), and it connects three collagen α-chains. The increasing concentration of PYD has been associated with increasing thermal denaturation temperature (Horgan et al., 1990).

PYD is one of the most used biomarker to quantify mature collagen crosslink content. Fujimoto et al. (1977) was among the first groups of researchers that isolated PYD from bovine tendon and categorized PYD as a heat-stable collagen crosslink. Dubost et al. (2013) evaluated and compared the total collagen content and PYD crosslink density in bovine longissimus thoracis (LT), SM, and BF grilled to 55°C, and they found that PYD crosslink density negatively affect sensory tenderness with muscles only cooked to 55°C. They concluded that PYD was a thermo-stable mature crosslink that could not be broken down easily with low end-point temperature (Dubost et al., 2013). Young et al. (1994) evaluated the PYD density of ovine SM, BF, and GM, and they concluded that PYD density was inversely correlated with collagen solubility and also noted that PYD crosslink was insoluble in the heat-dependent solubility test.

These studies demonstrated that PYD is a heat-stable mature crosslink that cannot be broken down easily even with extensive period of cooking time.

Animal maturity plays an important role in PYD density. Smith and Judge (1991) measured the collagen content as well as PYD density of SM from Holstein in different USDA maturities (A, B, C, D, and E) and found that the PYD density of E maturity cattle (0.14 mol/mol of collagen) was higher than the A maturity cattle (0.04 mol/mol of collagen). Additionally, there was a significant positive correlation between PYD density and maturity (r = 0.56). Smith and Judge (1991) also reported that collagen solubility decreased from 6.66% (A maturity) to 3.24% (E maturity), and there was a negative correlation between cattle maturity and collagen solubility (r = -0.47). Shiba et al. (2007) investigated the PYD density in caprine gastrocnemius and soleus from animals ranged from two weeks prenatal to 24 weeks of age and reported that there was a significant effect for age and muscle. Shiba et al. (2007) observed that PYD density increased along with animal age, and this was expected because the goats are still in growing stages. The PYD density from both muscles began to increase during the prenatal period and continued to increase when reached to the prepubertal period. At 20 weeks of age, Shiba et al. (2007) observed that caprine soleus muscle (0.22 mol/mol of collagen) had higher PYD density than gastrocnemius muscle (0.11 mol/mol of collagen), and they reported that the differences of PYD density in both muscles may due to soleus muscle was a slow twitch muscle that had high rate of collagen development for posture, which resulted in PYD accumulation more than the fast twitch muscle (gastrocnemius). Steinhart et al. (1994) evaluated the PYD density in sternomandibularis muscle from 2 to 11.5-year-old cows and found that the PYD density increased as animals age. However, Roy et al. (2015a) measured the collagen content, PYD density, and PYD concentration in bovine ST and GM from steers and mature cattle. They determined that PYD

density did not change with animal age in both muscles, but the PYD concentration increased with animal age due to increase in collagen content.

The PYD density vary depends on the muscle location. Bosselmann et al. (1995) measured the PYD density of bovine LD, ST, and extensor carpi ulnaris (ECU) and reported that the mature collagen crosslink density was closely related to the physiological functions of the muscles. LD is a supportive muscle which mostly carrying capacity of the vertebrae, and the animal utilizes this muscle during forward and backward movement (Bosselmann et al., 1995). On the other hand, ST and ECU were locomotive muscles, and these two muscles tend to experience more strain or force when the animal is standing or walking (Bosselmann et al., 1995). Hence, the epimysial PYD density of ST and ECU were found to be much higher than the LD epimysial PYD density. This was especially true for ECU due to its smaller muscle size, and its epimysium has more tendon-like function and texture (Bosselmann et al., 1995). Steinhart et al. (1994) measured the PYD density in bovine sternomandibularis (neck) muscle and ligamentum nuchae (backstrap) and found that the ligamentum nuchae had much higher PYD density compared to sternomandibularis. This is an interesting find as ligamentum nuchae is mainly elastin instead of collagen. This further demonstrated PYD density is expected to be higher in elastin than collagen. However, there is still a lot of unknowns in collagen crosslink analysis.

Conclusion

The structure and component of connective tissue has been well studied from many of the previous literatures because connective tissue provides the “background toughness” in meat. Perimysium is the major layer of connective tissue that concerns the most with meat tenderness, and it requires adequate temperature and cooking time in order for the collagen within the

perimysium to break down and dissolve. On the other hand, collagen content will vary in different muscles. Locomotion muscles tend to contain greater collagen content, while supportive muscles have less collagen content due to the differences in requirement for physical movement. Moreover, the crosslink formation in between collagen molecules also plays an important role in meat tenderness. These mature crosslinks are heat stable and their density will increase as animal age, while the collagen solubility decreases at the same time. Most of the previous studies focused on evaluating the palatability effect of connective tissue in meat based on Western consumers' point of view without putting much effort on the collagen crosslinks. Therefore, the purpose of this research is to investigate factors affecting Asian consumers' visual and eating preferences, as well as mature collagen crosslink densities and their relationship to cooked beef tenderness and connective tissue texture using stewed beef shanks.

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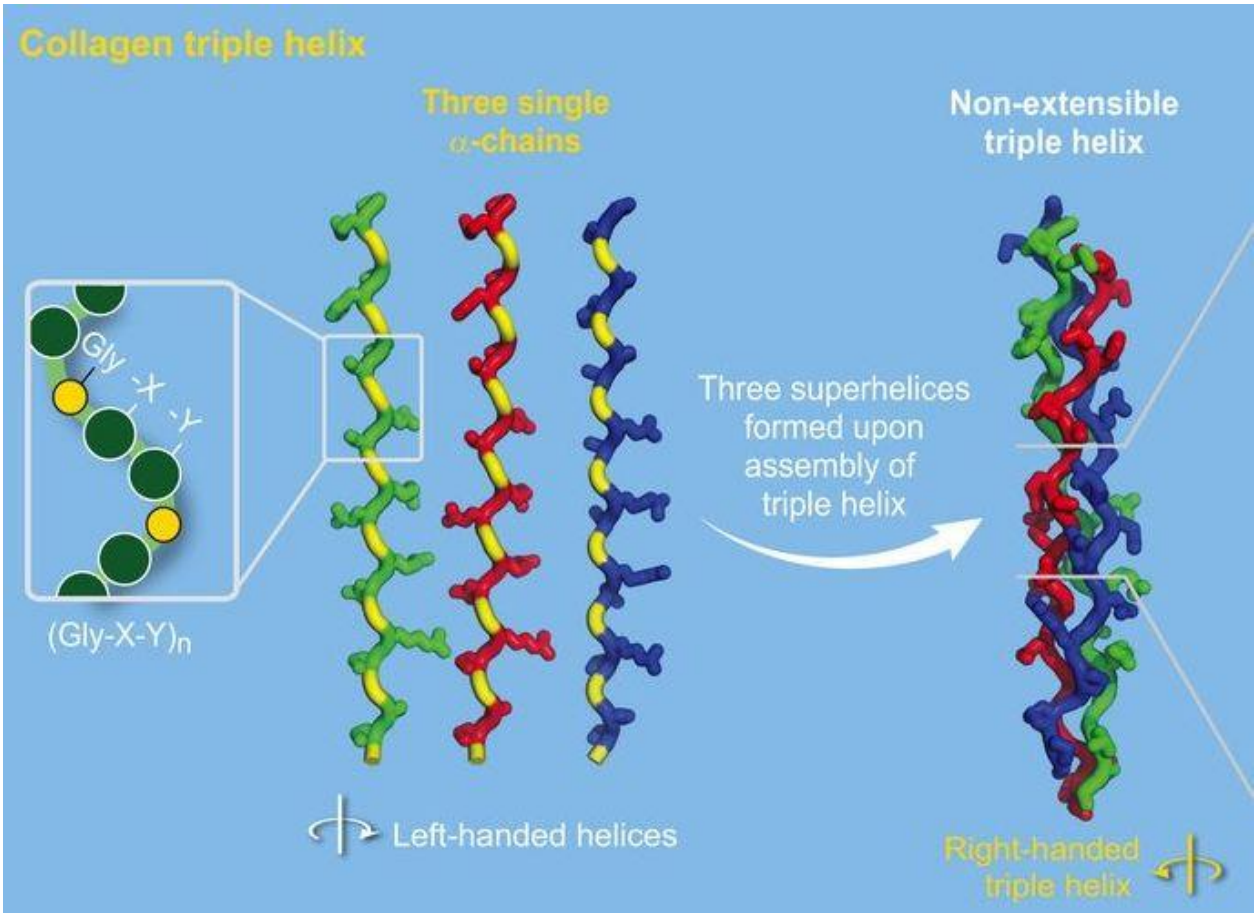

Figure 1.1 Collagen superhelix/triple helix structure: three amino acids (Gly - glycine, X - proline, and Y – hydroxyproline) made up to form a polypeptide or alpha chain, and three of these alpha chains wrap with each other to form a triple helix collagen structure (Fidler et al., 2018)

Chapter 2 - Factors affecting Asian consumers' visual and eating preferences of beef shank cuts

Abstract

The objective of this study was to evaluate factors affecting Asian consumers' purchasing decisions and eating preferences of six different beef shank cuts. Six beef shank muscles, three from forequarter [*biceps brachii* (BB); *deep digital flexor* - foreshank (DDF-F); *extensor carpi radialis* (ECR)], and three from hindquarter [*flexor digitorum superficialis* (FDS), *deep digital flexor* - hindshank (DDF-H)*;* a combination of *long digital extensor, medial digital extensor* and *peroneus tertius* (LMP)] were collected from 12 USDA Low Choice beef carcasses (n = 72). Shanks from the left sides were cooked using moist heat cookery for consumer panels using only Asian consumers, and the right sides were used for visual panels as well as objective color measurements and proximate analysis. The BB, FDS, and LMP showed higher ratings for tenderness, juiciness, and sensory overall liking, followed by ECR and DDF-H, with DDF-F had the lowest scores ($P < 0.01$). For portion size preference and visual overall liking, BB and ECR received the highest rating, followed by DDF-H, DDF-F and LMP, with FDS received the lowest rating ($P < 0.01$), but Asian consumers indicated that there was no visual difference in surface color among the shank cuts ($P > 0.10$). However, FDS had the highest L* value, followed by BB, DDF-F, ECR, and DDF-H, with LMP had the lowest L* value in cross-section area ($P < 0.01$). The BB, DDF-F, FDS, DDF-H, and LMP all had greater collagen and fat content, but less moisture compared to ECR ($P < 0.01$). These results indicated that tenderness and juiciness directly affected Asian consumers' eating preference, while shank size affected their visual preference for beef shank cuts. On the other hand, collagen, fat and moisture content of the raw shank cuts did not seem to have a direct relationship with Asian consumers' eating preference.

Key words: beef shank, connective tissue amount, shank size, collagen

Introduction

Beef shanks are general description of a list of locomotive muscles located in beef round and foreshank that represents approximately 3.3% of the carcass value (Grand View Research, 2019). Beef shank cuts are considered to be low value in the U.S. due to the high density of connective tissue within the cuts; therefore, the majority of beef shank meat produced in the U.S. are turned into ground beef (USDA, 2020). However, connective tissues will go through a series of conformation changes depending on the cooking time and temperature (Tornberg, 2005). Martens et al. (1982) reported that the initial collagen denaturation will occur when the cooking temperature reached between 53-63°C, and this was due to the breakage of hydrogen bonds which resulted in the contraction of collagen molecules. Furthermore, collagen solubilization/gelatinization will occur when temperature reached to 80°C (Palka, 1999; Ismail-Fitry et al., 2011). Based on cultural observations, high level of solubilized/gelatinized collagen in beef will significantly contribute to the tenderness and juiciness of beef which creating a sensory effect similar to the lubrication effect from marbling for stewed beef. Jeremiah and Gibson (2003) evaluated different muscles from the round with application of both dry and moist heat cookeries, and they concluded that moist heat cookery was ideal for muscles with high amount of connective tissue due to the collagen gelatinization effect.

Although beef shank cuts are not well sought after in the U.S., stewed beef shank is widely consumed in many Asian nations. The extended cooking time by stewing will soften the connective tissue, and the connective tissue texture is considered a desirable trait to a large portion of Asian palates. This is drastically different from the Western cultures' perception, which overall palatability scores tended to have a negative correlation with the total amount of

connective tissue in meat (Jeremiah et al., 2003a). Currently, the most popular beef shank cut in Asian markets is the banana shank, which is group of three inseparable muscles (*long digital extensor, medial digital extensor* and *peroneus tertius)* that are located on the most anterior end of the hindshank (Jones et al., 2001). It is roughly 900 g (~ 2 lb) in weight, eye appealing in shape, and most importantly, it contains a high concentration of connective tissue (personal experience). Based on a preliminary evaluation of the beef fore and hind shanks in our laboratory, there are at least 5 other shank cuts that demonstrated similar visual characteristics.

With the extensive economic and population growth for many Asian countries in the recent years, there are opportunities to export the low value beef shank cuts from the U.S. to Asian countries in order to meet the global demand and bring in additional profit by exporting these underutilized U.S. beef cuts (Nam et al., 2010). However, there are very few studies that aim to characterize the eating quality and visual appearance of beef shank muscles. Therefore, the objective of this study was to evaluate factors affecting Asian consumers' visual and eating preferences of six different beef shank cuts with the goal to identify other beef shank cuts that demonstrate similar or superior quality compared to the banana shank.

Materials & Methods

The Kansas State University (KSU) Institutional Review Board approved all procedures for use of human subjects in sensory panel evaluations (IRB #7440.5, August 2018).

Sample collection and preparation

At approximately three days postmortem, bone-in foreshanks (NAMP #117) with the humerus and *biceps brachii* attached, heel (NAMP #171F) and bone-in hindshanks (NAMP #157) from both sides of 12 USDA Low Choice beef carcasses were collected from a Midwestern beef processor (n = 72). Following collection, beef shanks were transported to the

KSU Meat Laboratory (Manhattan, KS), and stored in the cooler overnight at 2 ± 2°C. Six shank cuts (Figure 2.1) were fabricated from the subprimals the following day: three from the foreshanks [*biceps brachii* (BB); *deep digital flexor* from foreshank (DDF-F); *extensor carpi radialis* (ECR)], one from the heel [*flexor digitorum superficialis* (FDS)], and two from the hindshanks [*deep digital flexor* from hindshank (DDF-H)*;* a combination of *long digital extensor, medial digital extensor* and *peroneus tertius* (LMP)]. These cuts were determined based on their shape and size that meet the stewing beef shank preferences for Asian households. Both ends of tendons and accessary muscles were trimmed from all shank cuts.

All cuts with total of five days aging were vacuum packaged and stored in KSU Meat Laboratory freezer at -40°C until consumer sensory and visual panels. The left side of the beef shanks were designated for Asian consumer taste panels, and the right side of the beef shanks were used for Asian consumer visual panel, objective color measurements, collagen, fat and moisture analyses. One of the *bicep brachii* muscles from the right side of the carcass was misplaced and thus excluded from the visual panel and all laboratory analyses.

Consumer sensory panel evaluation

Asian consumers (n = 9*1*) were recruited from Manhattan, KS and the surrounding areas. Asian consumers from the Southeast Asia regions were selected if they had been exposed and had the habit of eating beef shanks to ensure the quality of the study. Each consumer was compensated with approximately 1 kg of beef shank meat following the completion of the panels for their participation. Panels were conducted on the KSU campus where consumers were seated into a lecture stadium-style classroom and 6 samples were served in a random order to each consumer. Consumers were supplied with napkins, plastic fork, expectorant cup, unsalted crackers, apple juice, and water to use as palate cleansers between samples. Before evaluation,

consumers were given verbal directions to explain the use of palate cleansers, evaluation procedures, and the digital survey.

Whole beef shank muscles from the left side were thawed for 72 h prior to the panels at 2 ± 2°C. Prior to cooking, raw weight was taken for each muscle. Each beef shank muscle was stewed for 90 min at 93 ± 5°C in a half size pan (32 cm x 27 cm) with 4,000 ml of boiling water using a countertop warmer (X*PRT Series W-3Vi; APW Wyott, Allen, TX). Peak temperature with an average of 91°C was measured using a probe thermometer at the geometric center of each beef shank cut (Thermapen Mk4; ThermoWorks, American Fork, UT), and cooked beef shank cuts were weighed to obtain cooked weight that were used in the cooking loss calculation. Beef shank cuts were placed into warming pots and placed on an electric ceramic glass cooktop (GoldSeries; Whirlpool, Benton Harbor, MI) at 50 ± 2°C to keep warm before serving. Immediately before serving, the dorsal end of each beef shank cuts was faced and cut into two 2.5 cm slices. Each slice was cut into 2.5 cm x 2.5 cm x 2.5 cm cubes, and 2 cubes were served to each consumer.

Each consumer was given an electronic tablet (Model 5709 HP Stream 7; Hewlett-Packard, Palo Alto, CA) with a digital survey (Version 2417833; Qualtrics Software, Provo, UT). Each survey contained a demographic questionnaire and 6 sample ballots in English with Chinese translation. Consumers were asked to evaluate each sample for amount of connective tissue, tenderness, juiciness, and flavor intensity on Just About Right (JAR) line scales. Anchors were set at 0, 50, and 100, with 0 anchored as too little, too dry, too tough, and too bland. At 50, it was the ideal score that anchored as JAR. At 100, anchors were too much, too wet, too soft, and too intense. In addition, consumers were asked to evaluate each sample for overall liking on 0 to 100 continuous line scale, with 0 anchored dislike extremely, 100 anchored like extremely,

and with 50 set as the midpoint anchored neither dislike nor like. Lastly, consumers were also asked to rate each sample as either acceptable or unacceptable with the answer "yes" or "no." Each consumer evaluated a total of six randomized samples with one sample from each beef shank cut. Consumers were first asked to taste the left side of all the beef shank muscles prior to conduct visual evaluation of the right side of the beef shank cuts from the same carcass in the display room.

Consumer visual panel evaluation

Beef shank cuts from the right side of each carcass were thawed 72 h prior to visual panels at 2 ± 2°C. Beef shank cuts were removed from vacuum packages, placed on a Styrofoam tray (#34 & 4S, white; Dyne-a-Pak, Ontario, Canada) with an absorbent pad, and overwrapped with PVC film (HIYG Gold Stretch Meat film, O_2 transmission rate = 1,191 cm^3/0.065 m^2/24 h; Berry Global Inc., Evansville, IN). A sticker with the randomized four-digit number was put on the lower right corner on the wrap for identification purposes. All samples were displayed in coffin style cases (Model DMF8; Tyler Refrigeration Corporation, Niles, MI) at 2 ± 2°C. The same group of Asian consumers that had finished the sensory panels were then (n = 84) visually evaluated each beef shank cuts under fluorescent lighting (Model F32T8, 32 Watt, Warm White 3000 Kelvin; Philips Lighting Company, Somerset, NJ) in the KSU Meat Color Laboratory. Consumers were provided an electronic tablet with a digital survey, and each survey contained 6 sample ballots in English with Chinese translation. The survey program was programed to assign the order that each beef shank was evaluated. Consumers were asked to visually evaluate each sample for size and color on JAR line scales. Anchors were set at 0, 50, and 100, with 0 anchored as too small and too light. At 50, it was the ideal score that anchored as JAR. At 100, anchors were too large and too dark. Moreover, consumers were asked to evaluate each sample

for overall liking on 0 to 100 continuous line scale, with 0 anchored dislike extremely, 100 anchored like extremely, and with 50 set as the midpoint anchored neither dislike nor like. Lastly, consumers were also asked to rate each sample as either acceptable or unacceptable with the answer "yes" or "no." Consumers evaluated a total of six randomized samples with one sample from each beef shank muscle. After the visual panels, all beef shank cuts from the right side were vacuum packaged and stored in -40°C freezer until further analysis.

Objective color measurement

Beef shank cuts from the right side of the carcasses were thawed for 72 h at 2 ± 2°C prior to secondary fabrication. Beef shank cuts were cut in half in the center, the dorsal-end half was overwrapped with PVC film (HIYG Gold Stretch Meat film, Berry Global Inc) and placed into coffin cases (Model DMF8; Tyler Refrigeration Corporation) at 2 ± 2°C for at least 30 minutes prior to objective color measurement. A Hunter Lab MiniScan EZ spectrophotometer (Model 4500L, Illuminant A, 2.54-cm aperture, 10° observer; Hunter Associates Laboratory Inc., Reston, VA) was used to obtain the color measurement on each sample cross-section by following the CIE L* (lightness), a*(green to red), b* (blue to yellow) system described in Meat Color Measurement Guidelines (AMSA, 2012). Objective color measurements were obtained at three random location on the cross-section of each sample, and the final value was acquired by averaging the three readings. The ventral half was vacuumed packaged and stored at -80°C and designated for laboratory analyses.

Moisture and fat analysis

Beef shank cuts designated for laboratory analysis (n = 72) were thawed for 24 h prior to pulverization at 2 ± 2°C. Each sample was diced, frozen in liquid nitrogen and pulverized using a commercial grade blender (Model S1BL32; Waring Products Division; Hartford, CT). The

moisture and fat percentages were determined using the AOAC 2008.06 method (AOAC, 2012) with a CEM Smart System 5 with Smart Trac Rapid Fat Analysis (CEM Corporation, Matthews, NC). Approximately 3.5 - 4.5 g of pulverized samples was spread evenly in between 2 CEM glass fiber square sample pads (Part #200150; CEM Corporation, Matthews, NC) and analyzed for moisture percentage using 100% power setting at 125 ^{o}C. After the moisture analysis, CEM pads containing the samples were placed on top of a Trac film (Part #159875; CEM Corporation, Matthews, NC), folded, and rolled into a cylinder and fit into the Trac tube (Part #160505; CEM Corporation, Matthews, NC) for lipid analysis. Both moisture and fat samples were measured in duplicates.

Collagen sample preparation

Sample preparation procedure was based on the protocol described by Avery et al. (2009) with modification. Approximately 500 mg of pulverized muscle tissue was weighed in a 15 x 125 mm glass tube, and mixed with 10 ml of 6N hydrochloric acid to each sample, capped with a PTFE coated cap, and placed samples into an oven (Isotemp, Fisher Scientific, Hampton, NH) for 24 hrs at 115°C. Samples were removed from the oven and cooled to room temperature. The 6N hydrochloric acid was evaporated and trapped using a Vacuum Evaporation System (RapidVap; Labconco Corporation, Kansas City, MO) with the following settings: 70 °C, 53% vortex speed, and 200 mbar vacuum until complete dryness (~24 h). After evaporation, samples were rehydrated with 0.5 ml of ultrapure water and stored at -80° until further analysis.

Collagen analysis

Collagen content was determined by measuring the amount of hydroxyproline and followed the protocol described by Bergman and Loxley (1963) with modification. Samples were diluted 1:200 with ultrapure water, and 0.5 ml of diluted sample was transferred to a clean

15 x 125 mm glass tube and diluted one more time in 1:4 with ultrapure water to a total volume of 2 mL. Hydroxyproline (1mM; trans-4-Hydroxy-L-proline, ≥ 99% purity; Sigma-Aldrich, St. Louis, MO) standards were made fresh on the day of analysis and the hydroxyproline standard curve final concentrations were: 0, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, and 10.5 µg/mL. For all standards and samples, 1 mL of chloramine-T hydrate buffer solution (6 mM chloramine-T hydrate, 140 mM citric acid monohydrate, 38 mM sodium hydroxide, 661 mM sodium acetate trihydrate, 29% of 1-propanol, at pH 6) was added, vortexed, and incubated for 20 min at room temperature. After incubation, 1 mL of 4-dimethylaminobenzaldehyde (DMBA) color reagent (67 mM of DMBA was dissolved in 21% perchloric acid, 35% 2-propanol, and 14% ultrapure water) was added to each sample. Samples were immediately covered with aluminum foil and incubated in water bath for 90 min at 60°C for full color development. Samples were removed from water bath, placed in cold water for 3 min, and 0.2 ml of sample was pipetted to a 96 wells plate (Nest Biotechnology Co., Ltd., Jiangsu, China) in duplicates. Hydroxyproline determination was performed using a spectrophotometer (Eon, BioTek Instruments Inc., Winooski, VT) with the absorbance at 558 nm. The hydroxyproline concentration was calculated using the standard curve, and the collagen content was determined by multiplying the hydroxyproline content by 7.14 and a final unit for collagen content was displayed as mg collagen per g of muscle tissue.

Statistical analyses

Statistical analyses were performed by using PROC GLIMMIX procedure of SAS (SAS Version 9.4; SAS Inst. Inc., Cary, NC), and treatment comparisons were considered as significant with an α of 0.05. Data were analyzed as a completely randomized design using a model with the fixed effect of beef shank cuts, and animal was considered as the random effect. For acceptability data, a model with binomial error distribution was used. The Kenward-Roger

approximation was used for all analyses to estimate the degrees of freedom. Separation of means was conducted using LSMEANS procedure with PDIFF option at $P < 0.05$.

Results and Discussion

Consumer panel demographics and consumption preference

The demographic profile of the 91 Asian consumers who participated in the consumer sensory panels are presented in Table 2.1. Participants were primarily Chinese (74.7%) with similar number of males (50.6%) and females (49.4%). The majority of the participants were married (72.5%), with 56.0% of consumers having a household size of 3-4 people. Moreover, most of the consumers had an annual household income of less than $50,000 (63.8%), but with an advanced degree (63.7%). When asked about beef shank meat tenderness preference, 36.3 % of consumers preferred their beef shank meat to be cooked to semi-tender (with slight amount of chewiness), and 57.1% of the consumers preferred the beef shank meat to be tender (Not chewy, but able to maintain structural integrity when bite into the shank meat), with only 6.6 % of the consumers preferred the shank meat to fall apart in their mouth. Finally, some consumers ate beef shanks few times a year (46.2%), followed by every week (28.6%), every month (24.1%), and 1.1% of consumer indicated they never had beef shank meat before.

In Asian cultures, tenderness is considered as part of the overall texture (Sasaki et al., 2014). In this study, we specifically measured the sensory attributes using the JAR scale because overly tender may not have a positive relationship with overall liking in Asian culture. As indicated by the consumer preference survey, only 6.6% of the panelists preferred the fall-apart - in-mouth texture. Therefore, differences between Asian and the typical American consumers' perceptions to beef tenderness should be taken into consideration when evaluating the results from this study.

Consumer sensory evaluation

Consumer palatability ratings for the six beef shank cuts are presented in Table 2.2. For the amount of connective tissue, BB, FDS, and DDF-H all received ratings close to JAR ($P > 0.05$). Consumers rated DDF-F with too much and ECR and LMP with too little ($P < 0.01$) connective tissue. For tenderness rating in this study, BB, FDS, and LMP received similar ratings close to JAR ($P > 0.05$), and ECR and DDF-H were tougher than those rated JAR ($P < 0.01$). DDF-F was the toughest among all for tenderness ($P < 0.01$). Following the same trend as tenderness rating, consumers indicated that BB, FDS, and LMP received similar ratings close to JAR for juiciness ($P > 0.05$), while ECR and DDF-H were rated less juicy, and DDF-F was the least juicy among all ($P < 0.01$). There was no difference ($P > 0.05$) in beef flavor intensity among the six beef shank cuts. Moreover, BB, FDS, and LMP received the highest sensory overall liking scores, followed by ECR and DDF-H, and DDF-F received the lowest overall liking score among all the shank cuts ($P < 0.01$). Lastly, BB, ECR, FDS, DDF-H, and LMP all received higher acceptability scores (>85%), and DDF-F was the only one received less than ideal acceptability scores (62%) compared to the others ($P < 0.01$). However, this was expected as DDF-F received lower ratings in other palatability traits.

In most past consumer studies, the amount of connective tissue in meat has always shown to have a negative relationship with consumer evaluation of tenderness and overall liking scores (Berry et al., 1988; Jeremiah et al., 2003b; Lorenzen et al., 2003). However, it is important to point out the uniqueness of this study as it specifically focused on Asian consumers, and the shank cuts were cooked through moist heat cookery. Asian consumers consider having connective tissue in stewed meat a desirable trait, as the softened/gelatinized connective tissue tends to create mouthfeel that is similar to the lubrication effect of marbling based on cultural

observations. However, it is also not the more connective tissue the better. Too much softened/gelatinized connective tissue may also create a sensation similar to greasiness, and consumers may lose their appetite after eating just a few pieces. In the case that if the connective tissues were not fully softened/gelatinized, excessive amount of connective tissue in meat will also make it difficult for the consumers to chew and has a direct effect on overall liking.

In this study, DDF-F was rated with the most connective tissue among the beef shanks, and DDF-F was also rated to be the toughest, least juicy, and least preferred beef shank cuts. Therefore, this was clearly the case that the connective tissues in DDF-F were not fully softened. All the beef shank cuts used in this study were cooked with the same method (moist heat cookery), cooking time and temperature, and DDF-F was not the largest of the beef shank cuts. Perhaps, instead of the amount of connective tissue, muscle functionality and collagen crosslink density should be considered as a contributor to the connective tissue toughness. On the other hand, too little connective tissue may not be able to provide the gel-lubrication effect for beef shanks as mentioned above, which may also be detrimental to the overall liking of beef shanks as the shanks contain very little fat. This may also explain why the Asian consumers rated ECR with lower scores for overall liking compared to the others. Although this concept has been long known within the Asian community, it is the first time to the authors' knowledge that it has been documented in a scientific study. Finally, this study demonstrated that beef shanks cuts that had the JAR amount of connective tissue, such as FDS, are the ones could provide the best eating experience for consumers, which also ended with the highest overall liking scores from Asian consumers.

Both Swanstrom et al. (2004) and Blunden et al. (2006) demonstrated that the forelimb, especially the muscle DDF-F and its tendon, endure greater amount of forces during galloping in

race horses compared to the other locomotive muscles. Shadwick (1990) further reported that the DDF tendon had twice the tensile strength compared to the surrounding extensor tendons in pigs as DDF tendon functions as an elastic energy storage element similar to a spring. It is possible that DDF-F may experience greater level of tension and force transmission in cattle, which may result in greater density of the heat stable mature collagen crosslinks in DDF-F. Both Bosselmann et al. (1995) and Smith and Judge (1991) measured the collagen solubility and mature collagen crosslinks density in bovine muscles and found an inverse relationship between them, which may result in the significant lower ratings for tenderness, juiciness, overall liking as well as acceptability score for DDF-F among all the beef shanks.

Kerth and Miller (2015) discussed that Millard reaction may be the main contributor of beef flavor intensity for cooked beef using dry heat cookery. Since this study utilized moist heat cookery which limited the interaction between amino acids and reducing sugar, only a tendency for beef flavor intensity differences was found among the beef shank cuts. The BB, LMP, and FDS tended to have stronger beef flavor intensity than ECR ($P = 0.06$). This was likely due to the fat content differences. O'Quinn et al. (2012) found that steaks that had greater beef flavor intensity also had greater fat content as lipid degradation products are main contributors to beef flavor. Perhaps, there were inherent differences in fat content among different beef shank cuts.

Consumer visual evaluation ratings and objective color measurement

Beef shank raw weight and visual evaluation ratings are presented in Table 2.3. The results showed that DDF-F, ECR, DDF-H, and LMP had the greatest raw weight, followed by BB, while FDS was the lightest among all the beef shanks ($P < 0.01$). On the other hand, Asian consumers indicated that BB was close to the ideal beef shank size. Asian consumers also rated ECR to be slightly too big and indicated that DDF-F, DDF-H, and LMP were bigger in size ($P <$

0.01). Finally, Asian consumers rated FDS was the smallest beef shank cuts ($P < 0.01$). Asian consumers indicated that there was no difference in surface color for all beef shanks ($P > 0.10$). Asian consumers also rated BB and ECR with the highest visual overall liking scores, followed by DDF-F, DDF-H, LMP, with FDS receiving the lowest score ($P < 0.05$). Following the same trend as overall liking, BB and ECR were most visually acceptable (> 95%), while DDF-F, FDS, DDF-H, and LMP were less acceptable than BB and ECR (> 70%; $P < 0.01$). Beef shank objective color measurements of the cross section are presented in Table 2.4. For objective color measurements, FDS had the highest L* value, followed by BB, DDF-F, ECR, and DDF-H, with LMP had the lowest L* value ($P < 0.01$). There were no differences found in both a* and b* among different beef shank cuts.

The evaluation of size was included in the current study because beef shanks are usually sold as a whole muscle cut in Asian countries and domestic Asian markets, and knowing the ideal beef shank size could help to identify Asian consumers' purchasing preferences. Based on these results, Asian consumers preferred shank size that is ~700-750 g. There are two explanations for Asian consumers' preference for medium size of beef shanks. First, Nam et al. (2010) reported that grains and vegetables are the major components in many Asian diets, while meat consumption is limited. This is very different from the western culture as Sweeter et al. (2005) showed that U.S. consumers tended to have greater demand for larger pieces of meat such as ribeye steaks. Second, the demographic survey from this study showed that most of the Asian consumers have 3-4 people in the household, and the "medium" size beef shank seem to be the ideal size for a smaller family size.

Goñi et al. (2008) compared instrument-based measurement and visual evaluation with the use of reference standards in beef color measurement, and they reported that the L* value

measured by colorimeter was closely correlated with the visual color evaluation scores. However, the current study showed a discrepancy between the objective and subjective color measurement. For this study, the epimysium, also referred as the "silver skin" was not removed from the surface of the beef shanks because this is how Asian grocery stores typically present these cuts. However, it may affect Asian consumers' ability to accurately determine the surface meat color of the beef shanks due to the presence of this silver skin. On the other hand, the objective color measurement was conducted using the cross-sections of beef shanks, which the silver skin was not in place to influence meat color evaluation. Based on the Asian consumers' visual evaluation results, the overall liking and acceptability were mostly determined by the beef shank size and weight, but not the color of the meat.

Collagen, fat and moisture content and cook loss

The collagen, fat and moisture content, and cook loss are presented in Table 2.5. The results from current study showed that DDF-F, FDS, DDF-H and LMP all had greater collagen content compared to ECR ($P < 0.01$), while BB was in between and was not different from DDF-F, DDF-H and ECR ($P > 0.05$). In addition, fat percentage followed the same trend as collagen content that BB, DDF-F, FDS, DDF-H and LMP all had more fat compared to ECR ($P < 0.01$). As expected, moisture percentage had an inverse relationship with the fat percentage measurement. The results showed that ECR had the greatest moisture percentage, followed by BB, DDF-F, and LMP, with FDS and DDF-H had the least moisture percentage out of all ($P < 0.01$). Finally, ECR and DDF-H had the greatest cooking loss percentage, followed by BB, and DDF-F, FDS, and LMP had the least cooking loss percentage among the six beef shank cuts ($P < 0.01$).

Rhee et al. (2004) reported that collagen content varied among muscles based on location and functionality, and locomotion muscles tended to have higher collagen content than supportive muscles. In the current study, the collagen content ranged from 11.1 to 19.5 mg/g muscle tissue, which was significantly higher than the collagen content of beef triceps brachii, semimembranosus, and semitendinosus (5.9 to 9.0 mg/g muscle tissue) reported by Rhee et al. (2004). However, this was expected as beef shank cuts had much greater amount of connective tissue than most other locomotion muscles due to their need to endure the extensive tension from weight and physical activities (Kiiskinen and Heikkinen, 1975; Kjaer et al., 2005). In the current study, ECR had the least amount of collagen compared to the other shank cuts. Loren and Lieber (1995) reported that ECR endured the least amount of strain among different human wrist muscles. Since the function of connective tissue is to provide support and protection to the skeletal muscle and allow the muscle to endure forces generated from muscle contraction (Mackey et al., 2011), it is expected to observe less collagen content in muscles that do not need to endure much strain.

Typically, the collagen content is closely related to connective tissue amount detected by sensory panels (Jeremiah et al., 2003a), and collagen content may significantly contribute to tenderness, juiciness and overall liking in meat cooked with moist heat cookery. In the current study, both DDF-F and FDS had similar collagen content; however, the tenderness, juiciness and overall liking scores for these two muscles were in great contrast of each other. Asian consumers rated FDS to be the most tender and juicy beef shank among all, while DDF-F was rated to be the toughest beef shank as well as least tender and juicy among all tested. LaRoche et al. (2020) conducted an Asian consumer sensory panel and investigated relationship between the collagen content and consumer overall liking of stewed goat meat, and they reported that there was no

correlation between the collagen content and Asian consumer overall liking for the stewed goat meat. These results indicated that collagen content is not a good indicator for Asian consumer overall liking for meat cuts cooked with moist heat cookery.

In the current study, BB, FDS, and LMP all had greater fat percentage than ECR ($P < 0.01$), perhaps these fat percentage differences might explain the tendency of greater beef flavor intensity from BB, FDS and LMP compared to ECR. In general, fat is considered to be a determinant for consumer preference in most meat cuts as it could increase the perception of juiciness and potentially reduce the toughness contributed by connective tissue (Thompson, 2001; Nishimura, 2010). However, in the current study, fat's influence on consumer preference may be limited as all of the shank cuts had less than 4 percent of fat, which was lower than the fat percentage found in USDA Select top sirloin steaks (5%) (Olson et al., 2019). The low percentage fat content indicated that beef shank cuts are healthy alternatives due to their lower content of fat. According to USDA's definition, beef that contains less than 10 g of fat in 100 g of beef is considered to be "lean", while beef that contains less than 5 g of fat within 100 g of beef is considered to be "extra lean" (USDA, 2014). Therefore, all of the beef shanks from this study met the USDA's definition of "extra lean" beef. Font-i-Furnols and Guerrero (2014) reported that global consumers' preferences for beef have changed over time, and consumers preferred leaner beef nowadays for health reasons. Zhang et al. (2017) further reported that this preference for lean meat trend can also be applied in many Asian communities.

Typically, lean meat contains approximately 75% water (Pearce et al., 2011). During the cooking process, meat will lose weight due to protein denaturation and losing its ability to hold water (Purslow et al., 2016). Olson et al. (2019) reported that the top sirloin steaks had ~21% of cooking loss in different USDA quality grades utilizing dry heat cookery. In the current study,

beef shanks cooking loss percentage ranged from 28 to 34%. Previous studies had demonstrated that higher cooking losses were sustained when moist heat cookery was utilized, which is in agreement with results from the current study (Moore et al., 1980; Jeremiah and Gibson, 2003). Moreover, Jeremiah et al. (2003a) investigated cooking loss and moisture content of different bovine muscles and reported that muscles with higher moisture content would also have higher cooking loss percentage. In the current study, all the beef shank cuts with the exception of DDF-F followed the trend as described by Jeremiah et al. (2003a). Ismail et al. (2019) measured soluble collagen and cooking loss of bovine semitendinosus muscle and reported that soluble collagen had a positive relationship with cooking loss percentage. Perhaps, the cooking loss discrepancy found for DDF-F was related to its collagen solubility, which indicated that DDF-F contained collagen that is more resistant to heat induced solubilization/gelatinization, resulting in less cooking loss as well as lower score in tenderness and juiciness.

Conclusion

This study provided missing knowledge gap and shed light on Asian consumers' visual and eating preferences of beef shank cuts. The results indicated that tenderness and juiciness of cooked beef shank affected Asian consumers' eating preference of beef shank cuts, while shank size was the main factor affecting their visual preference. As noted in the introduction, LMP was used as a control in this study in order to identify other beef shank cuts that exhibit similar palatability rating as LMP. With the mind that shank cuts should be priced based on their eating quality, we suggest that all the beef shank cuts evaluated in this study with the exception of DDF-F could be marketed in the domestic Asian markets and international markets based on their high consumer acceptability (>85%). However, DDF-H and LMP could be priced as the baseline shank cuts. BB and FDS could be priced with a premium, while ECR could be

discounted. Further investigation on the relationship between collagen crosslink density and beef tenderness should also be conducted to better understand the differences in connective tissue softening rate as observed in this study.

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Table 2.1 Demographic characteristics of consumers who participated in the Asian consumer panels (*n* = 91)

Characteristics	Response	Percentage of consumers
Sex	Male	50.6
	Female	49.4
Age	< 20	3.3
	20-29	29.6
	30-39	40.7
	40-49	22.0
	50-59	1.1
	> 60	3.3
Ethnicity	Chinese	74.7
	Taiwanese	17.6
	Japanese	2.2
	Other	5.5
Household size	1 person	15.4
	2 people	18.7
	3 people	31.9
	4 people	24.1
	5 people	6.6
	6 people	2.2
	>6 people	1.1
Marital status	Single	27.5
	Married	72.5
Annual household income, $	<$25,000	25.3
	$25,000-34,999	17.6
	$35,000-49,999	20.9
	$50,000-74,999	15.4
	$75,000-99,999	9.8
	$100,000-149,999	5.5
	$150,000-199,999	5.5
Highest level of education completed	High school graduate	11.0
	Some college/technical school	9.9
	College graduate	15.4
	Postgraduate	63.7
Beef shank tenderness preference	Semi-tender	36.3
	Tender	57.1
	Falls apart	6.6
Beef shank consumption frequency	Every week	28.6
	Every month	24.1
	Few times a year	46.2
	Never	1.1

Table 2.2 Least squares means for consumer (*n* = 91) ratings of palatability traits of six different beef shanks cooked with moist heat cookery (93°C ± 5°C for 90 min)

Beef shank	Connective tissue amount[7]	Tenderness[7]	Juiciness[7]	Flavor intensity[7]	Overall liking[8]	Acceptability %[9]
Fore shank						
BB[1]	47.4^{c}	51.0^{ab}	49.9^{a}	42.2	69.3^{ab}	95.0^{ab}
DDF-F[2]	66.1^{a}	30.3^{d}	38.3^{d}	38.7	45.6^{d}	62.3^{c}
ECR[3]	39.3^{d}	43.4^{c}	43.5^{bcd}	34.6	58.9^{c}	88.7^{b}
Hind shank						
FDS[4]	53.3^{b}	53.0^{a}	48.8^{ab}	39.9	73.1^{a}	97.0^{a}
DDF-H[5]	47.1^{c}	45.1^{bc}	41.2^{cd}	37.7	62.3^{bc}	91.9^{ab}
LMP[6]	43.8^{cd}	47.4^{abc}	47.3^{abc}	40.9	67.8^{ab}	93.9^{ab}
SEM[10]	2.35	2.35	2.31	2.67	3.10	3.19
P – value	< 0.01	< 0.01	< 0.01	0.06	< 0.01	< 0.01

[a-d]Least squares means in a column without a common superscript differ at $P < 0.05$.
[1]BB = biceps brachii
[2]DDF-F = deep digital flexor – foreshank
[3]ECR = extensor carpi radialis
[4]FDS = flexor digitorum superficialis
[5]DDF-H = deep digital flexor – hindshank
[6]LMP = long digital extensor, medial digital extensor, and peroneus tertius
[7]Sensory evaluation scores: 0 = too little/too tough/too dry/too bland; 50 = just about right (ideal score); 100 = too much/too soft/too wet/too intense.
[8]Sensory evaluation scores: 0 = dislike extremely; 50 = neither like nor dislike; 100 = like extremely.
[9]Acceptability % = percentage of people accept the muscle/total number of observation.
[10]SE of the least squares means.

Table 2.3 Least squares means for consumer (*n* = 86) visual evaluation ratings of six different beef shanks (raw)

Beef shank	Raw weight (g)	Size[7]	Color[7]	Overall liking[8]	Acceptability %[9]
Fore shank					
BB[1]	724.3[b]	52.5[c]	54.2	63.8[ab]	95.4[a]
DDF-F[2]	881.2[a]	67.5[a]	59.3	58.7[bc]	84.8[b]
ECR[3]	881.5[a]	59.9[b]	55.8	67.5[a]	96.5[a]
Hind shank					
FDS[4]	435.2[c]	32.1[d]	55.7	53.0[c]	74.1[b]
DDF-H[5]	936.1[a]	68.5[a]	53.3	59.1[bc]	84.8[b]
LMP[6]	864.8[a]	67.4[a]	51.0	59.2[bc]	84.8[b]
SEM[10]	35.43	2.00	2.51	3.06	3.58
P – value	< 0.01	< 0.01	0.21	0.02	< 0.01

[a-d]Least squares means in a column without a common superscript differ ($P < 0.05$).
[1]BB = biceps brachii
[2]DDF-F = deep digital flexor – foreshank
[3]ECR = extensor carpi radialis
[4]FDS = flexor digitorum superficialis
[5]DDF-H = deep digital flexor – hindshank
[6]LMP = long digital extensor, medial digital extensor, and peroneus tertius
[7]Visual evaluation scores: 0 = too small/too light; 50 = just about right (ideal score); 100 = too large/too dark.
[8]Visual evaluation scores: 0 = dislike extremely; 50 = neither like nor dislike; 100 = like extremely.
[9]Acceptability % = percentage of people accept the muscle/total number of observation.
[10]SE of the least squares means.

Table 2.4 Least square means for objective color measurements of six different beef shanks (*n* = 71)

Beef shank	L*[7]	a*[8]	b*[9]
Fore shank			
BB[1]	45.5[b]	24.4	16.1
DDF-F[2]	45.9[b]	24.5	16.5
ECR[3]	45.6[b]	25.3	16.2
Hind shank			
FDS[4]	47.7[a]	25.6	17.3
DDF-H[5]	45.8[b]	24.1	16.3
LMP[6]	43.4[c]	23.8	15.9
SEM[10]	0.65	0.83	0.56
P – value	< 0.01	0.54	0.47

[a-c]Least squares means in a column without a common superscript differ ($P < 0.05$).
[1]BB = biceps brachii
[2]DDF-F = deep digital flexor – foreshank
[3]ECR = extensor carpi radialis
[4]FDS = flexor digitorum superficialis
[5]DDF-H = deep digital flexor – hindshank
[6]LMP = long digital extensor, medial digital extensor, and peroneus tertius
[7]L*=lightness (0 = black and 100 = white).
[8]a*=redness (-60 = green and 60 = red).
[9]b*=blueness (-60 = blue and 60 = yellow).
[10]SE of the least squares means.

Table 2.5 Least squares means for collagen content, proximate analysis, and cooking loss of six different beef shanks (*n* = 72).

Beef shank	Collagen content, mg/g muscle tissue	Fat %	Moisture %	Cooking loss, %[7]
Fore shank				
BB[1]	14.7^{bc}	3.6^{ab}	73.8^{bc}	31.0^{b}
DDF-F[2]	18.0^{ab}	2.9^{bc}	74.4^{b}	29.0^{c}
ECR[3]	11.1^{c}	1.1^{d}	76.3^{a}	33.1^{a}
Hind shank				
FDS[4]	19.4^{a}	3.9^{a}	73.0^{d}	29.1^{c}
DDF-H[5]	17.4^{ab}	3.2^{abc}	73.6^{cd}	33.6^{a}
LMP[6]	19.5^{a}	2.7^{c}	73.7^{bc}	27.9^{c}
SEM[8]	1.48	0.28	0.31	0.82
P – value	< 0.01	< 0.01	< 0.01	< 0.01

[a-d]Least squares means in a column without a common superscript differ ($P < 0.05$).
[1]BB = biceps brachii
[2]DDF-F = deep digital flexor – foreshank
[3]ECR = extensor carpi radialis
[4]FDS = flexor digitorum superficialis
[5]DDF-H = deep digital flexor – hindshank
[6]LMP = long digital extensor, medial digital extensor, and peroneus tertius
[7]Cooking loss: [(raw weight – cooked weight)/raw weight] x 100.
[8]SE of the least squares means.

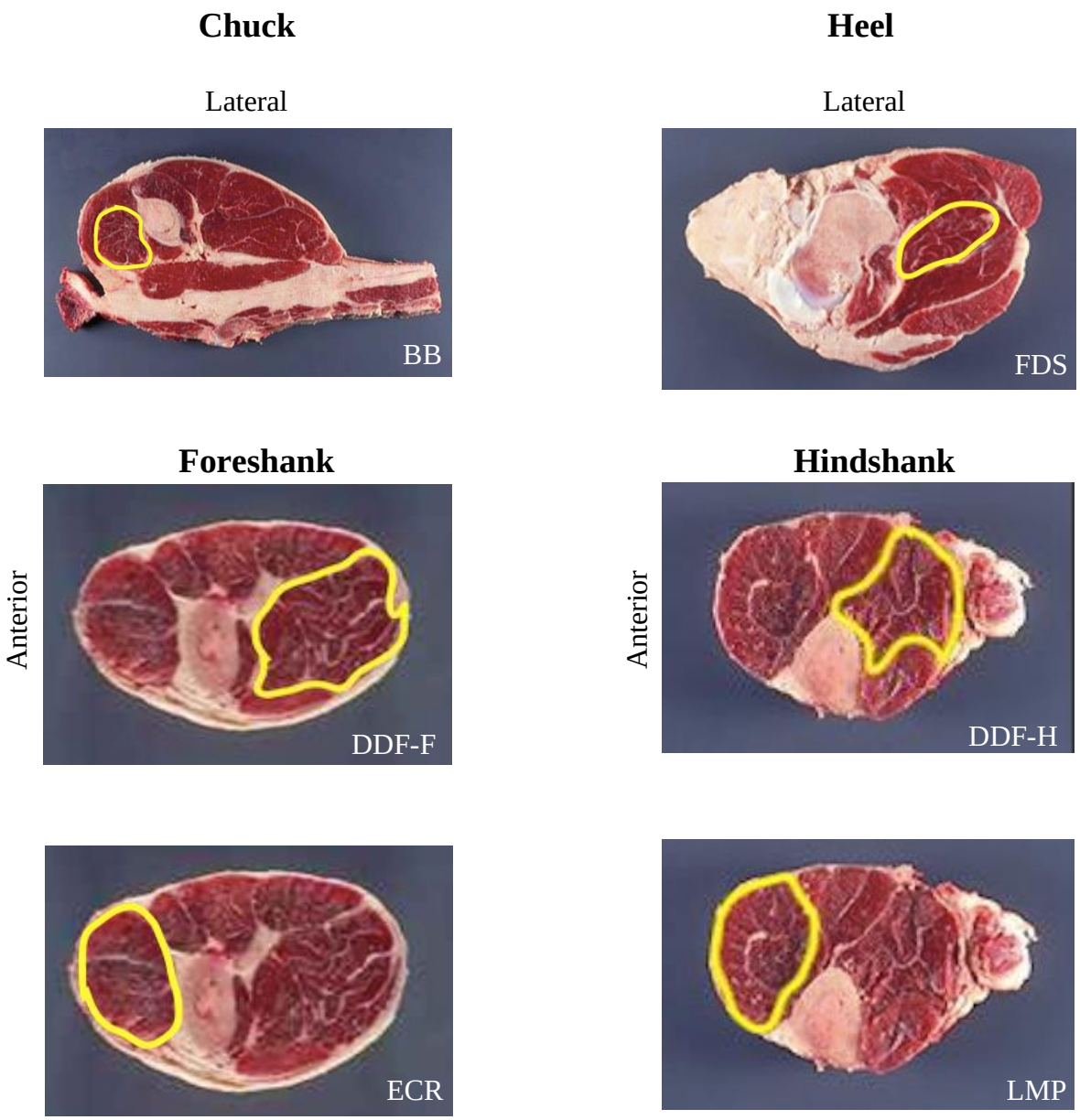

Figure 2.1 Cross-section of the anatomical location of six different beef shank cuts used in this study: *biceps brachii* (BB), *deep digital flexor* from foreshank (DDF-F), *extensor carpi radialis* (ECR), *flexor digitorum superficialis* (FDS), *deep digital flexor* from hindshank (DDF-H), a combination of *long digital extensor, medial digital extensor* and *peroneus tertius* (LMP). Cross section images were downloaded from http://bovine.unl.edu with permission. (Jones et al., 2004).

Chapter 3 - Investigating the contribution of mature collagen crosslinks to cooked meat toughness using a stewed beef shank model

Abstract

The objective of this study was to investigate mature collagen crosslink densities and their relationship to connective tissue texture using a stewed beef shank model. Connective tissue texture, Warner-Bratzler Shear Force (WBSF), collagen content and characteristics were measured for six different beef shank cuts from eight USDA Low Choice beef carcasses (n = 48). Deep digital flexor from the foreshank had the toughest connective tissue texture, greatest WBSF value, most cooked collagen content, greatest insoluble collagen percentage as well as greatest raw and cooked pyridinoline (PYD) densities among all the beef shank cuts ($P < 0.05$). Correlation analysis showed that cooked collagen content, insoluble collagen percentage as well as raw PYD densities had positive correlations with connective tissue texture (r = 0.550, 0.498 and 0.560 respectively; $P < 0.01$) and WBSF (r = 0.615, 0.392 and 0.730, respectively; $P < 0.05$). The results confirmed raw PYD density may be a great indicator for cooked beef connective tissue texture and ultimately, tenderness in beef cuts with high concentration of connective tissue prepared with moist heat cookery.

Key words: beef shank, moist heat cookery, insoluble collagen, pyridinoline, connective tissue texture, Warner-Bratzler shear force

Introduction

Connective tissue sheaths serve as scaffolds to allow for the growth and development of muscle tissue (Nishimura et al., 2002). Additionally, connective tissue has the strength and

elasticity to sustain the transmission of the forces generated by muscle contraction between muscles and bones during movement (Nishimura et al., 2002). However, the connective tissue also provides the "background toughness", which meat tends to be less tender when there is high amount of connective tissue present (Hill, 1966).

Collagen, a fibrous protein, is the main component of connective tissue, and collagen is stabilized by immature (divalent) and mature (trivalent) crosslinks (Roy et al., 2015b). The heat-labile immature crosslinks mainly exist in younger animals, and they transform into heat-stable mature crosslinks as animals age (Roy et al., 2015b). This replacement of immature crosslink with mature crosslink is known to decrease collagen solubility (Nishimura, 2010). Smith and Judge (1991) found an inverse relationship between mature collagen crosslinks density and collagen solubility in bovine muscles. Although tenderness was not measured in that study, it was likely this result can also be transcribed into similar relationship between mature collagen crosslink density and meat tenderness. Weston et al. (2002) concluded that the density of mature crosslinks in collagen is one of the key factors in collagen-related toughness in meat.

Pyridinoline (PYD) and deoxypyridinoline (DPD) are major mature collagen crosslinks. PYD is mainly found in muscles (Nakano et al., 1985; Young et al., 1994; Bosselmann et al., 1995), and DPD is mainly found in bones (Robins et al., 1994; Lietz et al., 1997). PYD density varies among muscles depending on the location and functionality of the muscle. Bosselmann et al. (1995) measured the PYD density of different bovine muscles and reported that the mature collagen crosslink density was closely related to the physiological functions of the muscles. They found that the locomotive muscles such as semitendinosus and extensor carpi ulnaris had higher PYD density than the supportive muscle such as longissimus dorsi (Bosselmann et al., 1995). Locomotion muscles tend to endure more strain or force than the supportive muscle (Bosselmann

et al., 1995). Therefore, locomotive muscles are expected to contain greater PYD density than supportive muscles.

Heat insoluble collagen that remains in meat after cooking is the main contributor of the background toughness in meat. However, the heat insoluble collagen is not well characterized, and the factors affecting collagen solubility are not well understood. Beef shank muscles are the perfect test subjects for this exploratory project as they are high in collagen content with expected differences in collagen mature crosslinks density due to their differences in physiological functions. Therefore, the objective of this study was to investigate mature collagen crosslink densities and their relationship to cooked beef tenderness and connective tissue texture using a stewed beef shank model.

Materials & Methods

The Kansas State University (KSU) Institutional Review Board approved all procedures for use of human subjects in sensory panel evaluations (IRB #7440.5, August 2018).

Sample collection and preparation

Bone-in foreshanks (Institutional Meat Purchase Specification #117) with humerus and *biceps brachii* attached, heel (Institutional Meat Purchase Specification #171F) and bone-in hindshanks (Institutional Meat Purchase Specification #157) from both sides of 8 USDA low choice beef carcasses were collected from a Midwestern beef processor at 3 days postmortem and transported back to KSU Meat Laboratory. Six shank cuts (Figure 3.1) were fabricated from the foreshank, heel and hindshank the following day: foreshank: [*biceps brachii* (BB); *deep digital flexor* from foreshank (DDF-F); *extensor carpi radialis* (ECR)]; heel: [*flexor digitorum superficialis* (FDS)]; hindshank: [*deep digital flexor* from hindshank (DDF-H); a combination of *long digital extensor, medial digital extensor* and *peroneus tertius* (LMP)]. Due to the nature of

LMP complex, there was no clear seam among *long digital extensor*, *medial digital extensor* and *peroneus tertius* in LMP. Therefore, LMP was kept as it is to maintain integrity for the connective tissue texture evaluation. Both ends of tendons and accessary muscles from each shank cut were trimmed.

All cuts were vacuum packaged and stored in a freezer at -40°C until analysis. The left side of the beef shanks were designated for collagen texture determination through consumer taste panel, Warner-Bratzler shear force (WBSF), cooked collagen content and mature collagen crosslink analyses, while the right side of the beef shanks were designated for raw collagen content and mature collagen crosslink analyses.

Consumer sensory panel evaluation

Beef shank cuts were thawed for 72 h prior to the panels at 2 ± 2°C and stewed for 90 minutes in 93 ± 5°C water in a countertop warmer (X*PRT Series W-3Vi; APW Wyott, Allen, TX, USA). After cooking, beef shank cuts were placed into warming pots and placed on an electric ceramic glass cooktop (GoldSeries; Whirlpool, Benton Harbor, MI) at 50 ± 2°C to keep warm before serving. Immediately before serving, dorsal end of each beef shank cuts was faced and cut into one to two 2.5 cm slices. Each slice was cut into 2.5 cm thick x 2.5 cm x 2.5 cm cubes, and 2 cubes were served to each consumer. The remaining beef shank of each sample was preserved for WBSF and laboratory analysis.

Asian consumers (n = 61) were recruited from Manhattan, KS, USA and the surrounding areas. Asian consumers from the Southeast Asia regions were selected if they had been exposed and had the habit of eating beef shanks to ensure the quality of the study. Panels were conducted on the KSU campus where consumers were placed into a lecture-style classroom and six samples were served to each consumer. Before evaluation, consumers were given verbal directions to

explain the evaluation procedures and the digital survey (Version 2417833; Qualtrics Software, Provo, UT, USA) on an electronic tablet (Model 5709 HP Stream 7; Hewlett-Packard, Palo Alto, CA, USA). Since connective tissue texture is an important component of many Asian cuisines; therefore, only Asian consumers were selected for the consumer panel of this study due to their ability to distinguish small differences in connective tissue texture. Consumers were asked to evaluate each sample for connective tissue texture on a Just About Right (JAR) line scale. Anchors were set at 0, 50, and 100, with 0 being too soft, 50 as the ideal score that anchored as JAR, and 100 being too hard. Each consumer evaluated a total of six randomized samples with one sample from each beef shank cut.

Warner–Bratzler shear force

One to two 2.5 cm slices were cut from the remaining cooked beef shank of each sample for WBSF evaluation. The procedure of WBSF was followed by using the American Meat Science Association Meat Cookery and Sensory Guidelines (American Meat Science Association, 2015). Six 1.27 cm diameter cores were taken from each cooked beef shank piece that were parallel to the muscle fiber orientation and sheared perpendicular to the muscle fiber orientation by using an Instron testing machine (Model 5569; Instron Corporation, Canton, MA) with a cross-head speed of 250 mm/min and a load cell of 100 kg. Measurements were averaged across all six cores per sample and recorded as the average peak force (kg). The remaining cooked beef shank cut was put into whirl-pak bag (4 oz, Nasco Inc., Madison, WI) and stored at -80°C for cooked collagen and collagen crosslink analysis.

Collagen & collagen crosslink sample preparation

Raw and cooked beef shank cuts designated for laboratory analysis (n = 48) were thawed for 24 h prior to pulverization at 2 ± 2°C. Each sample was frozen in liquid nitrogen, pulverized

using a commercial grade blender (Model S1BL32; Waring Products Division; Hartford, CT), transferred into whirl-pak bags (4 oz, Nasco Inc.) and stored at -80°C freezer until collagen sample preparation.

Collagen sample preparation procedure was based on the protocol described by Avery et al. (2009) with modifications. Briefly, approximately 500 mg of pulverized muscle tissue was weighed into a 15 x 125 mm glass tube and mixed with 2.5 ml of PBS containing 1% of sodium borohydride solution (100 mg of sodium borohydride per 1 ml of 0.01M of sodium hydroxide) as a reduction step to prevent the hydrolysis of crosslinks. The samples were incubated for 60 min at room temperature in a fume hood. Approximately 0.2 ml of glacial acetic acid was added into each sample to drop the pH (~pH 3) in order to terminate the reduction step. The samples were washed with 3 x 5 ml of ultrapure water and dried using a Vacuum Evaporation System (RapidVap; Labconco Corporation, Kansas City, MO, USA). After the drying process, 10 ml of 6N hydrochloric acid was added to each sample, and each sample was placed into a drying oven (Isotemp, Fisher Scientific, Hampton, NH) for 24 h at 115°C. After hydrolysis, samples were removed from the oven and evaporated in the Vacuum Evaporation System (RapidVap; Labconco Corporation) with the following setting: 70°C, 53% vortex speed, and 200 mbar vacuum until complete dryness (~24 hours). After evaporation, samples were rehydrated with 0.5 ml of ultrapure water and separated into two 250 µl aliquots. The samples were stored at -80°C until analysis.

Collagen content analysis

Both raw and cooked collagen contents were determined by measuring the amount of hydroxyproline described by Bergman and Loxley (1963) with modifications. Hydroxyproline standard curve was prepared on the day of analysis with the following concentrations: 0, 1.5, 3.0,

4.5, 6.0, 7.5, 9.0, and 10.5 µg/ml. Samples were diluted 1:800 with ultrapure water, and 2 mL of diluted sample and standards were mixed with 1 ml of chloramine-T oxidant reagent (6 mM chloramine-T, 140 mM citric acid monohydrate, 38 mM sodium hydroxide, 661 mM sodium acetate trihydrate, 29% of 1-propanol, at pH 6 and incubated in room temperature for 20 min. After incubation, 1 ml of 4-dimethylaminobenzaldehyde (DMBA) color reagent (67 mM of DMBA was dissolved in 21% perchloric acid, 35% 2-propanol, and 14% ultrapure water) was added to the samples and standards. Samples and standards were vortexed, covered with aluminum foil, and incubated in water bath for 90 min at 60°C for full color development. Samples and standards were removed from water bath, placed in cold water for 3 min, and 0.2 ml of sample and standards was pipetted to a 96 wells plate in duplicate. Hydroxyproline determination was performed using an UV/VIS Spectrophotometer (Eon, BioTek Instruments Inc., Winooski, VT) with absorbance set at 558 nm. Sample concentrations were quantified using the known standard curve, and the collagen content was determined by multiplying the hydroxyproline content by 7.14, with a final unit of mg collagen per g of muscle tissue in dry matter basis (DM). Both raw and cooked collagen content were adjusted to dry matter (DM) basis to account for moisture loss during the cooking process. Finally, relative percentages of soluble and insoluble collagen content were calculated from the collagen content measurements from raw and cooked shanks with the following equations:

Soluble collagen % = (raw collagen content– cooked collagen content) / raw collagen content

Insoluble collagen % = cooked collagen content/ raw collagen content

Collagen crosslink analysis

Sample pre-treatment

Mature collagen crosslink determination procedures followed the method described by Viguet-Carrin et al. (2009) with modification. Meat hydrolysates designated for mature crosslink analysis were first diluted 1:26 with ultrapure water. Four hundred µl of the diluted sample and 2.8 ml of sample buffer composed of acetonitrile and glacial acetic acid (6:1 *v/v*) were mixed resulting in a total volume of 3.2 ml of prepared sample. Solid Phase Extraction (SPE) cellulose cartridges (Bond Elut, 300mg/3 ml; Agilent Technologies, Santa Clara, CA) were first equilibrated with 2.5 ml of wash buffer consist of acetonitrile, glacial acetic acid, and ultrapure water in 8:1:1 ratio (*v/v/v*) using a PrepSep 24-Port Vacuum Manifold apparatus (Fisher Scientific, Hampton, NH). Immediately after loading the prepared samples, columns were washed with 4 x 2.5 ml of wash buffer to remove the interfering fluorophores. Cares were taken not to let the SPE column dry out between steps. Finally, crosslinks were eluted with 2 x 0.6 ml of 1% heptafluorobutyric acid (HFBA) and the columns were drained completely. Cleaned samples were transferred into 2 mL amber vials (*P*/N 5188-6535, Agilent Technologies) capped with a 9 mm pre-slit PTFE screw cap (*P*/N 5185-5865, Agilent Technologies).

Ultra-performance liquid chromatography (UPLC) conditions

Pyridinoline (PYD) and deoxypyridinoline (DPD) were separated on an UPLC system (Acquity H-Class; Waters Corporation, Milford, MA, USA) equipped with a degasser, a quaternary pump, a fluorescence detector, and data were processed with the MassLynx chromatography data software (Waters Corp.). Briefly, crosslinks were separated on a reversed phase column (Acquity UPLC HSS T3,1.8 µm, 2.1 x 100 mm; Waters Corp.) with an injection volume of 5 µL. Flow rate was set at 0.5 mL/min and the column temperature was maintained at 60°C throughout the run. The chromatographic separation was done using a gradient with mobile phase A consisting 0.2% of HFBA in ultrapure water and mobile phase B consisting 100%

acetonitrile. PYD and DPD were eluted with 85% solvent A and 15% solvent B at ~ 7.0 and 7.8 min, respectively. After PYD/DPD elution, mobile phase A was decreased to 0% with mobile phase B increased to 100% to elute the hydrophobic residues from the column from 10 to 15 minutes. At 15.01 min, mobile phase A was changed to 100% until the 20 min mark to equilibrate the column for the next run. The total run time for each sample was 20 minutes, and PYD and DPD elution was monitored by a fluorescence detector with an emission and excitation wavelength of 395/297 nm. The peak areas were used for PYD and DPD concentration calculations using the linear regression obtained with the standards, and the results were corrected with the dilution factor to obtain final concentration of PYD or DPD. The mature collagen crosslink density was calculated by dividing the molar concentration of PYD and DPD (using molar mass of 428.44 g/mol and 412.44 g/mol, respectively) by molar concentration of collagen (using molar mass of 300,000 g/mol). All mature collagen crosslink density results were expressed as mol of mature collagen crosslink per mol of collagen.

Standard curve and QC were prepared fresh on the day of the sample analysis using the PYD/DPD HPLC calibrator (*P/N* 4101, Quidel Corporation, San Diego, CA) diluted with the sample buffer. The standard curve contained a mixture of PYD at 0.02, 0.05, 0.09, 0.19, 0.37, 0.75 μM and DPD at 0.01, 0.03, 0.05, 0.10, 0.20, 0.40 μM. A set of QC containing a mixture of PYD at 0.07, 0.33, 0.65 μM or DPD at 0.04, 0.18, 0.35 μM was ran prior to sample analysis. In addition, QC containing PYD at 0.33 μM and DPD at 0.18 μM was inserted in between every 10 samples to ensure the quality of the analysis. Samples with concentrations out of the standard curve range were further diluted with 0.2% HFBA in 1:5 dilution and re-analyzed.

Statistical analyses

Statistical analyses were performed by using PROC GLIMMIX procedure of SAS (SAS Version 9.4; SAS Inst. Inc., Cary, NC), and treatment comparisons were considered as significant with an **α** of 0.05. Connective tissue texture, WBSF, soluble collagen percentage and insoluble collagen percentage were performed using a completely randomized design with beef shank cuts used as the fixed effect and animal was used as a random blocking factor. The Kenward-Roger approximation was used to estimate the degrees of freedom. For collagen content and crosslink (PYD and DPD) analyses, data were analyzed as a split-plot model, with beef shank cuts as the whole-plot factor and cooking treatment as the sub-plot factor of treatment. The Satterthwaite approximation was used to estimate the degrees of freedom. The PROC CORR procedure of SAS was used to determine Pearson's correlation coefficients between raw and cooked collagen characteristics to WBSF and connective tissue texture evaluated by Asian consumer panelists. Finally, to determine the significance of collagen characteristics contribution to connective tissue texture and objective tenderness, a multivariate regression model was constructed using PROC REG and the stepwise selection procedure, with variable required to be significant ($P < 0.05$) to remain in the final model.

Results and Discussion

Connective tissue texture, WBSF, collagen content and characteristics of six beef shank cuts

Collagen content results are displayed in Table 3.1. There was a significant muscle x cooking treatment interaction for collagen content ($P < 0.01$). In general, DDF-F, FDS, and LMP all had the greatest amount of raw collagen content, followed by BB and DDF-H, with ECR contained the least amount of raw collagen among all the beef shank cuts ($P < 0.01$). However, all the beef shank cuts had similar cooked collagen content except for DDF-F ($P < 0.01$), which

had the greatest cooked collagen content among all. In addition, collagen content reduced after cooking for all the beef shin/shank cuts ($P < 0.01$).

Past research has demonstrated that locomotive muscles tended to contain higher collagen content with less solubility in comparison to the supportive muscles (Wheeler et al., 2000; Torrescano et al., 2003). Although the current study only utilized beef shin/shank muscles (all considered locomotive muscles), there were still significant differences in the function and the force generation capacity by each of the individual muscle/muscle groups used in this study. Brown et al. (2003) studied the force generating capacities from different muscles in horses, and reported that DDF muscles had a peak isometric force at 9,504 N, while ECR had a peak isometric muscle force at 536 N. Collagen fibrils in tendon and muscles are essential for maintaining mechanical properties and force transmission during movements (Cen et al., 2008). With DDF having greater force generation capacity compared to ECR, it is expected to observe DDF to have higher collagen content than muscles with less force generating capacities.

Although the collagen molecules were made up of α-chains bound by hydrogen bonds in a triple helices structure making its structure extremely stable, heat treatment is able to break the hydrogen bonds to allow for the solubilization of collagen fibers (Weston et al., 2002). Therefore, it was expected to observe collagen loss for the beef shank cuts after cooking as collagen solubilization is expected to occur when the temperature reached 80°C (Palka, 1999; Ismail-Fitry et al., 2011). In the current study, all the beef shanks were stewed in water at 93°C for 90 minutes. With such cooking condition, any collagen that can be easily solubilized should be released, leaving only the “heat insoluble” collagen behind.

Results for soluble and insoluble collagen percentages, connective tissue texture evaluated by Asian consumers, and WBSF are shown in Table 3.2. The BB, DDF-H, FDS and

LMP all had the most soluble and least insoluble collagen percentage, followed by ECR, with DDF-F having the least soluble and most insoluble collagen percentage ($P < 0.05$). Among all the beef shank cuts evaluated in this study, Asian consumers rated DDF-F with the toughest connective tissue texture, followed by DDF-H, ECR and LMP, with BB and FDS having the softest connective tissue texture among all ($P < 0.01$). Finally, DDF-F was significantly tougher than the rest of shank cuts when measured by WBSF (86.79 N; $P < 0.01$), and all other beef shank cuts had much lower but similar WBSF values (~32 to 38 N; $P > 0.10$).

DDF-F was the only shank cut that retained connective tissue toughness after the extensive moist heat treatment, which likely also resulted in its high WBSF value among the shank cuts. Torrescano et al. (2003) investigated the insoluble collagen content and WBSF in bovine muscles and reported that muscles with greater insoluble collagen content also tended to have greater WBSF values. The mechanism behind this phenomenon was explained by Steinhart et al. (1994) who reported that the mature crosslinks tended to increase the mechanical and thermal stability of collagen fibers and their tensile strength, which would have a negative effect on collagen solubility. In addition, Yamauchi and Mechanic (1988) reported that collagen fibers with more mature crosslinks may require more extensive heat treatment to allow for the collagen solubilization as these heat stable mature crosslinks are known to withstand the heat and maintain the integrity of collagen fibers. These heat stable collagen fibers are known to contribute to the texture of connective tissue and meat toughness (Tanzer, 1973). Therefore, it is likely that DDF-F exhibited the toughest connective tissue texture and WBSF due to its greater proportion of insoluble collagen percentage and potentially greater density in mature collagen crosslinks compared to the other beef shank cuts used in this study.

Raw and cooked PYD and DPD density of the six different beef shank cuts are shown in Table 3.3. There was a significant muscle × cooking treatment interaction for PYD density. In raw beef shanks, DDF-F had the greatest PYD density, followed by FDS, with BB, ECR, DDF-H, and LMP had the least PYD density ($P < 0.05$). In cooked beef shanks, DDF-F again, had the greatest PYD density, followed by BB, FDS, and DDF-H, with ECR and LMP had the least PYD density in cooked shanks ($P < 0.05$). There was a cooking effect which cooking decreased PYD density for DDF-F ($P < 0.05$). Cooking also tended to increase PYD density for DDF-H ($P = 0.05$). However, cooking did not affect PYD density for the rest of the beef shank cuts ($P > 0.10$).

Raw PYD density may vary due to differences in species, diets, and muscle's intended functionality. Roy et al. (2015a) reported that collagen crosslink content in different beef muscles can be altered in response to growth promotant. Chun et al. (2020) investigated the mature crosslink densities of longissimus lumborum (LL), tensor fascia latae (TF), and gastrocnemius (GC; the heel) from young steers and found that the locomotive muscle GC had greater PYD density compared to the supportive muscles LL and TF. In addition, Bosselmann et al. (1995) measured the PYD density in different bovine muscles and observe greater PYD density in locomotive muscles such as semitendinosus (ST) and extensor carpi ulnaris (ECU) than the supportive muscle such as longissimus dorsi (LD). This was expected as locomotive muscles endure more strain or force because animals utilized them more extensively for movement than the supportive muscles. Furthermore, Swanstrom et al. (2004) investigated both FDS and DDF muscles of race horse and demonstrated that the linear stiffness (ability to resist deformation in response to an applied *force)* for FDS was 2.80 kN/cm, while DDF was 3.47 kN/cm. Although all of the beef shank cuts utilized in this study were considered as the muscles

of locomotion, it is likely that DDF-F had greater PYD density than the rest of the shank muscles due to the greater level of force it was required to sustain in living animal.

Previous studies demonstrated that as PYD density increased, collagen solubility would decrease in bovine muscles (Smith and Judge, 1991; Bosselmann et al., 1995). Furthermore, both Listrat et al. (2007) measured PYD concentration in raw and cooked bovine muscles and reported that the cooked meat expressed higher PYD concentration (nmol/mg of muscle tissue). It is important to note that when the PYD is expressed as concentration (based on weight of the muscle tissue), moisture loss during the cooking process needs to be accounted for. PYD was retained during the cooking process thus increased its concentration in meat. Therefore, the discrepancy among the results of this study from Listrat et al. (2007) was strictly due to differences in unit expression. Our findings confirmed that PYD is heat stable, and the network of connective tissue may require extensive heat treatment to solubilize in the presence of greater density of PYD in beef. Although cooking did not seem to affect PYD density for the majority of the beef shank cuts used in this study, a cooking effect was found for DDF-F. This phenomenon demonstrated that moist heat cookery with extended period of cooking time could still have an effect for PYD density, in particular, meat cuts with inherent high PYD density. However, the exact relationship between PYD density and heat treatment as well as the potential influence of other collagen crosslinks is still unknown.

There was also a significant muscle x cooking interaction for DPD density. DDF-F and ECR both had the greatest DPD density in raw beef shanks ($P < 0.01$), followed by FDS, with BB, DDF-H, and LMP had the least DPD density ($P < 0.01$). In cooked beef shanks, BB, ECR, and DDF-H had greater DPD density than DDF-F, FDS, and LMP ($P < 0.01$). There was a

cooking effect which showed that cooking decreased DPD density for DDF-F, ECR, and FDS ($P < 0.01$). Cooking did not affect DPD density for the rest of the beef shank cuts ($P > 0.10$).

It was expected that DPD density from all the beef shank cuts were significantly lower than the PYD density. DPD is the crosslink that predominate in bone and is known to be a minor component in connective tissue (Robins et al., 1994; Lietz et al., 1997; Cremers et al., 2008). Bosselmann et al. (1995) reported that bovine ECU contained about 0.010 mol/mol of collagen for DPD density, and that value was similar to the DPD density found in beef shanks for this current study. Yoshida et al. (2014) investigated the mature DPD crosslink and its relationship to mechanical properties in mouse cervical tissues and reported that DPD was positively correlated with mechanical properties such as ultimate stiffness, which demonstrated that DPD may be important to tissue tensile strength in certain tissues. Furthermore, Yoshida et al. (2014) also reported that the dihydroxylysinonorleucine (DHLNL) is an immature crosslink that may later be converted into the mature DPD crosslink, and they found a strong positive relationship between DHLNL and tissue stiffness. Perhaps the immature collagen crosslink DHLNL may play a role in bovine connective tissue texture, and further studies are needed to elucidate its relationship with beef tenderness.

Correlation coefficients and linear regression equations

Correlation coefficients of raw and cooked collagen content, soluble and insoluble collagen percentage, and different collagen crosslinks density with connective tissue texture and WBSF of six different beef shanks are presented in Table 3.4. The connective tissue texture and WBSF showed no significant correlation with raw collagen content ($P > 0.10$). However, cooked collagen content had a strong positive correlation with connective tissue texture ($r = 0.550$; $P < 0.01$) and WBSF ($r = 0.615$; $P < 0.01$). As expected, soluble collagen percentage had a negative

correlation with connective tissue texture (r = -0.498; $P < 0.01$) and WBSF (r = -0.392; $P < 0.05$), and insoluble collagen percentage had a positive correlation with connective tissue texture (r = 0.498; $P < 0.01$) and WBSF (r = 0.392; $P < 0.05$). Raw PYD density had a strong positive relationship with connective tissue texture (r = 0.560; $P < 0.01$) and WBSF (r = 0.730; $P < 0.01$). There was still a positive correlation between cooked PYD density and connective tissue texture (r = 0.375; $P < 0.05$) and WBSF (r = 0.324; $P < 0.10$), but the positive relationship was not as strong as for the raw PYD density. There was no significant correlation between raw DPD density and connective tissue texture. However, raw DPD density tended to have a positive correlation with WBSF (r = 0.321; $P < 0.10$). The connective tissue texture and WBSF showed no significant correlation with cooked DPD density ($P > 0.10$).

Many past research reported similar results as found in this current study which demonstrated at most a mild relationship between raw collagen content with WBSF (Crouse et al., 1985; DeVol et al., 1988). LaRoche et al. (2020) reported that there was no correlation found between raw collagen content and consumers' perception of tenderness in goat meat. Torrescano et al. (2003) evaluated collagen content and WBSF of "raw" bovine muscles and demonstrated that there was a positive correlation between WBSF and collagen content in raw beef. These results demonstrated that background toughness in raw meat is driven by the total collagen content as the raw collagen still retain its full tensile strength. On the other hand, background toughness in cooked meat is driven by the remaining "cooked" collagen that could not be solubilized during the cooking process. In agreement with the current study, Riley et al. (2005) reported that the insoluble or cooked collagen content had a positive correlation with WBSF in bovine LD muscle, and Jeremiah et al. (2003b) found that the insoluble collagen content was closely related with the palatability attributes from sensory panel. It was expected there was an

inverse relationship between collagen solubility with connective tissue texture and WBSF. As the collagen solubility increased, more collagen can be readily solubilized in meat and resulted in softer connective tissue texture during cooking, which would also decrease in WBSF.

Many previous studies demonstrated that the presence of mature collagen crosslinks was related to meat toughness (Bailey and Light, 1989; Lepetit, 2007) or tissue tensile strength (Yoshida et al., 2014) . In the current study, raw PYD density had a strong positive correlation with connective tissue texture and WBSF. Mature collagen crosslinks can retain the collagen structure by linking itself with the neighboring collagen fiber, which provided the structure and strength for collagen, at the same time, decreasing collagen solubility and preventing the softening of the connective tissue texture during cooking (Weston et al., 2002; Maynes, 2012). On the other hand, Dubost et al. (2013) and Chun et al. (2020) did not find a relationship between PYD density and meat tenderness in a variety of bovine muscles. Two factors can be used to explain this discrepancy. First, dry heat cookery with short cooking time aiming for an internal temperature of 55°C and 71°C respectively were applied for both studies. The dry heat cookery with short cooking time may not be enough to fully solubilize all the soluble collagen, which the soluble collagen continued to contribute to meat toughness, thus masking the PYD's relationship to meat toughness. Second, most of the beef muscles evaluated in both studies were supportive muscles, which contain very little connective tissue, collagen content, and collagen crosslinks. Therefore, it was difficult to find correlation between collagen crosslinks and meat toughness in supportive muscles as the collagen contribution to meat toughness in those muscles are likely minute compared to the beef shanks utilized in this study.

The cooked PYD density also had a positive correlation with connective tissue texture and WBSF, but not as strong as the raw PYD density. This could be related to the minor cooking

effect found in DDF-F for PYD density. PYD is known to be a heat stable mature crosslink, and yet the PYD density decreased in DDF-F from raw to cooked shank which demonstrated that moist heat cookery with extended cooking time could potentially release additional PYD. However, this is the first known attempt to the best of the authors' knowledge to specifically investigate the effect of extended moist heat cookery on mature crosslinks densities. Additional studies are needed to further confirm this observed phenomenon. Moreover, there was a tendency for positive correlation between raw DPD density and WBSF in this study. Yoshida et al. (2014) also reported that DPD was positively correlated with stiffness and mechanical strength in the cervical tissue from mouse. However, DPD's contribution to meat tenderness is still largely unknown within the meat science community.

Multiple linear regression analysis was conducted using a stepwise selection procedure to generate linear regression equations to predict the connective tissue texture and objective tenderness using these results (Table 3.5).The model for connective tissue texture was deduced as: connective tissue texture = 30.190 + 0.513 x cooked collagen content + 30.796 x raw PYD density with r^2 = 0.452. Based on this equation, connective tissue texture could be predicted by using the major factors, cooked collagen content as well as raw PYD density, which contributed for 45.2% to the connective tissue texture. The raw PYD density contributed 31.4% of the model, while the cooked collagen content contributed 13.8% of the model. Moreover, the model for objective tenderness was deduced as: WBSF = -1.072 + 0.103 x cooked collagen content + 8.622 x raw PYD density with r^2 = 0.673. Based on this equation, the objective tenderness could be predicted by using the major factors such as cooked collagen content and raw PYD density, and these major factors contributed for 67.3% to the objective tenderness. The raw PYD density contributed 53.3% of the model, and cooked collagen content contributed 13.9% of the model.

These equations further confirmed the potential of using raw PYD density as a biomarker to predict cooked beef tenderness.

Conclusion

Based on the results from this study, we found that cooked (insoluble) collagen is what contributed to the background toughness, and PYD is a heat stable collagen crosslink that may require extensive heat treatment to degrade and allow for the solubilization of collagen. As a result, raw PYD density may be a good indicator for cooked collagen content, cooked beef connective tissue texture and ultimately, tenderness in beef cuts with high concentration of connective tissue prepared with moist heat cookery. Although the current study demonstrated that the heat stable mature collagen crosslink – PYD can be released from meat subjected to extensive moist heat cookery, the influence of cooking time and temperature of the PYD degradation is still largely unknown. Future studies are needed to investigate the specific relationship among collagen crosslinks, cooking time and temperature to create an equation that can consistently predict the palatability of the beef cuts cooked with moist heat cookery.

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Table 3.1 Least square means of raw and cooked collagen content of the six different beef shank cuts evaluated in this study (*n* = 48).

Beef shank	Collagen content, mg/g (DM[7])			
	Raw	Cooked	SEM[8]	*P*-value
Fore shank			4.21	< 0.01
BB[1]	57.47^{Ab}	30.77^{Bb}		
DDF-F[2]	65.44^{Aab}	47.06^{Ba}		
ECR[3]	42.23^{Ac}	27.02^{Bb}		
Hind shank				
FDS[4]	75.78^{Aa}	32.31^{Bb}		
DDF-H[5]	57.14^{Ab}	31.19^{Bb}		
LMP[6]	75.77^{Aa}	35.51^{Bb}		

^{abc}Least squares means in a column without a common superscript differ ($P < 0.05$).
^{AB}Least squares means in a row without a common superscript differ ($P < 0.05$).
[1]BB = biceps brachii
[2]DDF-F = deep digital flexor - foreshank
[3]ECR = extensor carpi radialis
[4]FDS = flexor digitorum superficialis
[5]DDF-H = deep digital flexor - hindshank
[6]LMP = long digital extensor, medial digital extensor, and peroneus tertius
[7]Raw and cooked collagen content were adjusted to dry matter (DM) basis to account for moisture loss during the cooking process.
[8]SE of the least square means.

Table 3.2 Least squares means of soluble and insoluble collagen%, connective tissue texture evaluated by Asian consumers, and Warner-Bratzler shear force (WBSF) of six different beef shanks (*n* = 48).

Beef shank	Soluble collagen, %[7]	Insoluble collagen, %[7]	Connective tissue texture[8]	WBSF, N
Fore shank				
BB[1]	45.58^{ab}	54.42^{bc}	47.90^{c}	32.36^{b}
DDF-F[2]	27.70^{c}	72.30^{a}	75.54^{a}	86.79^{a}
ECR[3]	35.95^{bc}	64.05^{ab}	52.13^{bc}	32.46^{b}
Hind shank				
FDS[4]	57.16^{a}	42.84^{c}	45.23^{c}	38.25^{b}
DDF-H[5]	43.31^{ab}	56.69^{bc}	55.89^{b}	35.79^{b}
LMP[6]	51.94^{ab}	48.06^{bc}	51.55^{bc}	38.15^{b}
SEM[9]	5.38	5.38	2.60	2.75
P – value	0.01	0.01	< 0.01	< 0.01

^{abc}Least squares means in a column without a common superscript differ ($P < 0.05$).
[1]BB = biceps brachii
[2]DDF-F = deep digital flexor - foreshank
[3]ECR = extensor carpi radialis
[4]FDS = flexor digitorum superficialis
[5]DDF-H = deep digital flexor - hindshank
[6]LMP = long digital extensor, medial digital extensor, and peroneus tertius
[7]Soluble collagen % = (raw collagen content – cooked collagen content) / raw collagen content. Insoluble collagen % = cooked collagen content / raw collagen content – all in dry matter basis.
[8]Connective tissue texture scores: 0 = too soft; 50 = just about right (ideal score); 100 = too hard.
[9]SE of the least square means.

Table 3.3 Least square means of raw and cooked pyridinoline (PYD) and deoxypyridinoline (DPD) densities of six different beef shanks (*n* = 48).

Beef shank	PYD density, mol/mol collagen				DPD density, mol/mol collagen			
	Raw	Cooked	SEM[7]	*P*-value	Raw	Cooked	SEM[7]	*P*-value
Fore shank			0.04	< 0.05			0.001	< 0.01
BB[1]	0.14^{Ac}	0.23^{Ab}			0.008^{Ac}	0.012^{Aa}		
DDF-F[2]	0.54^{Aa}	0.42^{Ba}			0.016^{Aa}	0.008^{Bb}		
ECR[3]	0.19^{Ac}	0.14^{Ac}			0.019^{Aa}	0.013^{Ba}		
Hind shank								
FDS[4]	0.34^{Ab}	0.28^{Ab}			0.014^{Ab}	0.007^{Bb}		
DDF-H[5]	0.19^{Ac}	0.31^{Ab}			0.010^{Ac}	0.010^{Aa}		
LMP[6]	0.13^{Ac}	0.12^{Ac}			0.010^{Ac}	0.007^{Ab}		

^{abc}Least squares means in a column without a common superscript differ ($P < 0.05$).
^{AB}Least squares means in a row without a common superscript differ ($P < 0.05$).
[1]BB = biceps brachii
[2]DDF-F = deep digital flexor - foreshank
[3]ECR = extensor carpi radialis
[4]FDS = flexor digitorum superficialis
[5]DDF-H = deep digital flexor - hindshank
[6]LMP = long digital extensor, medial digital extensor, and peroneus tertius
[7]SE of the least square means.

Table 3.4 Correlation coefficient (*r*) of raw and cooked collagen content, soluble and insoluble collagen % and raw and cooked mature collagen crosslink densities with connective tissue texture and Warner-Bratzler shear force (WBSF) of six beef shanks.

Collagen components	Connective tissue texture	WBSF
Raw collagen content (DM[1])	-0.005	0.211
Cooked collagen content (DM[1])	0.550***	0.615***
Soluble collagen %	-0.498***	-0.392**
Insoluble collagen %	0.498***	0.392**
Raw PYD[2] density	0.560***	0.730***
Cooked PYD[2] density	0.375**	0.324*
Raw DPD[3] density	0.257	0.321*
Cooked DPD[3] density	-0.150	-0.220

[1]Raw and cooked collagen content were adjusted to dry matter (DM) basis to account for moisture los during the cooking process.
[2]Pyridinoline
[3]Deoxypyridinoline
* $P < 0.10$
** $P < 0.05$
*** $P < 0.01$

Table 3.5 Regression equations and coefficient of determination (r^2) for connective tissue texture and Warner-Bratzler shear force (WBSF) based on collagen characteristics measured from six different beef shank cuts.

Responses	Regression equation	r^2	*P*-value
Connective tissue texture	30.190 + 0.513 x cooked collagen content + 30.796 x raw [1]PYD density	0.452	< 0.01
WBSF	-1.072 + 0.103 x cooked collagen content + 8.622 x raw [1]PYD density	0.673	< 0.01

Stepwise procedure used require all variables in the model to be significant ($P < 0.05$).
[1]Pyridinoline

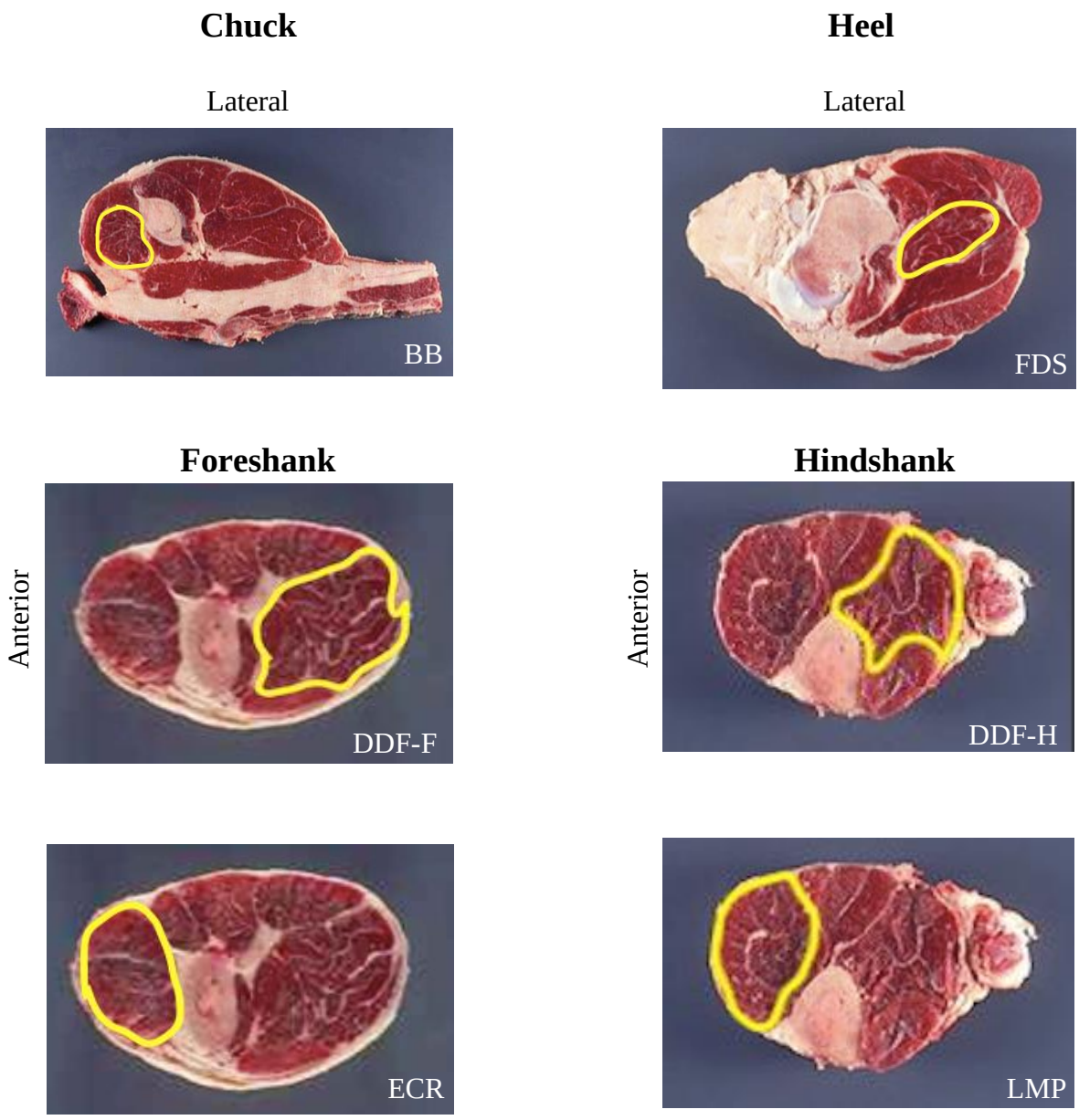

Figure 3.1 Cross-section of the anatomical location of six different beef shank cuts used in this study: *biceps brachii* (BB), *deep digital flexor* from foreshank (DDF-F), *extensor carpi radialis* (ECR), *flexor digitorum superficialis* (FDS), *deep digital flexor* from hindshank (DDF-H), a combination of *long digital extensor, medial digital extensor* and *peroneus tertius* (LMP). Cross section images were downloaded from http://bovine.unl.edu with permission. (Jones et al., 2004).

Appendix A - **Consumer Evaluation Form**

INFORMED CONSENT STATEMENT

1. I volunteer to participate in research involving Sensory Evaluation of Meat. This research will be conducted by personnel in the Department of Animal Sciences and Industry at Kansas State University.

2. I fully understand the purpose of the research is for the evaluation of beef steaks, pork chops, lamb chops, goat meat, poultry meat, ground meat, and processed meat products from the previously mentioned species for the sensory traits of tenderness, juiciness, flavor intensity, connective tissue amount, off flavor presence, odor, and color and sensory evaluation will last approximately one hour.

3. I understand that there are minimal risks associated with participating and that those risks are related to possible food allergies. All meat products will be USDA inspected and all ingredients are GRAS (generally accepted as safe) by FDA.

4. I understand that my performance as an individual will be treated as research data and will in no way be associated with e for other than identification purposes, thereby assuring confidentiality of my performance and responses.

5. My participation in this study is purely voluntary; I understand that my refusal to participate will involve no penalty or loss of benefits to which I am otherwise entitled and that I may discontinue participation at any time without penalty or loss of benefits to which I am otherwise entitled.

6. If I have any questions concerning my rights as a research subject, injuries or emergencies resulting from my participation, I understand that I can contact the Committee on Research Involving Human Subjects, 203 Fairchild Hall, Kansas State University, Manhattan, KS 66506, at (785) 532-3224.

7. If I have questions about the rationale or method of the study, I understand that I may contact, Dr. Travis O'Quinn, 247 Weber Hall, Kansas State University, Manhattan, KS 66506, at (785) 532-3469 or Sally Stroda, 107 Weber Hall, at (785) 532-1273.

I have read the Subject Orientation and Test Procedure statement and signed this informed consent statement, this _____ day of _____, _____.

_____ _____

Printed name Signature

Demographics questionnaire

KANSAS STATE UNIVERSITY

Panel#1 Red

Please tell us a little about yourself:

Panelist Number（小组成员号码）

Gender（性别）

- Male（男）
- Female（女）

Age（年龄）

- Under 20（20岁以下）
- 20 to 29 years old（20-29岁）
- 30 to 39 years old（30-39岁）
- 40 to 49 years old（40-49岁）
- 50 to 59 years old（50-59岁）
- Over 60（60岁以上）

Ethnicity（国籍）

- Chinese（中国人）
- Japanese（日本人）
- Korean（韩国人）
- Taiwanese（台湾人）
- Other（其他）

Marital Status（婚姻状况）

Single（单身）

Married（已婚）

Household Size（家庭人数）

1 Person（1人）

2 People（2人）

3 People（3人）

4 People（4人）

5 People（5人）

6 People（6人）

> 6 People（多于6人）

Annual Household Income（家庭年收入）

< $25,000

$25,000 - $34,999

$35,000 - $49,999

$50,000 - $74,999

$75,000 - $99,999

$100,000 - 149,999

$150,000 - $199,999

> $199,999

Highest Level of Education Completed（拥有的最高学历）

- Non-High School Graduate（高中以下）
- High School Graduate（高中毕业）
- Some College / Technical School（大学 / 技能学校）
- College Graduate（大学毕业）
- Post-College Graduate（除学士以外更高的学历）

When eating beef shank what texture do you prefer?（你比较喜欢什么质地的牛腱肉？）

- Semi-tender（软硬适中）
- Tender（软嫩）
- Falls apart（软烂）

How often do you consume beef shank?（你多久吃一次牛腱肉？）

- Every week（每星期）
- Every month（每月）
- Few times a year（一年几次）
- Never（从来不吃）

>>

Consumer sensory panel questionnaire

Sample Number

9628

Connective Tissue Texture （牛筋质地）

Too hard （太硬） 0 | Just about right （刚好） 50 | Too soft （太软） 100

Amount of Connective Tissue （牛筋量）

Too little （太少） 0 | Just about right （刚好） 50 | Too much （太多） 100

Juiciness （多汁度）

Too dry （太干） 0 | Just about right （刚好） 50 | Too wet（太湿） 100

Overall Texture （总体质地）

Too tough （太老） 0 | Just about right （刚好） 50 | Too tender （太软烂） 100

Flavor Intensity （味道强度）

Too bland （太淡）	Just about right （刚好）	Too intense （太浓）
0	50	100

Where any off flavors detected? （请问有任何异常味道吗？）

Yes（有）

No（没有）

Overall Liking （总体喜欢程度）

Dislike extremely（非常不喜欢）	Neither like nor dislike（一般）	Like extremely（非常喜欢）
0	50	100

Was this sample overall acceptable? （请问这个样品可否接受？）

Yes（可接受）

No（不可接受）

>>

Consumer visual panel questionnaire

Visual Panel# 1 Red

Please tell us a little about yourself:

Panelist Number（小组成员号码）

>>

Sample Number

3287

Size（大小）

Too small（太小）
0

Just about right（刚好）
50

Too large（太大）
100

Color（颜色）

Too light（太淡）
0

Just about right（刚好）
50

Too dark（太暗）
100

Overall Liking（总体喜欢程度）

Dislike extremely（非常不喜欢）
0

Neither like nor dislike（一般）
50

Like extremely（非常喜欢）
100

Was this sample overall acceptable?（请问这个样品可否接受？）

Yes（可接受）

No（不可接受）

>>

Appendix B - **Hydroxyproline Content Determination Protocol**

Modified from:

Bergman, I., & Loxley, R. (1963). Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Analytical Chemistry,* 35(12), 1961-1965.

Reagents:

Make the following three solutions prior to beginning hydroxyproline assay.

3000 µg/mL Stock Hydroxyproline Standard:

In a 50 mL volumetric flask, dissolve 150 mg (0.15 g) hydroxyproline in ultrapure water. Mix thoroughly and transfer to a 50 mL plastic conical tube, and store at 4°C for up to 2 months. (Make sure to get all of hydroxyproline into flask, weigh out hydroxyproline in flask to alleviate any problems with transferring)

Buffer solution:

1.) In a 1-L glass beaker filled with 500 mL of ultrapure water, dissolve while stirring:
    30g Citric acid monohydrate
    15g Sodium hydroxide
    90g Sodium acetate trihydrate
2.) Add 290 mL 1-propanol. Mix vigorously. At this point, if this solution is not mixed continually, it will separate into layers.
3.) Adjust the pH to 6.0 with concentrated HCl
4.) Transfer to 1-L volumetric flask and bring up to volume using ultrapure water. Store in a labeled, glass bottle covered in foil at 4°C for up to 1 month. Before using, make sure solution has not separated into layers again.

60% Perchloric acid: *Work in a fume hood and wear safety glasses.*

In a graduated cylinder measure out 85.7 mL of 70% perchloric acid. Bring up to 100 mL with ultrapure water. Transfer to labeled, 100 mL glass bottle. Store at 4°C for up to 1 month.

Make the following solutions same day, and just before adding the solution to the first set of tubes.

Chloramine-T Oxidant Reagent: *Wear a mask when weighing out the Chloramine-T*

Dissolve 1.41g chloramine-T in 100 mL of Buffer solution. Make fresh daily, approx. 20 mL over what is needed to pipette 1 mL into each tube

DMBA (dimethylaminobenzaldehyde) Color Reagent:

In a 100 mL beaker, dissolve 10 g of 4-dimethylaminobenaldehyde in 35 mL of cold 60% perchloric acid. Slowly add, with stirring, 65 mL of 2-propanol (isopropyl alcohol)

***Protocol*:**

1. Transfer 50 µL of the rehydrated sample to a 15 mL conical tube and dilute it with 9.95 mL of ultrapure water.

2. Transfer 0.5 mL of the diluted samples to 15 x 125 mm glass tubes.
3. Using the repeater pipet, pipet 1.50 mL of ultrapure water into all glass tubes containing the diluted samples.
4. Set water bath to 60°C and preheat prior to reading (takes approx. 30-45 min to get to correct temperature).
5. Prepare the 30 µg/mL working hydroxyproline standard: pipet 1 mL of 3000 µg/mL stock hydroxyproline into a 100-mL volumetric flask. Bring up to volume with ultrapure water. Mix thoroughly.
6. Prepare the standard curve following the table below:
    a. Order of the tubes for the standard curve is as follows:
    Blank, S-1, S-2, S-3, S-4, S-5, S-6, S-7

Standard Number	Volume of 30 µg/mL working hydroxyproline standard, mL	Volume of ultrapure water, mL	Final volume, mL	Hydroxyproline final concentration, µg/mL
Blank	0.000	2.000	2.000	0.000
S-1	0.100	1.900	2.000	1.500
S-2	0.200	1.800	2.000	3.000
S-3	0.300	1.700	2.000	4.500
S-4	0.400	1.600	2.000	6.000
S-5	0.500	1.500	2.000	7.500
S-6	0.600	1.400	2.000	9.000
S-7	0.700	1.300	2.000	10.500

7. Add 1.0 mL of Chloramine-T Oxidant Reagent to glass tubes containing the standards and diluted samples. Vortex to mix, and incubate at room temperature for 20 min.
8. During incubation of Chloramine-T, prepare sufficient amounts of DMBA Color Reagent to complete the assay
9. After incubation of chloramine-T, add 1 mL of DMBA Color Reagent to tubes. Vortex to mix, cover with aluminum foil, and incubate in a water bath set to 60°C for 90 min (timing is critical).
10. After incubation in water bath is complete, remove tubes and move them to a cold water bath for at least 3 mins
11. Pipet 0.2 mL of sample to 96 wells plate and read absorbance of samples on a Spectrophotometer set to 558 nm.
12. Total collagen content for each sample is determined by assuming 14% of collagen is hydroxyproline by weight.
13. Collagen content is calculated as: [concentration of sample in µg/mL x 4 mL x (10 mL/0.5 mL) x (0.5 mL / 0.05 mL) x 7.14] / muscle tissue weight in g

Appendix C - **Collagen Crosslink Sample Preparation Protocol**

Modified from:

Avery, N. C., Sims, T. J., & Bailey, A. J. (2009). Quantitative determination of collagen cross-links. In *Extracellular Matrix Protocols* (pp. 103-121): Springer.

Reagents:

1.) Phosphate buffered saline (0.15 M sodium chloride, 0.05 M sodium phosphate pH 7.4)
2.) Sodium borohydride solution (1 g of sodium borohydride dissolved in 10 mL of 0.01M of sodium hydroxide at 4^{o}C) **immediately before use. When react with water, it will produce an explosive gas (hydrogen), please make sure this step is conducted in fume hood**.
3.) 6N hydrochloric acid (HCl)

Sample Preparation Procedure:

1. Weigh out 0.5 g (500 mg) of pulverized muscle tissue in labeled 15 x 125 mm glass tubes.
2. Add 2.5 mL of phosphate buffered saline to each tube.
3. Add 0.05 mL (50 µL) of sodium borohydride solution to each tube in the fume hood and let the reduction process proceed for 1 hr – gas will be released
4. Add 0.2 mL (200 µL) glacial acetic acid to each tube to drop to pH 3.0 to terminate the reaction step
5. Centrifuge the samples at 1,000 g for 5 min, and carefully decant the reagent to hazardous waste.
6. Wash the samples with 3 x 5 mL of ultrapure water - (wash, centrifuge, decant)
7. Dry all samples in the evaporator with the following setting:

Temperature	Speed	Vacuum	Time
80^{o}C	53%	200 mbar	Run until dryness

8. Add 10 mL of 6N HCl to each tube
9. Heat in oven at 115°C (preheat) for 24 hrs
10. Take samples out from the oven and use the evaporator to evaporate all residual 6N HCl (**make sure the cold trap is turned on with enough ethanol added**) – setting:

Temperature	Speed	Vacuum	Time
70°C	53%	200 mbar	Run (non-stop)

Note: the evaporation process will take about 24 hrs

11. Rehydrate the samples in 0.5 mL ultrapure water and transfer 0.25 mL to each microcentrifuge tube (one for crosslink analysis and one for hydroxyproline content analysis).
12. Store in −80°C freezer until analysis.

Appendix D - **Collagen Crosslink Analysis Protocol**

Modified from

Viguet-Carrin, S., Gineyts, E., Bertholon, C., & Delmas, P. D. (2009). Simple and sensitive method for quantification of fluorescent enzymatic mature and senescent crosslinks of collagen in bone hydrolysate using single-column high performance liquid chromatography. *Journal of Chromatography B Analytical Technologies Biomedical and Life Science, 877*(1-2), 1-7.

Abbreviations

PYD: Pyridinoline
DPD: Deoxypyridinoline
HFBA: Heptafluorobutyric acid
W.S.: Working Standard

Sample Dilution

Tenderloin contains low concentration of collagen crosslinks. Therefore it is used as a negative control matrix to prepare standard curves and quality control samples.

Calculations:

Meat samples: 20 mg of meat sample → 0.5 mL ultrapure water
0.5 g of muscle tissue (= 500 mg)
Dilution: 0.5 mL * (500 mg / 20 mg) = 12.5 mL of ultrapure water

Dilution Procedure

1. Transfer 250 µL of concentrated sample to a 15 mL conical tube and dilute it with 6.25 mL of ultrapure water
2. Centrifuge the samples at 2,000 g for 20 min
3. Aliquot the supernatant (diluted sample) into microcentrifuge tube, discard the pellet, and stored the diluted samples at -80°C until analysis

Preparation of Clean-up Procedure

Reagents:

a. Sample buffer: mixture of acetonitrile and acetic acid (6:1, v/v)
b. Wash buffer: mixture of acetonitrile, acetic acid, and ultrapure water (8:1:1, *v/v/v*).

Prepare the following working standard, standard curve, and quality control samples on the same day of collagen crosslink analysis

Preparation of Working Standard (W.S.)

1. The PYD / DPD standard (P/N 4101) contains 5.91 µg/ml of PYD and 3.08 µg/ml of DPD in 0.2 M of acetic acid, and it is stored at -20 °C.
2. 3000 µL of W.S. is prepared by mixing 300 µL of PYD / DPD standard with 2700 µL of sample buffer. The total volume of W.S. for the standard curve and quality controls is 2705 µL, and the left over W.S. run as a standard control (blank).

Preparation of Standard Curve

In a 5 mL glass tube, add the following amount of W.S., sample buffer, and diluted tenderloin hydrolysate:

1. 25 µL W.S. + 2.88 mL sample buffer + 0.4 mL tenderloin
2. 50 µL W.S. + 2.85 mL sample buffer + 0.4 mL tenderloin
3. 100 µL W.S. + 2.80 mL sample buffer + 0.4 mL tenderloin
4. 200 µL W.S. + 2.70 mL sample buffer + 0.4 mL tenderloin
5. 400 µL W.S. + 2.50 mL sample buffer + 0.4 mL tenderloin
6. 800 µL W.S. + 2.10 mL sample buffer + 0.4 mL tenderloin

Vortex to mix, and the final volume for each glass tube should be 3.30 mL

Preparation of Quality Control (QC) samples

In a 5 mL glass tube, add the following amount of W.S., sample buffer, and diluted tenderloin:

1. 80 µL W.S. + 2.82 mL sample buffer + 0.4 mL tenderloin
2. 350 µL W.S. + 2.55 mL sample buffer + 0.4 mL tenderloin
3. 700 µL W.S. + 2.20 mL sample buffer + 0.4 mL tenderloin

Vortex to mix, and the final volume for each glass tube should be 3.30 mL

Preparation of Beef Shank Samples

In a 5 mL glass tube, mix 0.4 mL (400 µL) diluted sample with 2.8 mL sample buffer, vortex, and the final volume for each prepared sample should be 3.20 mL

Cellulose Clean-up Procedure

Make the following reagent daily prior to each clean-up process.
Reagent:

- *1% HFBA*: 1.2 mL of 1% HFBA per sample is needed.

Protocol:

1. W.S., standard curve, and QC should be prepared on the day of collagen crosslink sample analysis, while beef shank collagen crosslink samples could be clean-up and stored at -80°C until analysis.
2. Connect a Bond Elut Cellulose cartridge 300 mg, 3 mL (P/N 12102095) to a Vac Elut 20 Manifold apparatus to the vacuum.
3. Equilibrate the cellulose column with 2.5 mL of wash buffer. Let the buffer go through slowly without letting the cartridge dry.
4. Pipet sample into the column and allow it to go through slowly without letting the cartridge dry.
5. Wash the glass tube with 2.5 mL of wash buffer and load it into the column.
6. Wash the column with 4 × 2.5 mL of wash buffer to remove interfering fluorophores followed by 0.2 mL of ultrapure water. Discard the waste (step 2 to 6) of each sample.
7. Replace the waste tube with a clean, labeled 5 mL glass collection tube below the cellulose column.
8. Elute sample with 2 x 0.6 mL of 1% HFBA. Vortex and transfer the collected sample into amber vials with insert and apply a pre-slit screw cap. The total volume for each HPLC vial should be approximately 1.4 mL.

Ultra-Performance Liquid Chromatography (UPLC) Conditions

Mobile phase / solvents

A: 0.2% HFBA with ultrapure water (In a 500 mL volumetric flask, add 610 µl of HFBA and adjust the volume to 500 mL with ultrapure water).
B: Acetonitrile (HPLC grade)

UPLC parameters:
UPLC system: Waters Acquity H UPLC system equipped with a degasser, a quaternary pump, a sample manager, and a fluorescence detector.
Column: Waters HSS T3 column (2.1 × 100 mm, 1.8 µm)
Injection volume: 5 µL
Column temperature: 60 °C
Flow rate: 0.5 mL / min
FLD: Excitation at 297 nm and detection at 395 nm
Run time: 20 min

Inlet method set as the following:

Time (min)	%A	%B	%C	%D
Initial	100	0	0	0
10	85	15	0	0
15	0	100	0	0
15.01	100	0	0	0
20	100	0	0	0

Masslynx set up (sample order)

1. Blank
2. 25 µL
3. 50 µL
4. 100 µL
5. 200 µL
6. 400 µL
7. 800 µL

Standard curve

8. 80 µL
9. 350 µL
10. 700 µL

QC

11. Beef shank samples
12. End batch / wash (cool down column from 60 °C to 20 °C and wash out any residue in the column).

If there are more than 10 samples, insert the 350 µL QC into every 10 samples and run the standard curve again at the end of the batch (before the "end.batch/wash).

Note: samples with concentrations out of the standard curve range were further diluted with 0.2% HFBA in 1:5 dilution and re-analyzed