Influence of beef production practices on meat quality characteristics and expression of genes related to collagen synthesis and degradation in the bovine m. *triceps brachii*

by

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Abstract

Production factors such as cattle breed, genetics, age, feed, muscle activity and processing factor such post-mortem ageing may affect the contribution of collagen and collagen cross-links to beef toughness. Selection of low residual feed intake (RFI) cattle, which consume less feed than expected, to reduce the cost of production would only be beneficial if it has no negative effect on the quality attributes of beef. Two studies were conducted to examine the effects of breed type and RFI on meat quality and collagen characteristics of beef and the how the expression of genes related to collagen synthesis and degradation are related to collagen characteristics and meat quality attributes of ***triceps brachii***, a muscle from the chuck characterized with high intramuscular connective tissue. For the first study, seventy-one beef steers from Angus (n=23), Charolais (n=24) and Angus crossbred (n=24) genetics were used to examine the influence of breed type, residual feed intake (RFI) and post-mortem ageing on meat and carcass quality attributes and intramuscular connective tissue characteristics in the m. ***triceps brachii***. Each breed had high RFI and low RFI steers (n=12 each) to test the hypothesis that genetic selection for low RFI beef cattle may increase collagen content, reduce collagen heat solubility and increase beef toughness of the ***triceps brachii*** muscle. The effects of breed type and genetic selection of low RFI animals on ***triceps brachii*** muscle were limited although post-mortem ageing for 13 days reduced Warner-Bratzler shear force, a measure of toughness, and increased collagen heat solubility.

In the second study, twenty-four beef steers (Angus (n = 8), Charolais (n = 8) and Angus crossbred (n = 8) with low and high intramuscular collagen solubility at 3 days post mortem (dpm) were used to test the effects of breed type and solubility level on genes

involved in the synthesis and degradation of collagen. The hypothesis that phenotypic measurements of meat quality attributes and intramuscular connective tissue are related to differences in the expression level of target genes was tested. Expression levels of 27 candidate genes were evaluated using quantitative real time polymerase chain reaction (RT-qPCR) and the mean differences in expression between candidate and housekeeping (*18s ribosomal RNA*) genes were calculated (ΔCT). Surprisingly, Angus beef steers, a breed known for its superior marbling and tenderness had higher expression of collagen types genes *COL1A1, COL5A1, COL6A1,* and genes involved in collagen and collagen cross-links synthesis such as *FGF2, FGFR1, LOX, LH, ITGA1, ITGB1, P4HA1, SMAD2, SMAD3, SMAD 2, SMAD 6,* and *SMAD 7.* However, it also had higher expression of *MMP* genes and lower expression of *TIMP* genes. Expression of *FGFR1* and *ITGA1* genes were related to increased beef toughness of m. *triceps brachii* early post-mortem at day 3 of ageing. To increase the value and overall eating quality of m. *triceps brachii*, extending post-mortem ageing periods of the muscle is recommended. These results indicate that further research to understand mechanism controlling the expression of *FGFR1, ITGA1, TIMPs* and *MMPs* may be important to prevent excessive collagen and mature collagen cross-links accumulation.

Preface

This thesis contains two research works that investigated the effects of breed type, selection for low RFI beef cattle, post-mortem ageing and expression of collagen biochemical mechanisms genes on the eating quality of *Triceps brachii* muscle. Beef cattle were reared at University of Alberta Kinsella cattle herd according to Kinsella animal care protocol AUP00000777.

For the first study (Chapter 2), the data were provided by Agriculture and Agri-Food Canada's Lacombe Research and Development Centre and were analyzed by me. This study received research ethics approval from a University of Alberta Research Ethics Board under the project name "Genetics of the eating quality of high connective tissue beef", PRO00054386, Date: June 24, 2015.

For the second study (Chapter 3), the experimental design, data collection and data analyses were collected by me, with the assistance of Dr. Heather Bruce and Dr. Leluo Guan.

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Abbreviations

ADG	Average daily gain
ANC	Angus crossbred
ANOVA	Analysis of Variance
AFAT	Average backfat thickness
BF	Backfat thickness
BW	Body weight
CCW	Cold carcass weight
DMI	Dry matter intake
DOT	Days on test
EC	Ehrlich chromogen cross-link
FCR	Feed conversion ratio
FUFAT	Final ultrasound backfat
HOT	Hot carcass weight
HYP	Hydroxyproline
IMCT	Intramuscular connective tissue
LM	Longissimus muscle
LMY	Lean meat yield
MWT	Metabolic body weight
PYR	Pyridinoline cross-links
REA	Rib eye area
RFIf	RFI value adjusted for backfat thickness
SEM	Standard error mean
TB	m. *triceps brachii*
WBSF	Warner -Bratzler shear force
WHC	Water holding capacity

Chapter 1: Introduction and Literature Review

In this twenty-first century, the demand for meat of premium eating quality and high nutritive value is increasing in most countries; thus, the focus of the meat industry is to consistently produce quality meat that is acceptable to consumers to ensure continuous consumption of meat and meat products (Joo et al., 2013). Raw beef characteristics like colour, water holding capacity (WHC), pH, intramuscular fat (IMF, marbling), texture and beef palatability attributes such as tenderness, juiciness and flavour are considered to be the most significant factors affecting consumers beef purchasing or re-purchase decision (Smith et al., 2008; Alaa et al., 2014). These characteristics are used to predict the technological and organoleptic properties of meat to gauge the appropriate applications for the meat and its potential eating quality (Mullen 2002). Meat quality is defined as "a measurement of attributes or characters that determine the suitability of meat to be eaten as fresh or stored for reasonable period without deterioration" (Madruga et al., 2010). Eating quality of meat differs from meat quality in that eating quality refers to the quality of the sensory experience and satisfaction derived during the eating of the meat (Smith & Carpenter 1976). It is therefore of great importance to understand what meat quality measurements indicate, the scientific basis of these beef quality attributes, and ways to improve or control them, particularly as they affect the eating quality of the product.

Although eating quality of beef is important to the consumer, carcass quality is important to the beef producer, as it is on the basis of carcass quality rather than eating quality that producers are financially compensated. Generally, carcass quality is greatly influenced by multiple interacting factors which encompass the conditions under which the animal is raised

and the meat from their carcasses produced. These production conditions include the management system used during the raising of an animal, the age of the animal, its breed, genotype, and feeding (Henrik et al., 2005). However, the variability of beef quality emanates from the fact that quality attributes are altered at pre-slaughter handling and stunning, in the beef processing plant, in the retail store and even in the purchaser or consumer's home (Mullen, 2002). Hence, to reduce or eliminate the effect of the variability in cooked beef, post-harvest meat processing practices, particularly post-mortem ageing and cooking, play vital roles in the establishment of meat palatability (Kim et al., 2014).

Obvious improvements have been reported in the eating quality of aged beef through the action of proteolytic enzymes present in meat (Yuan et al., 2018). Teye & Okutu (2009) reported a significant impact of ageing on the juiciness of beef steaks, and others have reported its effects on beef initial colour and colour stability (Vitale 2014), on WHC (Lawson 2004), on beef flavour (Brewer & Novakofski, 2008), and on tenderness (Kim et al., 2014). However, questions as to why cooked beef tenderness varies in different muscles and/or why beef cut from matured cattle carcasses is tough still exist (Purslow 2014; Yuan et al., 2018). These questions persist because a solution to "background toughness" contributed by the variation in the composition and structure of intramuscular connective (IMCT) tissues and cross-links present in cooked beef, especially in retail cuts from round and chuck, is yet to be determined (Stolowski et al., 2006).

Muscle connective tissues primarily consist of proteins, glycoproteins, glycosaminoglycans and proteoglycans. These constituents are found in the three layers of connective tissue associated with muscle, namely, the epimysium, perimysium and endomysium (Purslow 2002). The epimysium, which is tough and thick, is usually separated

from cuts during fabrication (industrial processing of meat) or during consumption by the end consumer; therefore, only the endomysium and perimysium are regarded as IMCT that influence meat quality (Girard et al., 2012). Collagen is the most abundant protein present in IMCT, and it can constitute between 1.0-15% of the dry mass of meat (Leptit, 2008), and 0.2-2.5% of wet muscle (Patten et al., 2008). It is thought to have major influence on the background toughness in cooked beef between muscles (Purslow 2005). Collagen cross-links accumulate as the age of the animal increases (Leptit 2008). The accumulation of collagen cross-links over time results in the formation of trivalent cross-links from divalent cross-links (Roy et al., 2015). Trivalent cross-links are more complex, mature and thermally-stable, and thus resist protease hydrolysis during post-mortem ageing (Hill 1996). Pyridinoline and Ehrlich's chromogen/pyrrole cross-links are matured trivalent cross-links which have both been characterized to be directly involved in cooked beef toughness (Roy et al., 2015).

Compared to other conventional approaches, genomic applications provide a thorough observation of biological processes, which helps to identify novel genes, single nucleotide polymorphism (SNPs) and interactions (Picard et al., 2015). With the recent advancement of high throughput technologies such as the polymerase chain reaction (PCR) and real-time quantitative PCR, genomics analysis has been suggested as a method to potentially facilitate the manipulation of meat quality traits by revealing genes, proteins or metabolites whose expression level or abundance is associated with a phenotype of interest (D'Alessandro et al. 2012; Picard et al. 2012). Thus, for meat scientists, recent objectives have been focused on identifying meat quality biomarkers or genes that are quantifiable in live animals or during early post-mortem ageing to improve overall meat quality (Purslow 2005; Picard et al. 2015; Guo & Dalrymple 2017; Carlos et al. 2019).

Reducing the cost of production while maintaining or improving meat quality is crucial and beneficial to consumers who want to pay less for the best quality meat and to the beef industry that wants to be able to make profits. Provision of feed has long been recognized as the greatest input cost in most animal production systems, representing at least 60-75% with consideration to the significant variation in feed intake among individual animals. It is therefore important that the effectiveness of feed consumed by cattle will help to reduce the cost of production (Herd et al., 2003; Arthur et al., 2004). The most recently adopted feed efficiency measure employed is the selection of animals for low residual feed intake (RFI), first suggested by Koch et al. (1963). Compared to high RFI animals that consume the most feed, low RFI animals consume less but have a fast growth rate, indicating superior feed conversion rate, and are therefore referred to as efficient animals (Arthur & Herd, 2008). However, the effects of selection for low RFI on carcass quality traits and cooked meat quality remain contradictory (Baker et al., 2006).

This chapter will be reviewing how animal production and post-harvest factors affect carcass quality traits as well as beef quality. It will also encompass how myofibrillar proteins and intramuscular connective tissues contribute to the toughness of meat and how their toughening influence on meat can be reduced or eliminated by post-mortem storage (ageing) in the presence of calpains, cathepsins, lysosomal enzymes and metalloproteinases, the major enzymes responsible for protein and collagen degradation in meat. This review will also explore the mechanisms associated with collagen synthesis and degradation and how functional genomics can be used to obtain increased understanding of these mechanisms.

1.1 Beef eating quality

The gold standard of beef quality is its eating quality and the overall eating satisfaction derived by consumers (Markus et al. 2011). It is therefore important to evaluate factors that have inherent effects on quality attributes considered by consumers when purchasing fresh meat at retail (Smith et al., 2008). Factors associated with animal production and management such as breed, gender, genetics, age, feeding management and residual feed intake (Hocquette et al., 2007), and post-harvest factors such as post-mortem ageing, electrical stimulation, and cooking method (Mullen 2002), play vital roles on beef palatability. However, this chapter will only be reviewing the effect of breeds, age, residual feed intake, cooking and post-mortem ageing on beef eating quality.

1.1.1 Effect of breeds on beef eating quality

Breed is one of the most important factors that influence the characteristics of carcasses and hence of cooked beef quality (Cuvelier et al., 2006). It has been estimated that 50% of the difference in beef tenderness, an important palatability trait to consumers, between breeds is due to genetic effects (Wheeler et al., 2000). Differences in beef tenderness attributed to genetics have been to date associated with variation in the rate and extent of muscle proteolysis as estimated by calpain and calpastatin actvities during post-mortem ageing (Janse et al., 2013; Khan et al., 2016). The starkest differences in beef quality appear to exist between *Bos indicus* and *Bos taurus* cattle. *Bos indicus* breeds such as the Brahman originate from sub-tropical and tropical areas, while *Bos taurus* breeds such as Angus and Charolais are genetically distinct beef cattle originating from regions within temperate climates. This genetic diversity between *Bos indicus* and *Bos taurus* produces carcasses with different qualities (Lopez et al., 2012). Several studies have consistently reported that *Bos taurus* cattle

produce beef with a higher marbling score and greater tenderness than *Bos indicus* cattle (O' Connor et al., 1997; Burrow et al., 2001). Within the *Bos taurus* breeds there are differences in beef quality, with Angus beef having higher sensory tenderness ratings and drip loss compared to Charolais beef, and Charolais beef having higher juiciness ratings compared to Angus beef (Chambaz et al., 2003). The Angus breed has been shown to deposit more intramuscular fat (Greenwood et al., 2015; Krone et al., 2016; Dinh et al., 2010) than Charolais (Greenwood et al., 2015; Dinh et al., 2016), and Brahman (Krone et al., 2016). Some studies have also shown that there are no significant differences in beef flavour between the two breeds (Shackelford & Koohmaraie, 2001). Also, no differences in beef quality were observed between the two breeds when reared to the same age (Koch et al., 1976) and slaughtered at an equal back fat thickness (Laborde et al., 2001). This literature suggests that differences between breeds may be driven by physiological age rather than by actual genetic differences, and that slaughtering at the same back fat may eliminate differences observed (Aleksic et al., 2011).

1.1.2 Effect of animal age and sex on beef eating quality

Beef cattle are usually slaughtered between 9 and 30 months of age in most parts of the world and as a result large variation is observed in cooked beef tenderness (Purslow 2005). Harper (1999) concluded that the reason for tougher meat in older animals is due to the change in the intramuscular connective (IMCT) protein known as collagen which forms cross-links over time. The amount of collagen does not change, however, with an increase in the age of an animal, although collagen heat solubility does decrease, and the amount of insoluble collagen increases (Purslow 2005). Intramuscular fat and the colour of meat were reported to decrease with an increase in the age of the animal but there was no influence on palatability ratings (Maltin 1998). As a result of the influence of animal age on meat palatability, the

value of the carcass declines with animal age because of the assumption that cooked beef quality is decreased as well (Žgur 2003).

Although the age of cattle is among the most important criteria in the USA and Canadian beef grading systems (USDA 1997) it does not fully determine or explain the overall quality of beef (Harper 1999). Beef from bulls (matured male bovines) has lower quality than beef from steers (castrated male bovine) of the same age (Dransfield 1984; Rodbotten, et al. 2010). Field et al. (1966) observed that light bull beef was less tender when compared to that from light steers but was more tender than beef from heavy steers. Libouriussen et al. (1977) reported that bulls were of decreased meat quality, reduced intramuscular fat and decreased tenderness. These results suggest that sex of the animal and it maturity or growth rate, as well as the physiological age of the animal as indicated by the amount and rate of fat accretion may be more important than animal chronological age in the determining the eating quality of beef.

1.1.3 Selection for low RFI on beef eating quality

Residual feed intake (RFI) is used as a measure of feed efficiency and is defined as the difference between the actual and predicted feed intake for the observed gain (Herd & Bishop, 2000; Arthur et al., 2001a, b). RFI is moderately heritable (h^2 = 0.16 to 0.43; Herd et al., 2003), and the selection for parents with low RFI, referred to as 'efficient animals', resulted in progeny that were able to eat less feed while maintaining the same production levels and weights as high RFI animals at slaughter (Arthur et al., 2001, 2006; Crews 2005). Therefore, the breeding and selection for low residual feed intake (RFI) cattle has been investigated and concluded to reduce production cost and increase profitability (Baker et al., 2006).

However, it is important to understand the phenotypic relationships with carcass and beef quality before initiating the selection for low RFI in breeding programs to ensure no

unfavourable changes in cooked beef (Nascimento et al., 2016). There has been disagreement with the outcome from the few studies conducted, with Baker et al. (2006), who used muscle loin steaks from Angus cattle, reporting no differences between low and high RFI cattle for hot carcass weight, yield grade, colour, marbling score, calpastatin activity, intramuscular fat, and shear force value but reported reduced cooking loss in the beef from low RFI cattle. These results concurred with the results from RFI studies involving Angus and Charolais breeds reported by Herd and Bishop (2000), Herd et al. (2003), and Arthur et al. (2001a, b). Several other studies, notably Richardson et al. (2001) reported that progeny from Angus cattle selected for low RFI had less fat than cattle with high RFI (McDonagh et al., 2001). Baker et al. (2006) reported that steaks from high RFI cattle tended to be lighter and more yellow (b*) than steaks from low RFI cattle; however, McDonagh et al. (2001) reported no colour differences between longissimus muscle steaks from carcasses of cattle from both RFI levels. Using sensory panelists, Baker et al. (2006) also reported no differences in beef steak tenderness and flavour between both RFI levels, although the juiciness score showed a tendency to be lower ($p = 0.10$) in steaks from high RFI animals. Hence, further studies to validate the effect of genetic selection for low RFI on meat quality characteristics need to be performed.

1.1.4 Effect of muscle locations or types on beef eating quality

In general, muscles from the round and chuck of the beef carcass are known to be heavily involved in locomotion or movement, and thus tougher when compared to muscles of the loin and rib, which are involved in support (Mahesh et al., 2019). Several studies, notably McKeith et al. (1985) reported that muscle cut from the *Infraspinatus, Longissimus dorsi* (LD) and *Psoas major* (PS) had the highest fat contents and were rated as most tender and flavourful when compared to the *Gluteus medius* (GM) and *Triceps brachii* (TB). However,

the TB had a high amount of moisture and low amounts of collagen when compared to the *Infraspinatus* (McKeith et al., 1985). With this, several researchers identified the need to focus more on understanding and improving the palatability of individual muscles or group of muscles with current technology such as cooking, electrical stimulation and post-mortem ageing (Eilers et al., 1996; Purslow 2014).

1.2 Carcass quality measurement and beef eating quality

In order to regulate marketing and sale of beef, various countries including the European Union, United States, Australia, Canada, Japan, and Korea grade beef quality by observing and classifying carcass post slaughter attributes (Gotoh et al., 2018). The visual assessment of certain traits is utilized to classify carcasses due to the known and accepted scientific associations with beef eating quality (Aalhus et al., 2004). In Canada and USA, the grading systems identify both the youthful and mature carcasses and separate them from each other. For example, mature beef cattle above 30 months are separated from those under 30 months because as animals age there is a decrease in beef tenderness and juiciness most especially (Purslow 2005).

In general, the Canadian Beef Grading System measures carcass maturity, back fat thickness, muscling, colour, fat colour and marbling (Aalhus et al., 2004). To identify mature beef cattle carcasses the degree of bone ossification is assessed (López-Campos et al., 2012). For a carcass to qualify for the Canada A or B grades, cattle must have less than 50% ossification in thoracic and vertebral bones (Canada Gazette 2007; Canadian Beef Grading Agency 2014). Marbling is a major factor included in both the Canada and USA grading systems because of its positive relationship with flavour and juiciness (Anonymous 2009). To qualify for the Canada A grades, youthful carcasses must have a minimum of 2 mm of back

fat, and their rib eye at the 12th and 13th rib interface must have traces of marbling, be a bright red colour and firm, and have white or amber - coloured fat (Aalhus et al., 2004). Also, carcasses with a low or small amount of back fat are downgraded in the Canadian quality grading system because of its association with reduced tenderness (Aalhus et al., 2001).

Carcasses are then assigned to either Canada A, AA, AAA, or Prime grades based strictly on the amount of intramuscular fat or marbling present in raw meat which could be in traces, slight, small amount, or slightly abundant (Aalhus et al., 2004). Prior to January 2019, the yield grade estimation in Canada was based on the percentage of lean yield and the grades were assigned as Canada 1 or Y1 (greater than 59%), Canada 2 or Y2 (54% - 58%), and Canada 3 or Y3 (53 % or less). This yield was calculated as a prediction of retail yield using a a grade ruler, specifically:

Lean % = 63.65 + 1.05 x (muscle score: which is a combination of length and depth of the ribeye) – 0.76 x (grade fat: fat depth at the 12th and13th rib) (Canadian Beef Grading Agency 2014; Aalhus et al., 2014).

However, a new method for determination for lean meat yield in beef cattle was developed by Agriculture and Agri-Food Canada and implemented in January 2019. This was done to establish harmonize beef yield grade results between Canada and USA. Hence, the new method is closely related to that from the United States beef grading system. Each carcass is now assigned a yield grade of either Canada 1 or Y1 (52.4% or more), Canada 2 or Y2 (50.2% to 52.2%), Canada 3 or Y3 (47.7% to 50.1%), Canada 4 or Y4 (45.2% to 47.5%) or Canada 5 or Y5 (45.0 or less), and is calculated with a yield grade ruler using the fat classes and muscle scores:

Retail cut yield % = 53.13 + (0.44 × muscle score) – (0.32 × fat thickness, mm) (Canadian Beef Grading Agency 2019)

The value of beef is in the eyes, mouth and mind of the consumer, and beef producers are being paid based on carcass grade (Aalhus et al., 2014). It is therefore important that beef producers are able to understand the beef quality attributes upon which the grading system is based as well as the consumer's demand for consistency.

1.2.1 Raw meat colour

Fresh meat colour as one of the most important meat quality indices because this is the first feature that can be seen and used by consumers to determine the wholesomeness and freshness of meat. Therefore, great attention is paid to the colour of fresh meat and decisions on whether to purchase or not to purchase at retail are based on meat appearance (Ponnampalam et al., 2013). The colour of normal beef exposed to oxygen is bright cherry red and a deviation from this state is perceived as a deviation from freshness and a degradation of quality (Sawyer et al., 2009). Meat scientists and beef producers are aware of this and as such focus on optimizing beef colour appeal and stability throughout retail display.

Basically, meat colour is dependent on species, age and muscle type and differences in muscle colour are driven by myoglobin and its oxygenation state (Joo et al., 2013). Therefore, much research has been conducted and focused on optimizing myoglobin states (Bekhit et al., 2013). Myoglobin content in muscle is affected by various factors such as animal exercise, diet, genetics and environment (Joo et al., 2013) but the rate of myoglobin oxidation is highly dependent on the rate and extent of pH decline early post mortem (Faustman et al., 2010). Winkler et al. (1939) observed maximum red, green and blue light scattering around a pH value of 5 and a decline in scattering as the pH approached 7 in post rigor beef.

Dark or pale meat colour results in rejection by consumers and economic loss to beef producers compared to meat that is bright red (Viljoen et al., 2002). Whether meat is dark or pale is not only determined by myoglobin concentration and oxygen state but also by the amount of water present in muscle tissue and the structure and different muscle fibre types (Hughes et al., 2014). Dark, firm, dry (DFD) beef is therefore reported to be associated with increased pH (pH > 5.8) and myoglobin and minimal shrinkage in muscle fiber diameter (Mounier et al., 2006) while pale, soft exudative (PSE) meat is associated with low pH (< 5.5) (Swatland 2008) and low amounts of myoglobin oxidation (Abril et al., 2001).

Colorimetric instruments have been applied to objectively measure fresh meat colour and the information on the colour is presented in a colour space described by the International Commission illumination (CIE) colour coordinates (L*, a*, b*) (CIE, 1978). A LAB colour space has the dimensions L* for lightness, a* for redness to greenness and b* for blueness to yellowness (CIE, 1978). The most commonly used colorimetric instruments used to quantify beef colour are the HunterLab MiniScan™ (HUNTER) (Tapp et al., 2011) and the Minolta handheld which is portable and hence convenient, but recently the Nix Colour Sensor Pro™ (NIX) is of interest because of its affordability, connectivity and user friendly interface although with less history of use for meat colour measurement (Hodgen, 2016; Stiglitz et al., 2016).

1.2.2 Raw meat water holding capacity (WHC)

Muscle contains approximately 75% water and the capacity of a muscle to hold its water during the conversion of muscle to meat is referred to as water holding capacity (WHC) (Trout 1988). WHC is closely related to sensory juiciness and poor WHC results in increased cooking loss, purge loss, drip loss and lack of juiciness (dry meat) which can reduce eating quality as well as negatively affecting the texture of cooked beef (Aaslyng, 2002, Woelfel et

al., 2002). The loss of weight in carcasses and their cuts as a result of high drip loss and purge loss are of concern to beef industry because it affects the yield and quality of processed meat which subsequently reduces profitability and costs the beef industry millions of dollars annually (Wright et al., 2005).

Water that ultimately becomes purge or drip originates from the spaces between the muscle fiber bundles and escapes via the perimysial /endomysial network during rigor development and the conversion of muscle to meat (Offer & Cousins, 1992). Water content, water distribution and the mobility of water in muscle and meat changes as a result of numerous antemortem factors such as breed, muscle, stress level and type (Honikel 2004). However, excessive drip loss and soft texture are well documented to result mainly from rapid pH decline and high temperature during aging (Joo et al., 1999), which causes sarcoplasmic and myofibrillar protein denaturation and the release of water from within the proteins.

Estimating the water lost to gravity as drip loss is the main method adopted to measure WHC and it greatly contributes to the visual acceptance of meat by consumers thus directly influencing consumer purchase decision (Pearce et al., 2011). Other methods measuring WHC or moisture content in raw meat require the use of some sort of pressure device for fluid removal or centrifugation (Fennema 1990).

1.2.3 Raw meat pH

Normal meat is known to have an ultimate pH which ranges from 5.4 to 5.6 (Weglarz 2010). Tarrant (1989) suggested that to achieve a pH value of 5.5, the muscle must contain at least 57 μmoles of glycogen/g muscle. After animal death, there is a continuation of contraction and relaxation of muscle because of the presence of adenosine triphosphate (ATP) (Purslow 2005), but as soon as the muscle blood supply ceases, ATP becomes depleted, and the muscle metabolizes its glycogen to maintain homeostasis and preserve its ATP. This

means that anaerobic glycolysis is the primary energy producing pathway, and this leads to an increase in lactic acid and free hydrogen ions with a decrease in pH level to 5.5 (Matarneh et al., 2017; Rosenveld & Andersen, 2003). At pH below 5.7, myoglobin is able to maintain its oxygenation and hence a bright red colour is observed in fresh beef (Tarrant 1989).

However, due to factors including poor nutrition, increased animal age, high environmental temperature, hormone implant use, time spent in lairage and most animal stress during transport to the abattoir and during pre-slaughter handling, muscle glycogen may be depleted before slaughtering and there is consequently a decrease in post-mortem production of lactic acid and hydrogen ions (Schneider et al., 2007; Mach et al., 2008). With the depletion of glycogen ante-mortem, limited glycogen remains post-slaughter and the pH decline early post-mortem is limited. With a limited reduction in muscle pH, the beef remains dark, firm, and dry (DFD) and this can be observed at the muscle surface. Many studies suggested the use of the pH value of meat at 24-48hours post mortem as a benchmark for detecting DFD meat with a value between pH 5.8 and 6.0 indicating increased toughness (Pipek et al., 2003; Viljoen et al., 2002). At an ultimate pH between 5.8 and 6.2, the structure, taste and tenderness of the meat are negatively affected (Jeremiah et al., 1991). A reduction in proteolytic activity in this pH range has been hypothesized by various researchers to be the reason for the increase in beef toughness because the range is outside the optimal pH required for the calpain and lysosomal enzymes to degrade actin and myosin and other myofibrillar proteins (Lomiwes et al., 2013). Also, carcasses with high pH muscles have been observed to have an increased likelihood of microbiological growth (Egan & Shay, 1998).

1.2.4 Raw meat intramuscular fat (IMF) or marbling fat

Intramuscular fat, also referred to as marbling, is one of the most important palatability attributes used to evaluate meat quality in several countries including Australia,

Korea, Japan, and the United States. It is regarded as IMF because fat is deposited between muscle fiber bundles in the perimysial connective tissues (Moody & Cassens, 1968) and also within muscles bundles of high-quality grade beef (Smith et al., 2000). Marbling fat is reported by several studies to be positively correlated with meat juiciness, flavour, tenderness and palatability of beef (Piao & Baik 2015; Hunt et al., 2014). Hence, production of highly IMF beef is essential to meeting consumer expectations, and also to compete with less expensive imported beef that has a low marbling score (Seung et al., 2018). In relation to meat quality and the amount of beef, it is important to estimate the percentage of IMF in the ribeye, and subcutaneous backfat and ultrasound (antemortem) and colour imaging (post-mortem) are employed to do so (Nunes & Jose 2015).

1.2.5 Meat juiciness

Juiciness in meat is perceived as moisture released from saliva and meat during chewing and is one of the important attributes most valued by consumers. Low or high moisture content observed in meat is not only influenced by meat-related factors such as the amount of fat content but also physiological factors inherent to individual consumer (Thompson 2004). Juiciness can be felt and determined after being cooked and is thus difficult to measure before purchasing; therefore, WHC is sometimes used as an objective method to evaluate beef juiciness. To date, the only reliable and consistent measure of juiciness is achieved using sensory methods (Winger & Hagyard 1999).

1.2.6 Meat texture

Meat texture remains the most important eating quality traits of raw meat in Western countries such as North America (Brooks et al., 2000). The definition of meat texture encompasses the interaction of various sensory properties. Sensory properties of beef texture

include initial tenderness, that is the first bite using the incisors; overall tenderness (after multiple chews); and chewing and mouthfeel attributes such as fiber cohesiveness, adhesion, chew count, softness, hardness (Juarez et al., 2011). In the mouth, the given textural properties are dependent on all other meat quality characteristics, including WHC, fat content, juiciness because they are necessary for lubrication while chewing (Juarez et al., 2011).

Given this complexity, researchers sought to give a proper definition to the description, and early work by Harries et al., (1972) determined that the texture of roast beef could be satisfactorily described by only two attributes, "toughness and tenderness". Meat texture is therefore regarded as the force or work required for chewing and this is mostly measured by Warner–Bratzler shear force using a blade (Bratzler 1949). Several methods were developed for determining beef texture, but the most traditional measurement methods employed are geared towards being able to measure and reproduce the compressive and shear forces produced during chewing (Damez et al., 2011). As a result of that, the assessment by human sensory panelists and Warner-Bratzler shear force (WBSF) methods remain the benchmarks for meat texture (Lorenzen et al., 2010). The mechanical resistance to force is usually referred to as 'toughness' and this is used routinely by researchers. Hence, this chapter relies expansively on the use of 'beef toughness".

1.3 Beef tenderness

Of all beef quality attributes, tenderness is arguably the most important palatability determinant in providing a superior eating experience or satisfaction (Shackelford 2001), and therefore, the most significant challenge in terms of acceptability and re-purchase intent of meat purchased by consumers. It was documented that consumers are willing to pay more for guaranteed tender beef cut as long as the expectation with beef eating experience is

consistently met by beef producers (Shackelford 2001). Hence, to meet consumer expectations of consistency in beef tenderness, meat industries in different parts of the world especially in developed countries such as North America and Australia have identified solving the problem of meat toughness as a top priority (Koohmaraie et al., 2002).

Numerous studies have demonstrated that toughness of meat is influenced by variety of factors, starting from the farm where an animal is raised to when they are transported to the abattoir and being slaughtered (Hollung et al., 2007). The age of the animal, the feeds given to them on farm, the gender, the breed and their genetics also play important roles (Muchenje et al., 2009). However, it has been widely accepted that the major determinants of variation in cooked beef tenderness is generally associated with the amount myofibrillar and connective tissue proteins present in meat (Muchenje et al., 2009; Destefanis et al., 2008). Warner Bratzler shear force is used as a standard technique to differentiate between the impacts of myofibrillar proteins on meat toughness and the impacts of collagenous components in connective tissues on meat toughness (Leptit & Hamel, 1998).

Myofibrillar proteins have been identified as the major factor that explains most of the quality variation in raw meat (Hwang et al., 2003) because actomyosin cannot dissociate into actin and myosin without ATP (Lawrie, 1974). This is the cause of rigor mortis (stiffness) which causes muscle to become inextensible and the length of the sarcomere to be fixed (Purslow 2005). Hence, the toughness of cooked beef is reported to be dependent on the contraction state of sarcomere length (the basic unit of a skeletal muscle) by actin and myosin interaction during rigor mortis (Lawrie 2016). Myofibrillar proteins contribute about 50-55% of the total proteins in the muscle and are composed of the thin and thick filaments which are responsible for contraction in the muscle. The thin filament consists of actin with tropomyosin

and troponin, and the thick filament is primarily myosin (Tornberg, 2005). Other structural elements consist of other important connecting proteins such as titin, nebulin and desmin, β-actinin and α-actinin also help to maintain the framework of the myofibrils structure, while others include M protein found in only fast twitch muscles and C protein which is a myosin-binding protein (Tornberg, 2005; Heinz & Hautzinger, 2010).

Myosin and actin account for about 65% of the total myofibrillar proteins while tropomyosin and troponin account for just 5%, with the remaining 30% made up by the rest of the proteins (Heinz & Hautzinger, 2010). Myosin and actin are the major proteins directly involved in the contraction and relaxation of the sarcomere in live animals. The tropomyosin inhibits muscle contraction thereby blocking the interaction between the actin and myosin but with the action of troponin (Heinz & Hautzinger, 2010). Myosin which consists of about 45% of the total myofibrillar protein is activated by the presence of ATP, which it hydrolyzes once calcium is released from the sarcoplasmic reticulum. Once activated, a cross-bridge forms between actin and myosin, with about 20% of the total muscle protein forming actomyosin, causing the sarcomere to become shortened by moving closer to the Z disk (contraction), thereby increasing the toughness of the muscle (Belitz et al., 2009). However, the impact of myofibrils can be largely resolved by proper post-mortem aging of carcasses, stretching of the sarcomeres within the muscle fibers, and cooking (Wheeler & Koohmaraie, 1994).

This is not so with the effect of intramuscular connective tissues (IMCT) the contribution of which to beef toughness is not large, but minimally affected by the process of aging or application of heat (Purslow 2012; Lonergan 2010; Veiseth-Kent 2010). Thus, IMCT is thought to be the major contributor of variation that exists between cooked beef cuts (Purslow 2005, Nishimura 2010).

1.3.1 Improving cooked beef tenderness

1.3.1.1 *Post-mortem ageing influence on myofibrillar proteins and IMCT contributions to beef toughness.*

Post-mortem ageing is a common practice used by industry to improve beef tenderness and palatability (Mahesh et al., 2019). The United States National Beef Tenderness Survey showed that subprimals are aged for an average of 20 days in the beef supply chain and retail establishments (Guelker et al., 2013). Biochemical and cellular mechanisms that influence meat quality attributes undergo positive changes during post-mortem aging, hence there is improvement in overall cooked beef quality (Koohmaraie, 1996; 2006).

With a decline in muscle pH to around 5.5, endogenous proteases start to weaken and degrade myofibrillar proteins, thus, the increase in tenderness with ageing (Kemp et al., 2010). With the endogenous proteolysis, the stiffness associated with rigor mortis starts to decrease over time and hence rigor is resolved during post-mortem ageing storage (Wheeler & Koohmaraie, 1994).

Proteolytic enzymes such as calpain and lysosomal cathepsin enzymes, and the multi-catalytic proteinase complex (MCP) (Koohmarie & Geesink, 2006) have been identified to play important roles in muscle protein degradation during aging (Matarneh et al., 2017; Koohmaraie & Geesink, 2006). By incubating the muscles or carcass after the death of the animal for 24 hours, endogenous proteases activated by calcium degrade myofibrillar proteins such as titin, nebulin, troponin T, troponin I, tropomyosin, desmin, and M line proteins to the point that Z disks were destroyed, although desmin becomes degraded and titin is said to be degraded minimally (Boehn et al., 1998). Most importantly, myosin and actin do not undergo extensive degradation as reported in many studies (Colle & Doumit, 2017).

During post-mortem ageing, matrix metalloproteinases (*MMPs*) also degrade IMCT (Everts et al., 1996). *MMPs* are the enzymes associated with extracellular collagen turnover, hence any factor that will increase the activity of these enzymes during the life span of the animal will increase the rate of collagen turnover and this may be beneficial for the final beef tenderness (Birkedal-Hansen, et al., 2008). Through a consensus of research opinions, post-mortem ageing allows the effects of collagen cross-linking in meat to be completely offset by *MMPs* (Lin et al.,1976). Nishimura et al. (1998) observed a reduction in the perimysial network strength and also an increase in the heat solubility of intramuscular collagen was reported in meat after 13 days of ageing. Starkey et al. (2015) detected *MMPs* even after 21 days of post-mortem ageing in meat and they suggested that collagen may become increasingly soluble during post-mortem ageing with at least some collagen being completed degraded to hydroxyproline.

Most of the studies have been able to report that ageing was found to be associated with meat tenderization using muscles from the loin and rib (Eva et al., 2018), but its effect on cooked beef tenderness of raw meat from high connective tissue beef is still very much unclear (Stolowski et al., 2006). However, in the past 20 years, significant amounts of research have been conducted and dedicated to understanding the biochemical basis of post-mortem ageing in individual muscles as a means of improving cooked beef tenderness (Poltorak et al., 2017).

1.3.1.2 *Influence of cooking on myofibrillar protein and IMCT contributions to beef toughness*

Myosin and actin are minimally or not degraded during ageing (Colle & Doumit, 2017). However, with the application of heat, they are both denatured, although there are

contradicting results on the best temperature to use (Tornberg 2005; Baldwin 2012). Baldwin (2012) reported that there was shrinkage of myosin and actin at about 80°C temperature, while Tornberg (2005) in her review about the effect of heat on meat reported myosin was denatured between 54–58°C. Also, Tornberg (2005) from experiments on denaturing the same proteins in solution discovered that myofibrillar proteins began to unfold at temperatures between 30–32°C. Bircan & Barringer (2002) reported from their study that denaturation of myosin occurred at 62°C, whereas McGee (2004) suggested that the beginning of denaturation started at 50°C. In agreement with the report by Tornberg (2005), it was suggested by Martens & Vold, (1977) that myosin unfolds and loses its structure between 54–58°C. Additionally, Sikorski (2006) discovered that myofibrillar proteins were completely denatured at around 60°C and at 50°C by Potter & Ruhlman (2010), while actin was also suggested to be denatured at 66–73°C. In contrast, Bircan & Barringer (2002) suggested actin was denatured at 82°C, which confirmed reports from Wright et al. (1977) that actin can be denatured at a temperature between 80–83°C. Studies have also shown that titin in both pork and beef unfolds at 75.6°C and 78.4°C, respectively (Pospiech et al., 2002).

Bouton et al. (1984) concluded that the contribution of IMCT to beef toughness was high at low cooking temperature between 40°C to 60°C and decreases at above 60°C. Lewis & Purslow, (1989) showed that the strength of perimysial network in meat cooked at 50°C increased and decreases above this temperature. Heat labile, divalent IMCT cross-links are denatured during cooking at 60 to 75ºC, and collagen subsequently becomes gelatin (Bejerholm et al., 2014). However, no reduction is observed in cooked beef toughness especially in high connective tissue beef and that from mature cattle as the collagen in that

beef is composed of a high proportion of trivalent cross-links, which are heat-stable (Nishimura et al., 2010).

1.4 Intramuscular connective tissues

Perimysium and endomysium networks in muscles are referred to as intramuscular connective tissues (IMCT), and the structure, composition and the amount vary among muscles, within muscles, and among breeds of cattle (Nishimura 2010). The relationship of IMCT content with meat toughness was first established by Lehmann (1907), who reported that the toughness of raw meat and cooked meat depended greatly on the total amount of collagen.

Compared to endomysium which surrounds single muscle fibers, perimysium accounts for about 90% of the total intramuscular connective tissue and the amount varies from muscle to muscle (Purslow 1999; Von et al., 2005). It was reported by Lewis & Purslow (1990) that the strength of the endomysial-perimysial junction is very low when compared to the strength of perimysium. Several studies have shown a better correlation between the amount of perimysium and variations in meat toughness than to the amount of endomysium (Purslow, 1999). Perimysium, which is a three-dimensional collagen network surrounding large and small bundles of muscle fibers, determines the level of difficulty in breaking cooked beef apart (Torrescano et al., 2003; Purslow 2018). High correlations were observed between perimysium thickness and raw meat shear force in pork ($r = 0.98$) by Fang et al. (1999), in poultry ($r^2 = 0.95$) by Liu et al. (1996), in beef ($r = 0.95$) and Brooks & Savell (2004) however observed a low correlation of ($r = 0.13$) in cooked beef. Hence, the perimysial network is regarded as a key structure and factor in determining the effect of intramuscular connective tissue toughness in cooked beef (Torrescano et al., 2003).

The IMCT is composed of extracellular matrix (ECM) and includes mainly collagens, proteoglycans (PGs), and glycoproteins (Nishimura 2015). Several studies have reported, notably by Pfeiffer et al., (1972), that beef toughness was closely related to the chemical nature of the covalent intermolecular and intramolecular cross-links collagen (Leptit 2007). The importance of the proportion of thermally stable to labile bonds in the collagen molecule to toughness was emphasized by Jeremiah (1978). Hence, in recent studies, the major concerns of meat scientists are focused on the total amount of collagenous component present in IMCT and the tensile strength of collagen cross-links which dictates how tough cooked meat would be (Weston et al., 2002). Both are related to an increase in age of the animal and muscle locations or types (Roy et al., 2015) because young beef cattle have muscles that are not vigorously involved in locomotion of a heavy weight and as a result have less collagen and fewer heat-stable cross-links and thus produce tender meat. Hence, the measurement of the total amount of collagen and collagen cross-links is considered a predictor of cooked beef toughness (Leptit 2007).

1.4.1 Collagen

The word collagen was coined from ancient Greek in the 1800's from the word known as 'kola' which is either referred to as a 'gum' or 'gel' (Silvipriya et al., 2015). It is a fibrous structural protein present in the extracellular matrix (ECM) and connective tissues of animal (Ramshaw et al., 2009). It comprises about 25-30% of the total amount of protein present (Muller & Werner, 2003). Collagen can literally be found in every part of mammalian body such as in corneas, bones, blood vessels, cartilage, tooth dentin, as well as in skin, tendons, and ligaments, appearing as an elongated fibril when viewed microscopically (Szpak & Paul, 2011). Its major function is to provide strength and structure to the muscle and it also plays a

significant role in cell survival, proliferation and differentiation (Buehler 2006), and it is formed by fibroblasts associated with connective tissue and other epithelial cells (Lullo et al., 2002; Kadler et al., 2007).

In a study conducted in chicken and beef by Liu et al., (1996), it was observed that the resistance of raw meat is highly correlated with the total amount of collagen present (r^2 = 0.94 in chicken and r = 0.95 in beef) (Liu et al., 1996). Several other researchers were also in agreement with their results, as Dransfield et al. (2003) reported a positive correlation between beef toughness and collagen content (r = 0.72). In cooked meat, Dransfield (1977) observed a high positive correlation of r = 0.70, but subsequently other researchers including Dransfield et al. (2003) observed lower values of correlations between the range of r = 0.46 (Ngapo et al., 2002), r = 0.20 (Dransfield et al., 2003) and r = 0.10 (McKeith et al., 1985). It was however reported that the different values were observed from different muscle types or locations; therefore, variation in the toughness of meat is highly dependent on muscle type and location. The more the muscle is used for physical activities or locomotion, the more collagen is present in raw and cooked meat (Leptit 2007).

Bulls (mature male bovines) have also been identified to have muscles with greater collagen content when compared to that of steers (castrated male bovines) (Dransfield et al., 1984). Hunsley et al., (1971) reported higher collagen contents in IMCT from bull beef also, and this agreed with Libouriussen et al. (1977), who reported that beef from an early maturing breed of beef bulls had more stable collagen. However, there has been little substantiation of the differences between bulls and steers with regard to collagen in beef cooked at low temperatures like 50^{o}C (Liboriussen et al., 1977), 60^{o}C (Hunsley et al., 1971), and 63^{o}C (Hawrysh et al., 1979).

1.4.1.1 Structure of collagen

After so many decades of research, the first description of connective tissue structure was provided by Bowman (1840), and since then there has been an interest in its structure, composition and contribution to the textural qualities of muscle when cooked and eaten as meat. The molecular structure of collagen eluded scientists for many years; however, the first evidence that it possessed a regular structure and packing pattern was presented in the mid 1930's. Collagen is composed of three polypeptide chains that are arranged in the form of a right-handed triple helix known. Type I collagen is referred to as hetero-polymers because two chains are identical (α1) while the third one differs in its amino acid composition (α2). Collagen displays a distinct repeating trimer in the helical portion of its molecule, specifically glycine-proline-X and glycine-X-hydroxyproline (van der Rest & Garrone, 1991), where X is any other amino acid other than glycine. Proline or hydroxyproline are common amino acids in collagen with hydroxyproline or proline constituting about 1/6 of the total sequence (McCormick 1999).

Hydroxyproline and proline play a major role in collagen stability (Nelson & Cox, 2005) and they also allow sharp twisting of the collagen peptide to accommodate the formation of the triple helix (Brinckmann et al., 2005). However, hydroxyproline differs from proline because of the presence of a hydroxyl (OH) group attached to the gamma carbon atom. It is roughly about 4% of all amino acids found in animal tissue which is a greater amount than every other amino acid (Gorres et al., 2010). Hydroxyproline is unique to collagen and elastin, but because collagen is substantially more abundant than elastin (Bendall 1967), hydroxyproline is used to quantify or determine the total amount of collagen present in meat. The use of hydroxyproline for this purpose was first reported by Dakin (1920) & Bergmann (1935), after its discovery by Hermann Emil Fisher in 1902 when he isolated

hydroxyproline from hydrolyzed gelatin, and its synthesis in 1905 by Hermann Leuchs, who synthesized a racemic mixture of 4-hydroxyproline (Plimmer et al., 1912).

1.4.1.2 Types of collagen

To date, there are about 29 forms of collagen derived from more than 30 genes and the different types of collagen have been categorized into families with different roles to play in biological systems (Ricard-Blum & Ruggiero, 2005). Although all of them have a characteristic triple helix, the structure of these collagens varies from one another in length, size and nature (Miller 1984), from a mesh-like network of Type IV collagen to the long filamentous fibers of Type I, II and III collagens (Nimni & Harkness, 1988). Among the most common in muscle are Types I, II, III, IV and V, and 90% of the collagen present in muscle ECM of mammals is Type I and this is due to its wide prevalence in almost all connective tissue layers but mostly in the endomysium (Cheah, 1985) followed by Type III, with lesser amounts of Type V and Type IV collagens associated with perimysium and basement membranes respectively (Bailey & Light, 1989). The ratio of collagen type I and III vary between bovine muscles and also in the diameters of collagen fibrils (Light et al., 1985). Burson & Hunt (1986a) reported that Type I is less heat stable than type III, however contradictory results between the amounts of type III and effect on meat toughness were also observed (Burson & Hunt, 1986b; Light et al., 1985).

1.4.1.3 Collagen synthesis

Fibroblasts are the major cell responsible for the formation of collagen and the synthesis of collagen occurs inside and outside of the cell undergoing extensive post-translational modifications (Du et al., 2010). The process of collagen synthesis begins with the transcription mRNA (messenger RNA) inside of the cell, and this involves the promotion

of gene expression, typically alpha 1, 2, or 3. There are approximately 34 genes identified to be associated with collagen formation. Once the genes are turned on and the final mRNA exits from the cell nucleus into the cytoplasm to link up with the ribosomal units, the process of translation occurs. A new or first peptide known as the signal sequence is formed on the N-terminal and C-terminal which then directs it into the endoplasmic reticulum for post-translational processing leading to the formation of procollagen from the alpha peptide (Myllharju 2003; Elizabeth et al., 2005). The C-propeptides plays a significant role in procollagen chains (Bulleid et al., 1997).

For the formation of an alpha peptide, the signal peptide is dissolved, leaving a collagen pro-peptide. The enzymes prolyl hydroxylase and lysyl hydroxylase produce hydroxyproline and hydroxylysine, respectively, on the pro-peptide, hence providing the structures necessary for the formation of hydrogen-bonds and covalent cross-linking of the alpha peptides, respectively (Myllyharju & Kivirikko, 2004). Vitamin C is required in this enzymatic step as a cofactor because studies have shown that in the absence of Vitamin C the hydroxylation of prolines and lysines does not occur and the triple helix that is formed will be loosely bonded, hence leading to scurvy (Elizabeth et al., 2005). After which, glycolysation occurs either by the addition of glucose or galactose monomers onto the hydroxyl groups placed onto lysines but not on prolines. The hydroxylated and glycosylated pro-peptide twists towards the left very tightly until three pro-peptides form a right handed triple helix known as procollagen. However, the procollagen is not yet properly formed as it has large telopepetides, which are non-helical random peptides at each end of the triple helix. The procollagen is transferred into Golgi apparatus for the last post-translational modification, specifically the addition of oligosaccharides, before being secreted out of the cell. Immediately upon the pro-collagen molecule entering into the extracellular space, enzymes

known as N- and C- peptidases remove the large telopeptide ends, resulting in the formation of tropocollagen, which is less soluble than pro-collagen and thus more stable (Elizabeth et al., 2005).

1.4.1.4 Collagen cross-links in muscles

The formation of collagen cross-links begins with collagen fibril aggregation, which is initiated by lysyl oxidase acting on lysines or hydroxylysines residues producing the aldehydes allysine and hydroxyallysine, respectively. These residues eventually establish covalent intermolecular bonds between tropocollagen molecules essential for the mechanical strength, stability, and stiffness of collagen fibers (Bailey et al., 1998).

The amounts and nature of cross-links formed in IMCT change both at prenatal development and postnatal maturation (Avery et al., 2009). Collagen cross-links are of two types; immature or divalent cross-links and mature or trivalent cross-links. Divalent cross-links consist of ketoamines (heat stable), and the aldimines (heat liable) which over time tends to become condensed with others provoking them to disappear from almost all tissues; therefore, the ketoamines are related to decreased solubility rate in cooked beef (Allain et al., 1978). They are replaced by non-reducible mature trivalent cross-links, hence contributing to the toughness of meat from aged animals (Nishimura 2015).

Trivalent cross-link numbers and collagen mechanical stability increase with animal age because collagen reacts with glucose and aldehydes, and there is an increase in the heat stability of cross-links (Bailey & Light 1989; McCormick 2009). The increase in the number of heat-stable collagen cross-links per mole of collagen with age has been documented to explain the increase in cooked meat toughness observed in older animals. Shimokomaki et al., (1972) reported that there was a steady decrease in divalent cross-links in bovines up to 5 years of age, but the rate decreased with time and divalent crosslinks were virtually absent in

the muscle of a 10 year old bovine. It has been therefore concluded that mature cross-links and the total amount of collagen have a strong effect on cooked meat toughness leading to an increase in both time and temperature required for collagen denaturation (McCormick 1999). However, the number of cross-links per mole of collagen in relation to meat toughness greatly varies from relatively high (r = 0.82, Bailey 1989) to non-significant values among different muscle types or animals or breeds of animals (Avery et al., 1998).

In meat, pyridinoline cross-links (hydroxylysylpyridinoline (HP) or lysylpyridinoline (LP), a fluorescent cross-links (Fujimoto et al., 1977), and Ehrlich chromogen, a pyrrole, are two known condensation products of divalent ketoamines (Light & Bailey, 1985) which link three collagen molecules and therefore are referred to as trivalent cross-links (Eyre & Wu, 2005). They are important in determining the texture of cooked meat (Bailey & Light 1989) because their functionality is 1.5 times higher than the functionality of a divalent cross-link such as hydroxylysinorleucine (HLNL) and dihydroxylysinorleucine (DHLNL) which link two cross-links (Erman & Mark, 1997). The functionality of collagen cross-link is referred to as the number of chains or strands meeting at the network junction (Mark & Erman, 1988). Pyrrole cross-links cannot be directly analyzed by acid or base hydrolysis due to its inherent lability, thus, the only method to determine the quantity is ensuring samples are being solubilized by enzymatic digestion, although this is not completely accurate, and thereafter the colour yield of the supernatant with Ehrlich's reagent is measured (Hanson & Eyre 1996).

Horgan et al., (1991) have shown that no change was observed in the amount of pyridinoline or Ehrlich chromogen cross-links in their study when meat was heated to 80 C for 45 min. However, HLNL and DHLNL cross-links were completely destroyed when meat was heated to 73 C for 10 min (Allain et al., 1978). The means of total collagen content and HP cross-links were observed to rank in order of increasing tenderness as Biceps femoris

(BF), Triceps brachii (TB), Semimembranosus (SM), Longissimus dorsi (LD), Gluteus medius (GM) and Psoas major (PM) (BF<TB<SM<LD<PM) according to Segers et al. (1974). The same meat tenderness rankings were reported by Wheeler et al. (2000) and Wulf et al. (2002) who reported the SM muscle was slightly more tender than BF. This shows that meat tenderness ranking among various muscles can be slightly different (McCormick 1999).

1.4.1.5 Collagen turnover or collagen degradation in meat

The total amount of collagen content and collagen cross-links are positively correlated to each other, whereas collagen turnover also known as collagen degradation is negatively correlated with collagen cross-links (Archile-Contreras et al., 2010). Generally, over the life of the animal, collagen synthesis and collagen degradation in muscles remain in an equilibrium state; however, any external factor may affect this state by either increasing or decreasing net collagen turnover.

Cooked beef tenderness is highly dependent on the rate of collagen turnover during ageing (Bailey 1989), thus if animals are slaughtered during this stage, background toughness may be reduced significantly. Also, as the age of the animal increases, collagen turnover decreases, leading to a steady increase in collagen cross-linking which in turn reduces collagen solubility (Mays et al., 1991). Purslow et al. (2012) suggested that with the inducement of collagen turnover throughout the lifespan of an animal less mature collagen will be formed. Hence, to dramatically reduce background toughness in cooked meat, increasing the rate of turnover is the key (Archile-Contreras et al., 2011).

1.4.1.6 Mechanisms involved in collagen synthesis and collagen degradation

Several key enzymes or mechanisms and biochemical processes are involved in the synthesis or formation of collagen, collagen cross-linking and the reduction of cross-links

during aging (Guo & Dalrymple 2017). Beef toughness is as a result in part of the collagen content in muscles and this can be predicted from the activities of these key enzymes whose candidate genes have been identified. Therefore, it will be of great benefit if researchers and the beef industry understand these mechanisms to reduce collagen and collagen cross-links and improve beef tenderness and quality without compromising the health and function of cattle.

1.4.1.6.1 Lysyl hydroxylase (LH) and lysyl oxidase (LOX)

Collagen and collagen cross-linking depend solely on enzymatic actions of intracellular modifications of procollagen alpha chains by *LH* and extracellular modifications by *LOX* (Trackman 2016). *LH* also known as *PLOD* (procollagen-lysine-2-oxoglutarate 5-dioxygenase) determines the type of cross-links formed (Eyre & Wu, 2005).

Three known isoforms of *LH* (*PLOD*) such as *LH1* (*PLOD1*), *LH2* (*PLOD2*) and *LH3* (*PLOD3*) hydroxylate collagen sequences (Van der slot et al., 2003). *PLOD1* (*LH1*) is known to favour lysine residues as substrates at the two triple helical sites of cross-linking and *PLOD2* with its splice variant (*LH2a* and *LH2b*) has been identified as a specific hydroxylase which is believed to act on telopeptide lysines (Eyre & Wu, 2005). As more telopeptide lysines are hydroxylated, the degree of pyridinium cross-links production increases (Van der slot et al., 2003). LH isoforms have been demonstrated to increase total collagen synthesis in muscles in several studies (Mercer et al., 2003). LH isoforms have been identified in human (Hautala et al., 1992; Passoja et al., 1998), mouse tissues (Sipila, 2000) and *PLOD1* was identified in rat (Armstrong & Last, 1995), chicken (Myllyla et al., 1991), and cow with an accession number of AF054274 (Norman & Moerman, 2000).

LOX is the most critical enzyme responsible for initiating and regulating covalent cross-linking of collagen fibrils during fibrillogenesis by oxidatively deaminating lysine and

hydroxylysine to produce allysines (Huang et al., 2012). The formation of cross-links between telopeptides and the helix of collagen is thereby enhanced and further interact to form trivalent cross-links during maturation (Kagan & Li, 2003). The *LOX* family consists of five members which are the *LOX* and the *LOX* homologs like enzymes, *LOX 1, LOX 2*, *LOX 3* and *LOX 4* (Mohar et al., 2003). A study by Huang et al., (2012) observed a positive correlation between the expression of collagen cross-linking and LOX.

1.4.1.6.2 Prolyl-4-hyroxylase

P4HA1 is a gene that codes for prolyl-4-hydroxylase, which is an enzyme that controls the formation of 4-hydroxyproline for the conversion of proline residues to hydroxyproline residues (Kivirikko & Myllyharju, 1998; Myllyharju, 2003). Hydroxylation of proline is essential for the proper folding of newly synthesized procollagen chains (Pajunen et al., 1990).

1.4.1.6.3 Insulin growth factor (IGF), fibroblast growth factor (FGF) and transforming growth factor (TGFβ)

Insulin-like growth factor (IGF*)*, fibroblast growth factor (FGF*)*, and transforming growth factor β (TGFβ) are all important growth factors involved in controlling the fibrogenic proliferation and differentiation and aid the stimulation of connective tissue synthesis by muscle cells (Seung, 2018). Kanematsu et al. (2004) observed an interaction between collagen type I and FGF and TGFβ, a key regulator of fibrogenesis (Mio et al., 2015) that increases collagen content and pyridinoline cross-links in skeletal muscle of lambs (Huang et al., 2010). Reiser et al. (1996) observed IGF increased collagen synthesis but decreased collagen cross-links. Montaseri et al. (2011) showed TGFβ and IGF suppressed the activity of *MMP* during

interleukin induction (Huang et al., 2011). TGFβ isoforms are activated by the activities of Sma and Mad related proteins (Letterio & Roberts, 1998).

1.4.1.6.4 SMAD related proteins

The SMAD family consists of a common *SMAD* (Co SMAD) known as SMAD 4, receptor SMADs (R SMAD) which include *SMAD1, SMAD2, SMAD 3*, and *SMAD 5,* and two inhibitor SMAD (I-SMAD) known as *SMAD 6* and *SMAD 7* (Moustakas et al., 2001). *SMAD 2* and *SMAD 3* are phosphorylated and bind to *SMAD 4* (Suwanabol et al., 2011), with the resulting complex translocating into the nucleus and activating the expression of fibrogenic genes including procollagen and enzymes catalyzing collagen cross-linking (Massague & Chen, 2000).

1.4.1.6.5 Fibronectin and Integrins

Fibronectin (*FN*) is a major component responsible for the binding of ECM like collagens (Frantz et al., 2010; Ulmer et al., 2008). It was reported that the expression of FN gene was found to be highly expressed in the Wagyu breed when compared to the Angus cattle breed. No significant differences in the expression of FN were observed in the *longissimus thoracis* of bulls and steers (Seung et al., 2018).

Integrins are major receptor for cell adhesion to ECM proteins and they activate many intracellular signaling pathways (Hynes, 2002). Four integrins binding to collagens have been identified, namely α1/β1 (*ITGA1*), α2/β1(*ITGA3*), α10/β1(*ITGB1*) and α11/β1(*ITGA11*) (Popova et al., 2007). Collagen types I and III bind to α1/β1 and α11/β1 respectively (Zeltz et al., 2014). Seung et al., (2018) reported an increase of α1/β1 integrin in steers ($p = 0.06$) when compared to bulls but no changes in mRNA levels of α11/β1 integrin were observed between steer and bulls.

1.4.1.6.6 Pituitary homeobox 2 (PITX2)

PITX2, also known as paired-like homeodomain transcription factor 2, is a protein which acts as a transcription factor and regulator of procollagen lysyl hydroxylase (*PLOD*) gene expression (Logan et al., 1998).

1.4.1.6.7 Metalloproteinase matrix (MMPs)

Members of the *MMP* family, which are important enzymes responsible for degradation of ECM constituents such as collagen, also play a major role in fibrogenesis involved in the development of connective tissues (Purslow 2005; Christensen & Purslow, 2016). Currently, there are about 24 different types of *MMPs* that have been identified among vertebrates (Ajay et al., 2010) and about 11 *MMPs* have been observed to be expressed in bovine muscle (Blacerzak et al., 2001).

Collagenase *MMPs* such as *MMP1, MMP8, MMP13,* and *MMP18* are identified to denature collagen types I, II and III, whereas gelatinases *MMPs* like *MMP 2* and *MMP 9* act on denatured collagen (Christensen & Purslow, 2016). *MMP 9* and *MMP 13* were also observed in *longissimus thoracis* (LT) muscle to be involved in fibrogenesis (Seung et al., 2018). Steers showed a higher mRNA level of *MMP9* when compared to bulls, *MMP 9* also degraded collagen type I and type III (Seung et al., 2018). Purslow et al. (2012) summarized that several studies have suggested the possibility of reducing mature cross-links in IMCT by increasing the activities of *MMPs*. The increase in the activities is expected to influence meat quality (Christensen & Purslow, 2016). However, the activities of MMPs are inhibited by the activity of tissue inhibitor of metalloproteinase (*TIMP*) family (Blacerzak et al., 2001; Seung et al., 2018).

1.4.1.6.8 Tissue inhibitor metalloproteinase (TIMPs)

TIMPs are the major protein family that controls the activities of MMPs and there are four known types, specifically *TIMP 1, TIMP 2, TIMP 3*, and *TIMP 4* (Christensen & Purslow, 2016; Visse & Nagase, 2003). It was concluded by Baker et al. (2002) that these enzymes, other than their inhibitory capacities, regulate *MMP* activity during tissue remodeling and other biological activities. The normal functions of *MMP*s are dependent on the balance between them and their inhibitors (Kessenbrock et al., 2010). *TIMPs* may also serve as activators of proMMP 2 and proMMP 9, which are the gelatinases responsible for proteolysis of denatured collagens or ECM turnover (Christensen & Purslow, 2016; Huang et al., 2010).

1.4.2 Reduction of IMCT, collagen and collagen cross-links in cooked beef

Post-mortem ageing has been reported by several researchers to consistently reduce the strength of IMCT and collagen cross-links of muscles in the raw state (Purslow 2005). With the elevated activity of protease enzymes such as the *MMPs* in a live or slaughtered animal, biochemical degradation occurs during post-mortem ageing causing tenderization (Birkedal-Hansen, et al., 2008). Stanton & Light (1990) observed damage of perimysial collagen in raw beef and partial solubility during post-mortem ageing. Thermal shrinkage of bovine IMCT decreases by 7-8^{o}C within 7 days post-mortem aging (Judge & Aberle, 1982). Liu et al. (1995) also observed that the integrity of IMCT decreases during post-mortem aging of chicken, in beef (Nishimura et al., 1995) and in pork (Nishimura et al., 2008).

However, it is controversial as to whether collagen solubility is most affected by temperature or the time of post-mortem ageing. This is because there have been major concerns as to what that means for cooked meat toughness (Jeremiah 1978), as no effect on cooked meat toughness has been observed especially in high IMCT beef (Purslow 2014).

Bouton & Harris (1972) concluded that IMCT is unaffected by extensive post-mortem ageing time when followed by cooking.

Cooking of meat between 20-50ºC enables the increase of IMCT strength but with higher temperatures and longer cooking times its contribution to toughness decreases, and so conclusive links between mature cross-links and cooked beef toughness have been difficult to prove (Purslow 2005). Lewis et al. (1991) observed a reduction in the strength of IMCT with post-mortem aging, but the effects were annulled after cooking the high IMCT muscle to temperatures of 60ºC and above because both aged and unaged perimysial IMCTs had the same strength after being cooked.

Purslow (2014) hypothesized that there are two populations of collagen molecules, a weak pool which can be easily degraded by post-mortem ageing and cooking, and a strong pool which is more resistant to both. The resistance of the thermally stable collagen and its cross-links during protease hydrolysis, post-mortem ageing especially in muscles with greater amounts leaves its reduction or elimination as a choice to alleviate background toughness in cooked beef (Lepetit 2008).

Recently, scientists have identified the need to investigate mechanisms involved in degradation or turnover (Purslow 2018). One possible alternative to investigate is the mechanism or manipulation required to affect the characteristics of IMCT and understand how to use naturally occurring gene coding through the application of genomics has been suggested (Purslow 2018).

1.4.3 Application of functional genomics to meat quality

Expressional genomics is a science application that helps to study the structure, function and the evolution of genomes (Picard et al., 2015). The knowledge of the genomes and development of sequencing tools have allowed the expansion of functional or

expressional genomics. Expressional or functional genomics studies the function of genomes, and thanks to the availability of thousands of genetic polymorphisms (SNPs with chips), transcripts (transcriptomics), proteins (proteomics) and metabolites (metabolisms) (D'Alessandro et al., 2011; Picard et al., 2015). The whole genome sequences of livestock such as chicken (Wallis et al., 2004), cattle (Elsik et al., 2009), sheep (Jiang et al., 2014) and goat (Dong et al., 2013) have been documented and provide new hypotheses for meat science.

Currently, the use of quantitative real-time PCR (RT-qPCR) have been employed and utilized to reveal the genes, enzymes, proteins or metabolites whose expression level or abundance or absence is related with a phenotype of interest such as meat quality (D'Alessandro et al., 2012; Picard et al, 2012). Although expensive, this technology will provide adequate knowledge needed to understand the interactions between genetic and environmental factors that influence the development of phenotypes such as meat quality attributes (Picard et al., 2015; Picard et al., 2013). Hence, the recent objective of meat scientists is focused towards identifying and expressing meat quality candidate genes that are quantifiable on live animal or early post mortem on carcass, because such knowledge would help to manipulate breeding behaviors of animals to increase meat potential and to improve high IMCT beef quality (Picard et al., 2013).

Several studies have applied this tool to improve meat quality in relation to the expression of genes associated with marbling (Childs et al., 2002), muscling characters (Lehnert et al., 2006), and for calpain and calpastatin activities involved in myofibrillar degradation during post-mortem ageing (Hocquette et al., 2005). To date, little research has been done investigating the expression of genes related to IMCT, collagen and collagen cross-link formation in beef (Purslow 2014). Recently, Christensen et al. (2017) identified single

nucleotide polymorphisms (SNPs) for *MMP1* associated with the composition in bovine muscles, but the expression of the gene was not investigated.

1.5 Conclusion

This literature review investigated the influence of production factors such as breeds, muscle location, animal age, and selection for low RFI on carcass traits and beef quality attributes. The biggest challenge faced by beef industries and meat scientists is to be able to solve the problem of beef quality inconsistency between breeds, and between and among various muscles. Beef tenderness is the most important palatability trait to consumers, and intramuscular connective tissue (IMCT) is thought to be responsible for the background toughness in cooked beef because of the amount of perimysial network and the collagen cross-linking over time. Significant amounts of research have been conducted to understand the mechanisms affecting meat quality attributes and beef tenderness especially in muscles from the loin and rib, but limited study exists on high connective tissue muscles from the chuck and round.

In addition, post-mortem ageing is utilized to tenderize muscles originally from the loin and rib but its effect on cooked beef cuts, especially from the chuck and round is unclear. It is therefore of great importance to fully investigate and understand the effects of cattle breed (Angus and Charolais) and Angus crossbreds, selection for low RFI, and post-mortem ageing on beef quality attributes. The application of genomics in the identification and expression of biochemical processes and mechanisms associated with IMCT characteristics needs to be fully investigated to be able to manipulate beef tenderness through genetic selection and post-mortem ageing, which will be beneficial to both the beef producers and consumers.

1.6 Summary and thesis structure

Based on the literature review, being able to improve the overall beef eating quality of steaks from the chuck and round is crucial for the beef industry as it will add value to the overall carcass. In comparison to steaks from the loin and rib, primal cuts from the chuck and round are classified as underutilized because of the perceived toughness associated with them (Belew et al., 2003). However, evidence suggests that specific muscles from the chuck might have greater eating quality compared to steaks from the round and similar to the ribs (Kukowski et al., 2004; 2005; Nyquist et al., 2018).

The *triceps brachii* (TB), a muscle from the chuck and part of the clod, is a locomotive muscle found behind the shoulder at the base of the scapula and is perceived to be tough either because of the amount of IMCT present or the presence of matured trivalent collagen cross-links. The retail cut is therefore sold as ground product or as a stew beef or a roast in order to improve its marketability, but it is consequently sold at a less expensive price when compared to the price of roasts or steaks. It is therefore important to investigate beef production factors that may exists to further add value to TB muscles to improve its acceptability or superior eating quality

Most studies on significant factors like breed and post-mortem ageing investigating their effect on physio-chemical factors of meat such as intramuscular pH, temperature, proximate analysis, colour, intramuscular fat, shear force, as well as the structure and composition of intramuscular connective tissues in meat have largely focused on the muscles from the loin and the rib of the carcasses. Works of literature on the effects of those significant factors on the TB muscle are limited, and demonstrate the attempt to determine the differences in tenderness and other properties among the beef muscles. Also, selection for low

RFI may be a powerful tool to reduce the cost of production, but no conclusive results have been reported on its effect on beef quality.

In this study, we hypothesize that breed type and selection for low RFI do not have an effect on the physio-chemical properties of TB muscles from beef cattle raised to the same back fat depth and physiological age. The primary objective is to examine the influence of popularly consumed breed types of beef cattle in Canada, specifically the Angus (purebred), Charolais (purebred) and the University of Alberta's beef synthetic the Kinsella composite (Angus crossbred), and selection for low residual feed intake combined with post-mortem aging on the meat quality attributes and intramuscular connective tissue characteristics of TB muscles. Chapter 2 also shows the relationship between carcass and meat quality attributes and intramuscular connective tissues characteristics such as endomysium, perimysium, the total amount of collagen, and mature collagen cross-links (Ehrlich chromogen and pyridinoline) concentrations.

As reviewed in this chapter, no study has reported the application of genomics in the identification or expression of specific mechanisms or biochemical processes associated with synthesis and degradation of collagen and collagen cross-links in relation to carcass and beef quality. We, therefore, hypothesize that the genes of enzymes associated with IMCT characteristics will be differentially expressed among Angus, Charolais and Kinsella composite breeds of beef cattle and solubility level (low and high). In Chapter 3, the objective is to apply functional genomics to express specific genes of enzymes and proteins associated with IMCT characteristics of TB muscles.

Chapter 2: Effects of breed type, residual feed intake and post-mortem ageing on physio-chemical properties of the m. *triceps brachii* and their relationships with beef toughness.

2.1 Introduction

The overarching goal of the beef industry is to increase the value of underutilized carcass muscles and increase production efficiency through the enhancement of feed and forage utilization through increased feed efficiency (BCRC, 2018). Underutilized carcass muscles are mostly found in primal cuts from the chuck or round, which represent half the weight of the whole carcass and are characterized by high intramuscular connective tissue (IMCT) and matured trivalent cross-links, hence, sold at half the price of cuts from the loin or rib (Shand et al., 2016).

The level of difficulty in breaking cooked beef apart is determined by the strength of the perimysial network present in IMCT (Latorre et al., 2018). However, the toughness of raw and cooked meat depends on the total amount of collagen and its cross-links (Purslow 2005; Nishimura, 2010). Collagen, the most abundant protein in IMCT, forms cross links, which become thermally stable over time and their stability during heating is related to beef background toughness (Nishimura et al., 2010). Pyridinoline (PYR) and Ehrlich's Chromogen (EC) are two mature cross-links identified to be positively correlated with beef toughness, although the contribution of EC declines with cattle age (Bruce and Roy, 2019).

Production factors such as age of animal at slaughter, muscle activity, genetics and cattle breeds may affect the contribution of collagen and its cross-links to beef toughness (Bruce and Roy, 2019), but studies performed to understand the impacts of these factors on

the physio-chemical properties of carcass and meat quality attributes, especially tenderness (Veiseth-Kent et al., 2018), have being largely focused on low IMCT muscles. As a result, with proper production and processing practices such as post mortem ageing, retail cuts from the loin and rib are now classified as "tender" or "very tender" due to low intramuscular connective tissue (IMCT) content (Savell et al., 2016), but the effect of post-mortem ageing on high IMCT beef from the chuck is unclear.

Over 50 years ago, Koch et al., (1963) proposed residual feed intake (RFI) as a method to measure the efficiency of feed utilized in cattle independent from their body weight (BW) and level of production. This method has since been adopted, and to increase feed efficiency rate of animal, the genetic selection of low residual feed intake (RFI) animal, also referred to as efficient animal, has been severally suggested. Efficient or low RFI animals tend to consume less feed without a compromise in growth rate performance and thus selection for low RFI may be a means of reducing the production cost of meat (Seabury et al., 2017). However, the selection for efficient or low RFI animals will only be effective if it does not have a negative impact on the carcass and meat quality end-product. Studies on the effect of selection for low RFI animals are limited, are contradictory and inconclusive (Baker et al., 2006) and do not consider the impact on the quality of high IMCT muscle.

Hence, there may be significant differences among breeds and RFI levels on the physio-chemical characteristics of the *triceps brachii,* a muscle from the chuck on the beef carcass. The genetic selection for low residual feed intake beef steers may have increased concentration of mature collagen crosslinks and significant effects on WBSF value. Therefore, the aim of this study was to characterize the effects of breed type, residual feed intake (RFI) and post-mortem ageing on carcass measurements and the meat quality and

IMCT characteristics of the *triceps brachii* in three cattle breed types. This study examined the relationship between carcass measurements and meat quality, IMCT, and collagen and collagen cross links characteristics and beef tenderness.

2.2 Materials and methods

The study was performed by following the guidelines of the Canadian Council on Animal Care (CCAC, 1993). Steers (castrated male beef cattle) were reared at the University of Alberta Kinsella cattle heard and ethics approval for their use in this study was obtained from a research ethics committee at the University of Alberta (AUP00000777).

2.2.1 Experimental design and animals

A 3 x 2 factorial design was utilized to investigate the effects of breed types and RFI levels on carcass, beef quality and collagen measurements of *Triceps brachii* muscle. Calves were born in April or May 2014, into the University of Alberta Kinsella cattle herd, castrated within 8 weeks of birth and were uniquely identified using ear tags. A total number of seventy-one (n=71) beef steers were selected based on their growth performances, physiological age and back fat. Breed type included purebred Angus (n=23) and Charolais (n=24), and Angus crossbreds (n=24)) also known as Kinsella composite. The Angus crossbred steers were produced by crossing a hybrid maternal cow line bred at the University of Alberta with Angus bulls as described by Jiang et al. (2012). The hybrid dam line was approximately 33% each of Angus and Charolais and 20% Galloway, with the remainder consisting of other beef breeds as described in detail by Nkrumah et al. (2007).

2.2.2 Management of cattle and determination of RFI

At approximately 6 months of age, the steer calves were weaned and placed on a forage diet from which they were gradually introduced to barley grain. At approximately 13-

14 months Angus and Charolais were assessed for RFI, while Angus crossbreds were assessed at about 11-12months. As describe by Barasab et al., (2003), beef steers were grouped into feedlot pens by breed type and the Growsafe feeding systems (Growsafe Systems Inc., Airdrie, Alberta, Canada) was used for RFI evaluation. The Growsafe feeding system was monitored daily for feed intake by individual steers and their feeding frequency during the finishing period monitored to ensure proper function of the feedlot test system. The feeding test periods were for Angus steers 66 days, for Charolais steers 72 days and for the Angus crossbred 75 days. As described by Mao et al. (2013) growth performance and ultrasound traits including back fat and ribeye area were measured. Body weight (BW) of all steers was taken and recorded every two weeks and ultrasound measurements of the fat depth at the 12 - 13th rib and the longissimus thoracis area (LM) were performed every 28 days during the feedlots test period with an Aloka 500V diagnostic real-time ultrasound through a 17cm, 3.5-MHz linear array transducer(Overseas Monitor Corporation Ltd., Richmond, BC, Canada). Metabolic BW (MWT) was estimated as midpoint $BW^{0.75}$, where the midpoint BW was computed as the sum of initial body weight (BW) and average daily gain (ADG) multiplied by half of the days on a test (DOT). The final ultrasound back fat (FUFAT) and LM area (FUREA) of each steer were predicted at the end of tests from the regression equation for ultrasound fat depth measurements over time (d).

As described by Mao et al. (2013), three measures of RFI were performed and included RFI, RFIf estimated as the adjusted for ultrasound back fat the end of feedlot period, and the RFIr which was estimated from the RFI adjusted for both the ultrasound back fat thickness and the REA (carcass rib eye muscle area) at the end of the feedlot test. RFI, RFIf and RFIr were then computed as the differences between the standardized daily DMI and the

DMI predicted using a regression coefficient which resulted from the following three linear regression models:

$$Y_i = \beta0 + \beta1ADGi + \beta2MWTi + ei \quad [1]$$

$$Yi = \beta0 + \beta1ADGi + \beta2MWTi + \beta3FUFATi + ei \quad [2]$$

$$Yi = \beta0 + \beta1ADGi + \beta2MWTi + \beta3FUFATi + \beta4FUREAi + ei \quad [3]$$

Where; *Yi* is the standardized daily DMI for the ith steer,

β0 is the regression intercept,

β1 is the partial regression coefficient on ADG,

β2 is the partial regression coefficient on MWT,

β3 is the partial regression coefficient on FUFAT,

β4 is the partial regression coefficient on FUREA,

ei is the residual error for the ith steer.

Individual steer RFI values were calculated as the difference between an animal's actual dry matter intake (DMI) and the predicted DMI based on average daily gain (ADG), MWT and RFIf (value adjusted for back fat thickness). With the availability of RFI values, animals were grouped into positive (high) regarded as inefficient animals (greater than 0.3 from the mean) or negative (low) RFI groups regarded as efficient animals (greater than -0.3 from the mean) within each of the breeds and balanced for body weight. Thereafter, steers were fed to a finished back fat greater than 2 mm at the 12^{th} -13^{th} rib site, as this is the minimum required for a beef carcass to qualify for Canada A quality grades.

2.2.3 Animal slaughtering and muscle removal

From July to September 2015, steers were slaughtered by breed group at the Meat Research Laboratory abattoir at the Agriculture and Agri-Food Canada Science and

Technology Branch, Lacombe, Alberta. Prior to slaughtering, cattle received at the abattoir were rested for approximately 2 h prior to slaughter with *ad libitum* access to water. Cattle were then inspected by Canadian Food Inspection Agency personnel to ensure the fitness of the steers for human consumption. Just prior to slaughter, live weight was recorded. In accordance with federal regulations (Canada Gazette Part II, 2001), humane slaughtering of animals was performed using a captive bolt pistol. Steers (n = 4 or n = 6) from each RFI group were processed by breed per kill day, with the exception of the Angus, where one steer died prior to slaughter. The mean kill ages were 452, 533, and 511 days for Angus crossbred, Angus, and Charolais, steers, respectively. The right sides of the carcasses were fabricated and the *Triceps brachii* (TB) muscle was removed at 72 hours post-mortem for meat quality and proximate analysis. Steaks 2.5 cm thick were removed proximal to distal, with the first steak used for sarcomere length estimation and proximate analysis. The second steak was used for Warner-Bratzler shear force on day 3 post mortem, the third steak was used for Warner-Bratzler shear force at day 13 post mortem, the fourth steak was sued to estimate collagen heat solubility and quantify pyridinoline and Ehrlich chromogen cross-links on day 3 post mortem, the fifth was used to measure collagen heat solubility on day 13 post mortem, the sixth steak was used for sensory evaluation for another thesis, and the seventh steak was used to estimate drip loss during retail display. Each of the steaks were weighed and vacuum packaged individually following the appropriate ageing period and then frozen at about -20^0C until further analysis.

2.2.4 Chemical analysis of carcass and meat quality traits determination

2.2.4.1 Proximate analysis determination (moisture, crude protein and crude fat content)

The analysis was performed at Agriculture and Agri-Food Canada Meat Research Laboratory in Lacombe, Alberta. Epimysium and surface/excess fat was removed from the 2.5cm steak used for proximate analysis and the steak was then ground (Robot Coupe Blixir BX3; Robot Coupe USA Inc., Ridgeland, MS, USA). Moisture content was determined using microwave and infrared heating using a Smart Turbo Moisture Analyzer (Model 907990, CEM Corporation, Matthews, NC, USA). The percentage of intramuscular fat (IMF) was determined using a Smart Trac Fat Analyzer (Model 907955, CEM Corporation, USA) using method 2008.06 as described by Leffler et al., (2008). Crude protein was determined using a CEM Rapid Analyzer System (Sprint Protein Analyzer Model 558000, CEM Corporation, USA). Compositional contents were expressed as a percentage of sample weight.

2.2.4.2 Carcass yield and quality grade

Hot carcass weight (HCW), cold carcass weight (CCW), average back fat thickness (AFAT), carcass ribeye area (REA), lean meat yield (LMY), muscle score, and marbling scores at the 12^{th} and 13^{th} rib muscle were recorded as described in the Canadian beef grading system (Canada Gazette, 2007). Carcasses with marbling scores between 100 to 199 indicated that they had trace marbling and were graded as Canada A, while those with marbling scores between 200 to 299 had slight marbling and were graded Canada AA, those with marbling scores between 300 to 399 had small to moderate marbling and were graded Canada AAA , and those with slightly abundant or more marbling with scores of 400 to 499 were graded as Canada Prime.

2.2.4.3 Meat quality measurements

Physio-chemical properties such as intramuscular pH and temperature, drip loss, colour, cooking loss, Warner-Bratzler shear force (WBSF), and sarcomere length were measured as described by Girard et al. (2012) and Holdstock et al. (2014).

For intramuscular temperature and pH measurement, a Fisher Scientific Accumet AP72 pH meter (Fisher Scientific, Mississauga, ON) equipped with an Orion Ingold electrode (Udorf, Switzerland) was used. Care was exercised to properly standardize or calibrate the pH meter and fresh/standardized buffer solution of pH 4 and 10 were used. Values were collected in triplicate and the means were calculated and statistically analyzed.

Drip loss was determined by weighing the steak and its initial trimmed muscle weight was recorded. The trimmed steak was then kept for four days at 2°C in a polystyrene overwrapped tray with a Driploc® pad (Crovac Inc., Charlotte, North Caroline). Samples were re-weighed, and this weight recorded as the final trimmed muscle weight. Drip loss was expressed in milligrams of water lost per gram of muscle and calculated as:

Drip loss (mg/g) = (initial trimmed muscle weight (g) – final trimmed muscle weight (g))/initial weight (g) x 1000).

For sarcomere length measurements, two grams of each muscle trimmed of connective tissue and fat were hand-minced, immersed in 20 mL of 0.02 M EGTA, 0.25 M sucrose solution in a 50 mL centrifuge tube, and homogenized for 10 s at 6000 rpm (Polytron Homogenizer PT3100 with a 2 cm generator, Brinkmann Instruments Inc., Mississauga ON). A small amount of sample was placed on a microscope slide, covered with a cover-slip to prevent dehydration and twelve images of sarcomeres per steak sample were captured with the use of a phase contrast microscope (Axioscope, Zeiss, West Germany) equipped with a Sony DXC

930 Color Video Camera (Sony Corporation, Japan) and Image Pro-Plus software V4.0 (Media cybernetics, Silver Spring, MD). Thereafter, the mean sarcomere length value of each of the steak was calculated from the best ten images and expressed in micrometers in statistical analysis.

Meat colour was determined by the method described by the Japanese Meat Grading Association (JMGA) where 1 = pale and 8 = extremely dark and classification using the JMGA method performed by a certified beef grader. A Minolta CR-300 with Spectra QC-300 software was used (illuminant C and 2 observer, 3 mm aperture; Minolta Canada Inc., Mississauga, ON, Canada) was used for objective colour measurements (CIE *L**: brightness; *a**: red-green axis; *b**: yellow-blue axis; Commission Internationale de l'Eclairage 1978). Colour measurements were collected in triplicate on the surface of the steak used for colour and L*, a* and b* values used to calculate hue [h_{ab} = arctan(b*|a*)] and chroma [$C^*_{ab} = (a^{*2} + b^{*2})0.5$].

The analyses for shear force, cooking loss, and cooking time were performed using steaks aged 3 and 13 days post-mortem (dpm). Steaks for non-aged (day 3) and aged (day 13) were cut, weighed and grilled at approximately 210ºC (Garland Grill ED30B, Condon Barr Food Equipment Ltd., Edmonton, AB). A spear point temperature probe placed in the geometric center of each of the steaks was used to monitor the internal temperature at 30s intervals (Type T copper-constantan,10 cm in length, AllTemp Sensors Inc., Edmonton, AB). The steaks were turned at an internal temperature of 35.5ºC, and cooked to a final temperature of 71ºC (monitored with a Hewlett Packard HP34970A Data Logger, Hewlett Packard Co., Boise ID). The cooking time was recorded and expressed as seconds per gram cooked meat. Thereafter, steaks were removed from the grill, sealed in polythene bags and allowed to cool

in an ice bath. After cooling, bagged steaks were transferred to the refrigerator and stored at 2°C for 24 hours, and the final weight was taken afterward for cooking loss estimation, which was calculated as:

Cooking loss (%) = ((initial raw steak weight – cooked steak weight)/initial raw steak weight) x 100

For shear force determination, six 1.9 cm diameter cores were removed parallel to the muscle from cooked steaks aged 3 and 13 dpm. The shear force peak values of the cores were measured using a Texture Analyser (Model TA.XT plus, Texture Technologies Corp, New York) equipped with a Warner-Bratzler shear force head. The crosshead speed was 200mm/min and peak shear force values were determined by averaging the values of the six cores from each steak. Shear force values were recorded in kilograms (Texture Exponent 32 Software, Texture Technologies Corp., Hamilton, MA).

2.2.5 Intramuscular connective tissue/collagen characteristics

2.2.5.1 Isolation of intramuscular perimysium

Intramuscular perimysium was isolated and determined by using the method described by Roy et al. (2015). About 100g of cubed muscle were homogenized using a laboratory blender (Waring, New Jersey). Deionized water (5 volumes) at 4°C was added and blending was performed at low speed for 10s and increased for 10s to high speed. The homogenate slurry was filtered through a metal sieve of pore size 1 mm^2 and the material left on the sieve was collected as the IMCT. The IMCT was returned to the blender and deionized water was added again and blended as before, filtered and this step was repeated twice. Isolated IMCT was dried using Whatman No.4 filter paper (Fisher Scientific, Mississauga, Ontario). The blood vessels remaining in the material were removed prior to drying with filter paper. The

wet isolated IMCT was weighed and was considered wet perimysium and stored at -20°C until dried and de-fatted. For drying and de-fatting, the perimysium was thawed and fresh chloroform/methanol (2:1, v/v) was added to the perimysium in a glass beaker. The perimysium was extracted with new chloroform/methanol mixture until the mixture was no longer cloudy. The perimysium was then removed from the mixture and allowed to dry in a fume hood overnight at room temperature in the dark. De-fatted perimysium was then weighed as dry perimysium and stored at -20^{O}C away from light until analysis for total collagen, Ehrlich's chromogen and pyridinoline crosslinks.

2.2.5.2 Ehrlich's chromogen cross-link concentration

As described by Horgan et al. (1991), Ehrlich's chromogen (EC, pyrrole) cross-link concentration in the lyophilized IMCT was determined by tryptic digest. Dried perimysium (0.1g) was suspended overnight in 50mM Tris-HCl, 1mM calcium chloride at 4ºC. Trypsin was added the following day and samples were incubated at 37ºC for 4 hours (mixed by vortex every 15minutes). The incubated trypsin solution was heated in a dry bath for 20 minutes at 65ºC, cooled at room temperature and centrifuged for 30minutes at 28000g. The supernatant was filtered through a 0.45 µm nylon filter in a 5 mL Eppendorf tube and 1mL of filtrate from each of the sample was added to 5% p-dimethylaminobenzaldehyde in 4 M perchloric acid (containing 0.01% mercuric chloride) and centrifuged at 14.000 for 2 minutes. Immediately, the absorbance of the supernatant for each of the samples at 572 and 640nm was measured and the 640 nm absorbance reading was subtracted from 572 nm reading as a baseline measurement for each of the sample.

As reported by Roy et al. (2015), the EC or pyrrole crosslink concentration was calculated using a molar extinction coefficient of 25000 and expressed as mol/mol collagen and nmol/g muscle as follows ;

A/ε = molar concentration, where A = absorbance and ε = the molar extinction coefficient.

To estimate the total collagen content, the remainder of the tryptic digest was stored at -20ºC for the determination of hydroxyproline content and assays were performed in duplicate.

2.2.5.3 Pyridinoline cross-link determination

Pyridinoline (PYR) quantification was performed in semi-darkness to avoid the destruction of the pyridinoline ring by UV light (Wu and Eyre, 1984). About 0.20g (±0.003 g) of freeze-dried perimysium sample was hydrolyzed with 6 mL of 6 M hydrochloric acid for 20 hours at 110ºC in a labeled Teflon® glass tube placed in a dry bath, previously flushed with nitrogen and capped tightly. After the completion of hydrolysis, the tubes were allowed to cool at room temperature in an ice water bath for approximately 10minutes. The hydrolysates were filtered (Whatman No. 4 filter paper, Fisher Scientific, Mississauga, Ontario), tubes and glass were rinsed with 1 mL de-ionized (DI) and the rinse filtered. The total hydrolysate volume was 8 mL and 2 mL aliquot for each sample was retained for total collagen quantification from hydroxyproline (HYP) measurement and the remaining 6 mL was used for PYR measurement.

Pyridinoline quantification was performed using the method described by Robins, Duncan, Wilson, and Evans (1996), ensuring no or minimal exposure of PYR hydrolysates to light (Sakura, Fujimoto, Sakamoto, Mizuno, & Motegi, 1982). The remaining 6 mL hydrolysate was evaporated to dryness using a rotary evaporator at 40ºC and 690 mmHg (Heidolph Collegiate rotary evaporator, Brinkmann, equipped with a DistiVac Ultra auto-purge vacuum system, Brinkmann, Mississauga, ON) and reconstituted with 1.5 mL of 10% acetic acid (v/v). Thereafter, PYR cross-links were separated by gel filtration (size exclusion chromatography) using polyacrylamide beads (Bio-gel P2, Bio-Rad, Canada) in a gravity

column (Econopac, 20 mL bed volumes, 1.5 cm x 12 cm column, Bio-Rad, USA) equilibrated with 10% (v/v) acetic acid. Elution was performed with 10% (v/v) acetic acid according to Robins et al., (1996). Fractions (6 mL x 5) were collected for each of the subsamples in 15 mL plastic graduated tubes (Thermofisher, Korea). The presence of PYR was confirmed using 1.0 mL from each of the 5 mL fractions collected by its natural fluorescence with excitation at (λex) 295 nm and emission at (λem) 395 nm using a fluorescence spectrophometer (Lumina, Thermofisher, USA). Fractions containing PYR were pooled and their volume measured . The pooled fractions were evaporated and reconstituted with 1.5 mL of 0.1M HCL and stored in a 2.0 mL Eppendorf tube (Fujimoto & Moriguchi, 1978).

Further purification of reconstituted PYR fractions was performed by using cellulose phosphate cation exchange column chromatography previously equilibrated with 0.1 M HCL. Fractions (5 mL x 4) were collected and the presence of PYR monitored by fluorescence spectrophometry as described previously. The column was washed extensively with 0.1 M HCL between samples and PYR was eluted with 1.0 M HCL (Robin et al., 1996). Fractions containing PYR for each sample were pooled and their volumes measured. Pooled fractions were dried using rotary evaporation and reconstituted with 1.5 mL of 1% aqueous heptafluorobutyric acid (HFBA) (Sigma-Aldrich Canada, Ltd) and filtered (Acrodise LC 13mm syringe filter with 0.2 µm PVDF membrane, Life Sciences). For quantification of PYR, 1.0 mL was loaded into an HPLC auto-sampler vial (amber type) for reverse-phase high performance liquid chromatography HPLC.

2.2.5.4 Total collagen content quantification

The total collagen content of muscle was determined by measuring hydroxyproline (HYP) content using a modified version of the method reported by Bergman and Loxley

(1963). A 2 mL aliquot of each 6 mL hydrolysate retained for PYR quantification was neutralized with NaOH, confirmed with pH paper, evaporated, reconstituted with 5 mL deionized water and stored at -20ºC until use. Reconstituted samples were thawed at room temperature and 0.05 mL duplicate aliquots were taken with 0.95 mL of distilled water added (1 mL in total) for each sample in 20mL glass tubes. Blanks were prepared with 1 mL deionized water and standards were generated by dilution of a stock solution of 400 µg/mL trans-4-hydroxy-L-proline (Sigma-Aldrich Canada Ltd, Oakville, ON) to 2.5, 5.0, 10.0, 20.0 and 40.0 µg/mL. The tubes were allowed to stand for 4 minutes after adding 2 mL of isopropanol and 1 mL of oxidant in each of the tubes for all samples (n = 71 by 2). In each tube, 13 mL of Ehrlich reagent were added, the tube then capped and mixed by vortex, the tube then placed in a water bath at 60ºC for 25 minutes, and then cooled in an ice bath for 5 minutes. Tube contents were transferred to 50 mL volumetric flasks that were filled to 50 mL with isopropanol. The absorbances of the final experimental samples were measured at 558 nm against a blank and the HYP concentration was derived from the regression of expected trans-4-hydroxy-L-proline standards concentrations on to means of their absorbance values. The percentage of the total collagen content was estimated by multiplying HYP concentration by 7.14, assuming that the HYP concentration of collagen is 14% (Etherington & Sims, 1981).

2.2.5.5 Soluble collagen content and collagen heat solubility measurement

The method of Hill (1966) was used to determine heat-soluble collagen. Freeze dried powdered meat (1.0 ± 0.003g) from each sample was weighed, transferred to a labeled 50 mL centrifuge tube with a stopper, and 12 mL of ¼ strength Ringers solution was added. The mixture was gently mixed by vortex, and heated for 63 minutes at 77ºC with each tube gently shaken every 5-10 minutes. After heating, tubes were cooled in ice water for 15 minutes,

centrifuged (Avanti J-E Centrifuge, Beckman Coulter, Mississauga, ON) at 3500 ×g for 10 minutes at 4 °C, and the supernatant removed and stored at -20°C. The pellet was washed with 8 mL of ¼ strength Ringers solution and centrifugation was repeated. The supernatant was also removed and added to the previous supernatant. From the pooled supernatants, 1.8 mL was subjected to centrifugation for 25 seconds and then 1 mL of this was transferred into a clean labeled glass tube. Hydrolysis was performed on the 1 mL by adding 12 N HCl and 3 mL of 6 N HCl to the 1 mL, Hydrolysis was performed in duplicate with two 1 mL for each sample as described in Section 2.2.5.3, except no filtering was performed, to determine an average of collagen in the solution. Heat solubility was expressed as a percentage as follows:

Collagen heat solubility (%) = (soluble collagen ÷ total collagen) x 100

2.3 Statistical analysis

The general linear model (GLM) procedure of R software (Version 3.5.1) was used to fit the appropriate mixed models and data analysis was performed as a split plot. Breed type, RFI and their interaction were sources of variation in the whole plot, where animal is the experimental unit, while post-mortem ageing and its interactions with sources of variation in the whole plot were the effects tested (at the sub plot level, with steak as the experiment unit. The slaughter group within each breed type was included as a random effect. Least square means of each treatment with their residual errors were compared, with the differences significant at $P<0.05$ using Tukey's Honestly Significant Difference test (HSD). Non-significance level was set at $P > 0.05$ for the model and interactions at $P > 0.05$ were removed. Correlation analysis was performed to calculate the correlation coefficients and determine linear relationships between carcass, meat quality, and connective tissue characteristics using R software (Version 3.5.1) with the Hmisc package (Version 4.0-2). The

Bonferroni correction was used to compensate for the likelihood of Type I error. Thirty two comparisons were made, and the significant adjusted P value of correlations was 0.0016 and calculated by dividing the level of significance by the number of comparators 0.05/32 (Mahmood et al., 2016).

2.4 Results

2.4.1 Carcass traits

Breed types significantly differed in mean final weight and carcass dressing percentage ($P < 0.005$) (Table 2.1). Mean final weight was significantly greater for Charolais than Angus and Angus crossbred steers ($P = 0.02$). Mean dressing percentage was higher in Charolais than Angus crossbred and Angus carcasses ($P = 0.03$). No significant effects of breed type were observed on carcass shrink, marbling and rib eye area measurements ($P > 0.05$). The selection for residual feed intake (RFI) had limited effect on the measurements ($P < 0.05$) (Table 2.1). Grade marbling score was greater for high RFI than for low RFI steer carcasses ($P < 0.0001$). There were no significant interactions between breed type and RFI ranking ($P > 0.05$).

2.4.2 Meat quality characteristics and proximate analysis measurements

Breed type had limited effects on meat quality measurements ($P > 0.05$). Mean temperature at fabrication was greater in Angus crossbred than in Angus and Charolais steer carcass muscles ($P = 0.02$) (Table 2.2). There were no effects of RFI ($P > 0.05$) (Table 2.2) and no significant interactions between breed type and RFI ($P > 0.05$) on any measurements.

2.4.3 Intramuscular connective tissue and collagen characteristics measurement

There were no effects of breed type or RFI on intramuscular connective tissue (IMCT), or collagen and collagen crosslinks measurements ($P > 0.05$) (Table 2.3). There were

no significant effects of breed type and RFI level ($P > 0.05$) observed on the amount of wet perimysial connective tissue recovered as a percentage of wet tissue, the percentage of dried perimysium connective tissue, or the total amount of collagen present in the TB muscle. Additionally, no significant effect of breed type and RFI level ($P > 0.05$) was observed on the density of PYR and Ehrlich's chromogen cross-links in TB collagen (mol/mol collagen) or in the concentration of there cross-links in raw TB muscle (nmol/g raw muscle). There were no significant interactions between breed type and RFI levels ($P > 0.05$) on any of these measurements.

2.4.4 Effects of breed type, RFI level and post-mortem ageing on cook loss, cook time, shear force (WBSF) and core standard deviation

Breed type and genetic selection for RFI level had no significant effect on cooking loss, cooking time, WBSF and WBSF core standard deviation (StdDev) ($P > 0.05$) (Table 2.4); however, there was an interaction between breed type and post-mortem ageing ($P < 0.05$) (Figure 2.1). Compared with the other breeds Angus beef cooking time decreased at 13 dpm, while an increase of cooking time at 13 dpm was observed for beef steaks from both Charolais and Angus cross-bred steers.

Post-mortem ageing significantly decreased WBSF and WBSF core StdDev value ($P < 0.05$) (Table 2.4). Mean WBSF value at 3 dpm was significantly greater than at 13 dpm ($P < 0.001$). Core StdDev value was greater at 3 dpm than at 13 dpm ($P = 0.03$).

2.4.5 Effects of breed type, RFI level and post-mortem ageing (dpm) on heat soluble collagen, insoluble collagen, collagen heat solubility measurements

There were no significant effects of breed type and RFI level on heat soluble collagen, insoluble collagen and percent collagen heat solubility ($P > 0.05$) of TB from beef steer

carcasses (Table 2.5). No interaction was observed for any of the measurements ($P > 0.05$). However, post-mortem ageing had significant effects on all the measurements ($P < 0.05$) (Table 2.5). Heat-soluble collagen at 13 dpm was greater than at 3 dpm ($P < 0.001$). At 3 dpm, insoluble collagen was significantly greater than at 13 dpm ($P = 0.005$). Collagen heat solubility was increased at 13 dpm compared to at 3 dpm ($P < 0.001$).

2.4.6 Pearson correlation analysis between carcass and meat quality traits, intramuscular connective tissue and collagen characteristics

The correlation analysis for all the measurements is presented in Tables 2.6a, b, and c. Temperature was positively correlated with moisture content ($P < 0.0001$) and a negative correlation with final weight, dressing percentage, and marbling ($P < 0.0001$). Moisture had a strong negative correlation with intramuscular fat, final weight, dressing and marbling ($P < 0.0001$). Intramuscular fat had slight strong negative correlation with marbling ($P < 0.0001$). Crude protein had a positive correlation with shrink ($P < 0.0001$). Drip loss showed a positive correlation with cooking loss at 3dpm, final weight, and rib eye ($P < 0.0001$). Drip loss had a negative correlation with dressing percentage ($P < 0.0001$). Cooking loss at 3dpm had a strong positive correlation with cooking time at 13dpm and WBSF at 3dpm ($P < 0.0001$). Cooking loss at 13 dpm had strong positive correlation with cooking time at 13 dpm, WBSF at 13 dpm, and positive correlation with wet perimysium ($P < 0.0001$). Cooking time at 3 dpm had a positive correlation with WBSF at 3 dpm ($P < 0.0001$) and cooking time at 13 dpm had a positive correlation with WBSF at 13 dpm, dry perimysium, and wet perimysium ($P < 0.0001$). WBSF at 3dpm had significant negative correlation with rib eye area ($P < 0.0001$) and a positive relationship with pyridinoline in raw meat ($P < 0.0001$) and pyridinoline in

collagen (P = 0.001). WBSF at 13 dpm had a strong positive correlation with insoluble collagen at 3 dpm (P < 0.0001).

Final weight had strong positive correlation with dry perimysium and wet perimysium (P < 0.0001). Dressing percentage showed a positive correlation with dry perimysium (P < 0.0001) and a weak negative correlation with pyridinoline in raw meat (P = 0.001). Rib eye had a positive correlation with dry perimysium, and wet perimysium (P < 0.0001). Shrink had a weak negative correlation with pyridinoline in raw meat (P = 0.001).

In Table 2.9c, wet perimysium had a strong positive correlation with insoluble collagen at 13 dpm, and EC in raw meat (P = <0.0001), and a negative correlation with total collagen (P < 0.0001). A strong positive correlation was observed with total collagen and insoluble collagen at 13 dpm, and EC in raw meat (P < 0.0001). Total collagen also had a negative correlation with solubility at 3 dpm, solubility at 13 dpm, and EC in collagen (P < 0.0001). Soluble collagen at 3 dpm had a strong positive correlation with solubility at 3 dpm (P < 0.0001), and a negative correlation with insoluble collagen at 3 dpm (P < 0.0001). Soluble collagen at 13 dpm had a strong positive correlation with solubility at 13 dpm (P < 0.0001) and a weak negative correlation with insoluble collagen at 13 dpm (P = 0.001). Insoluble collagen at 3 dpm was negatively correlated with solubility at 3 dpm (P < 0.0001). Insoluble collagen at 13 dpm had a negative correlation with solubility at 3 dpm, solubility at 13 dpm, EC in collagen (P < 0.0001) and a positive correlation with EC in raw meat (P < 0.0001). Solubility at 13 dpm had a weak negative correlation with EC in collagen (P = 0.001).

2.5 Discussion

The variation between breeds with regard to carcass quality traits is an important genetic resource for the improvement of beef production, carcass composition and feed efficiency. The values obtained for the final mean weights for Charolais and Angus steers were similar to those reported by Barton et al., (2006) and Chambaz et al., (2001), when breeds were raised to the same age and slaughtered at a target level of intramuscular fat. In general, the Angus breed produces lighter carcasses than the Charolais breed and this may be because the average daily live weight and carcass gained is highest in the Charolais breed (Pesonen and Huuskonen, 2015; Brown et al., 2004). The low dressing percentage of Angus and Angus crossbred steers found in this study can be explained by their low slaughter weight or final weight compared to the Charolais breed. Moreover, O'Farrell and Keane (1990) reported in their study that this trait increased with slaughter weight and this has since been established by others (Barton et al., 2006). These results confirm that Charolais beef steer carcasses are high yielding carcases, more so than Angus and Angus crossbred steers. The value of a carcass in Canada is determined not by dressing percentage however but by quality grade as well, and so a high yielding carcass with high level of marbling would be the most valuable.

High RFI or inefficient animals had greater marbling compare to low RFI animals ($P < 0.05$) (Table 2.1). In 133 Angus beef steers, Ahola et al. (2011) reported the same for their study, which is not in agreement with several other studies (Baker et al., 2006; Barsarab et al., 2003). It was however reported by those authors that there is a tendency for inefficient animals to have greater marbling compared to efficient animals. Nkrumah et al., (2007) suggested there was a link between carcass fat content and RFI classification because in all of

the studies, although limited, carcass grade fat and lean meat were found to be lower in high RFI animals. Hence, to produce carcasses with increased marbling, genetic selection for high RFI may be the most desirable. However, no differences in the final weight between RFI classification was observed and this agrees with most other studies, which concluded that the weight of beef cattle in different phases does not differ across RFI levels (Baker et al., 2006; Gomes et al., 2012, Cruz et al., 2010).

Meat quality characteristics were similar among the breeds except for temperature at fabrication (Table 2.2). The results obtained do not agree with several other studies because of the differences known to exist between the Angus and Charolais breeds; however, this could possibly be due to the fact that the beef steers were raised to approximately the same physiological age and back fat thickness and raised on the same diet. Koch et al. (1967) reported there was limited differences among different breeds raised to a constant age, constant weight and constant fat in longissimus muscle colour, texture, firmness, and tenderness. Breeds of cattle fed the same diet showed no difference in drip loss, WBSF, and fat colour (Xie et al., 2012). However, the breeds used in that study were different breeds from those used in this present study, and no literature is available comparing the meat quality of Angus, Charolais and Angus crossbreds based on approximately the same age and feed resources. The results obtained may also be specific to the type of muscle used (Purslow 2005; Guerrero et al., 2013). Bianchi et al. (2006) in their research reported that consumers could not detect the differences in meat sensory tenderness between the breeds and the authors concluded that age of animal and muscle type were more important factors than breed. As mentioned earlier, the muscle type used in this study is a muscle that has more insoluble collagen (Blanco et al. 2013) and this may have overridden the effects of breed type on the

quality attributes of the meat. The difference on temperature at fabrication due to breed type might be a result of variation in carcass cooling rate due to differences in carcass weight (Pike et al., 1993), as heavy carcasses would be most likely to warm more slowly than light carcasses after chilling.

Selection for low RFI had no effects on meat quality measurements (Table 2.2), which agrees with several other studies (Baker et al., 2006; Ahola et al., 2011; McDonagh et al., 2001). The authors all concluded that there was no significant relationship between RFI levels and meat quality attributes (Ahola et al., 2011). Furthermore, regardless of the slaughter age or feeding, most of the carcass and beef quality attributes are considerably affected by a decline in pH during slaughter and in this present study, muscles from the carcasses of the different breeds and RFI levels were characterized by normal pH ranging from 5.5 to 5.7. Adequate pre-slaughter condition could have contributed to the optimal pH levels of the muscles, which in turn positively improved the quality traits of the *triceps brachii* muscle.

In this thesis, breed type and RFI ranking had no effects on IMCT and collagen characteristics (Table 2.3). Although many studies have reported that breed has a significant effect on total collagen (Sañudo et al., 2004; Serra et al., 2008), other authors observed no effect of breed on total collagen (Waritthitham et al., 2010). Results may be equivocal because the amount of IMCT and collagen characteristics is largely dependent on the type of muscle and the age of the animal. Stolowski et al., (2006) found significant effects of muscle type on the amount of IMCT content ($P < 0.001$) and no effects of breed ($P > 0.05$). In that study, *Biceps femoris* and *Vastus lateralis* had high amount of total collagen and they also demonstrated the highest WBSF values. Geesink et al. (1995) demonstrated that *triceps brachii* muscle contained a greater amount of IMCT and endomysium than the *Longissimus*

dorsi muscle. Other studies have also reported that muscles from the round had higher shear force values than muscles from the loin and chuck (Prost, et al., 1975; Nishimura et al., 2010; Anderson et al., 2012; Mahesh 2019). These findings clearly show that more studies are required to understand factors that could positively influence the toughness of individual primal cuts from the beef carcass rather than continuing to examine the differences among breeds.

The significant effects of post-mortem ageing on the mean value of WBSF and core standard deviation which decreased as ageing days increased may be due to the presence of calpain and matrix metalloproteinase (*MMP's*) enzymes early post-mortem ageing. The activities of these two enzymes may have been aided by the normal pH obtained in this study. With that, calpains enzymes are able to degrade proteins of the Z-disks, desmin and nebulin, which accounts for about 90% or more of the tenderization that occurs (Hopkins and Thompson, 2002). Matrix metalloproteases (*MMP's*) have been associated with the degradation of collagen present in muscle (Purslow et al., 2012; Nishimura et al., 2010). Both calpains and *MMP's* are found to be dependent on the type of muscle (Cha & Purslow, 2010; Delgado et al., 2001), and understanding how the two enzymatic system work might explain some variations in tenderness between different bovine muscles (Veiseth-Kent et al., 2018).

As expected, with the increase in storage time or days of ageing heat soluble collagen increased and insoluble collagen was reduced, and this could be used to explain the decreased in WBSF and core standard deviation observed at 13 days post-mortem ageing due to proteolytic activities early post-mortem. Stolowski et al. (2006) reported there was an increase in the percentage collagen heat solubility among six various beef muscles with an increase in ageing days. It was concluded in that study that the differences in the amount of collagen

present in each muscle and their solubility rate seem to explain the variation of WBSF value obtained. This result is reasonable because the amount of IMCT increases with the use or location of each muscle while its solubility depends upon the activities of *MMPs* and their inhibitory tissue metalloproteinases (*TIMPs*).

Although there are multiple low to moderate and high correlations observed in this thesis, several linear relationships between measurements and WBSF value were observed. As mentioned earlier, beef tenderness is the most important of the palatability traits and is related to consumer willingness to re-purchase a particular beef cut (Destefanis et al., 2008). The toughness of *triceps brachii* muscle as measured using peak shear force was associated with an increase in cooking loss and cooking time. It is well known that the action of heat on meat is significant in improving the palatability of beef as its greatly influences muscle proteins through denaturation of myosin, gelatinization of collagen, and reduction in the water holding capacity of meat (Juarez 2012). Interestingly, cooking had the most influence on peak shear force values in this study, as shear force was positively correlated with cooking time, regardless of post-mortem ageing time. Cooking for a longer time was correlated with increased cooking loss, indicating that as would be expected myofibrillar protein denaturation increased with cooking time. Cooking loss for steaks aged 3 days post-mortem was positively correlated with drip loss, suggesting that steaks that lost the most moisture during cooking also had more moisture to lose. For tenderisation to maintained in beef, the loss of water has to be prevented because higher water holding capacity of meat greatly promotes the solubilisation of collagenous content (Leptit 2008). It is however important to mention that, grilling or heating of beef to an internal temperature of 71^{o}C as performed in this study with the use of a grill was associated with increased in beef toughness as the high heat of the grill

leads to significant rapid dehydration, therefore suppressing the solubilisation of collagenous content and preventing gelation (Palka & Daun 1999; James & Yang 2012). Hence, understanding and manipulating the best cooking parameters such as time, temperature and atmosphere which influences the structural changes of raw meat might help to further improve the tenderness of *triceps brachii* post-mortem ageing.

The linear relationship observed between the amounts of perimysium, insoluble collagen and Ehrlich chromogen cross-links in the raw meat indicates the contribution of perimysium to beef toughness of *triceps brachii* muscle. This result agrees with several other studies, which conclude that the perimysial network influences the toughness of beef (Lewis & Purslow 1990; Lewis et al., 1991; Brooks & Savell, 2004). However, it has been established that the influence of perimysium on beef toughness is dependent of muscle type and Brooks and Savell (2004) reported that muscles with high amounts of perimysium had increased values of WBSF. The tensile strength of perimysium was reduced by post-mortem ageing and cooking in this thesis because there was no correlation with WBSF. Harris & Shorthose (1988) concluded that the contribution of perimysium to cooked beef toughness can be reduced by cooking at a temperature above 60^{o}C and this agreed with the report of Lewis & Purslow (1991).

The total amount of collagen present in steaks is believed to determine beef toughness of different muscles after post-mortem ageing (Purslow 2005). High, low and no correlations between collagen content and meat toughness after cooking beef have been reported in several studies (Dransfield 1997; Dransfield et al., 2003; Ngapo et al., 2002; Christensen et al., 2011). Positive correlations between total collagen content and insoluble collagen content and Ehrlich chromogen cross-link concentration indicated that as the amount of collagen increased

so too did the amount of Ehrlich's chromogen and collagen that was resistant to heat. These results also indicated that the presence of Ehrlich chromogen was not linked to beef toughness before or after post-mortem ageing and cooking. Lepetit (2007) concluded that WBSF differences between different types of beef muscles when cooked to temperature above 60ºC was due to both a contribution from myofibrils and matured trivalent collagen cross-links other than Ehrlich's chromogen, a view shared by others (Christensen et al., 2000; Christensen et al., 2011: Bruce & Roy 2019). The concentration of pyridinoline was moderately negatively correlated with shear force of the *triceps brachii* muscle at day 3 post-mortem, with the magnitude of the correlation declining by day 13 post mortem. This result supports the hypothesis that pyridinoline cross-links are more important in explaining the variation in the background toughness of unaged cooked beef. The concentration of pyridinoline was not correlated to the amounts of total collagen or insoluble collagen, indicating that its concentration was not a function of collagen concentration like the density of Ehrlich chromogen cross-links. For this to be, the formation or presence of pyridinoline would have to be purposefully manufactured rather than simply a function of opportunity and time. The concentration of Ehrlich chromogen in raw meat appears to be still important however as it was associated with increased insoluble collagen and decreased collagen solubility at 13 days post-mortem ageing. Hence, these results suggest that being able to reduce the amount of pyridinoline and Ehrlich chromogen in raw meat may improve the overall eating quality of beef.

2.6 Conclusion

Selection for low RFI cattle reduced ribeye visual marbling score; however, with no effect of RFI on any other meat quality measurement, cattle production costs can be reduced by selecting for low RFI animals without sacrificing product quality. Additionally, the toughness of the *triceps brachii* can be decreased with post-mortem ageing, and increased collagen solubility contributes to this decrease, meaning that the collagen of the *triceps brachii* is susceptible to post-mortem proteolysis and so background toughness may not be as intransigent as previously thought. This result suggests that muscles from the chuck may be utilized to provide consumers with a satisfactory eating experience, thereby increasing the value of carcass. As a result, it would be advantageous for beef producers to be able to minimize the contribution of collagen concentration and cross-linking to beef toughness through modification of good production and processing practices.

2.7 Tables and Figures

Table 2.1 Effects of breed type and residual feed intake (RFI) on least squares means for steer growth and carcass quality measurements

Measurement	Breed				RFI			P value[3]	
	Angus	Charolais	ANC[1]	SEM[2]	Low	High	SEM[2]	Breed	RFI
Final weight (kg)	553[b]	682[a]	568[b]	9.58	605	597	7.87	0.02	0.48
Shrink (%)	2.71	4.86	4.64	1.26	3.96	4.19	1.24	0.09	0.60
Dressing (%)	57.78[b]	61.07[a]	58.20[b]	0.31	59.31	58.73	0.26	0.03	0.54
Marbling[4]	417	421	370	34.94	384[b]	421[a]	19.82	0.59	<0.001
Ribeye area (cm^2)	81.80	102.0	86.17	7.82	91.25	88.69	4.36	0.33	0.12

[a, b] Least square means with different letters within a row and treatment differ at $P \leq 0.05$.
[1]ANC: Angus crossbred.
[2]SEM: Standard error of the mean.
[3]P value: Significance level of main effects for breed and RFI.
[4]Marbling: 100-199 = trace (Canada A grade); 200-299 = slight (Canada AA grade); 300-399 = small to moderate (Canada AAA grade); 400-499 = slightly abundant or more (Canada Prime).

Table 2.2 Effects of breed type and residual feed intake (RFI) on least squares means for meat quality characteristics and proximate analysis of the m. *triceps brachii.*

Measurement	Breed				RFI			P value[3]	
	Angus	Charolais	ANC[1]	SEM[2]	Low	High	SEM[2]	Breed	RFI
Sarcomere length (μm)	2.30	2.17	2.21	0.05	2.19	2.26	0.04	0.42	0.23
Ultimate pH	5.55	5.55	5.58	0.04	5.56	5.56	0.03	0.88	0.46
Temperature (^{O}C)	2.77[a]	1.41[b]	3.15[a]	0.43	2.49	2.40	0.41	0.02	0.54
Moisture (%)	74.81	73.89	74.99	0.27	74.57	74.55	0.23	0.07	0.90
Crude protein (%)	19.87	20.56	20.11	0.41	20.30	20.06	0.38	0.22	0.14
Intramuscular Fat (%)	3.63	4.07	3.06	0.26	3.50	3.67	0.19	0.16	0.38
Drip loss (mg/g muscle)	29.53	40.52	35.08	1.97	33.92	36.16	1.47	0.11	0.24

[a, b] Least squares means with different letters within a row within a treatment differ at $P \leq 0.05$.
[1]ANC: Angus crossbred.
[2]SEM: Standard error of the mean.
[3]P value: Significance level of main effects for breed, RFI.

Table 2.3 Effects of breed type and residual feed intake (RFI) on least squares means of intramuscular connective tissue and collagen characteristics of the m. *triceps brachii.*

Measurements	Breed				RFI			P value[3]	
	Angus	Charolais	ANC[1]	SEM[2]	Low	High	SEM[2]	Breed	RFI
Perimysium dry weight (%)	4.21	3.30	7.72	1.21	4.93	5.22	0.69	0.21	0.46
Perimysium wet weight (%)	15.45	22.09	11.91	4.52	16.00	16.95	2.51	0.43	0.44
Total collagen (mg/g muscle)	7.25	7.18	6.04	0.73	6.87	6.87	0.57	0.38	0.81
EC[4] (nmol/g muscle)	7.43	7.53	6.40	0.39	7.20	7.05	0.34	0.24	0.57
EC[4] (mol/mol collagen)	0.32	0.34	0.34	0.02	0.34	0.33	0.02	0.74	0.77
PYR[5] (nmol/g muscle)	1.80	1.12	1.16	0.26	1.34	1.40	0.23	0.13	0.68
PYR[5] (mol/mol collagen)	0.18	0.11	0.14	0.01	0.14	0.15	0.01	0.09	0.46

[a, b] Least square means with different letters within a row within a treatment differ at $P \leq 0.05$.
[1]ANC: Angus crossbred.
[2]SEM: Standard error of the mean.
[3]P value: Significance level of main effects for breed, RFI.
[4]EC: Ehrlich chromogen.
[5]PYR:Pyridinoline.

Table 2.4 Effects of breed type, RFI levels and days post-mortem ageing (dpm[3]) on least squares means for cooking loss, cooking time, shear force (WBSF), and shear force core standard deviation of the m. *triceps brachii.*

Measurements	Breed				RFI			Ageing			P value[2]			
	Angus	Charolais	ANC[1]	SEM	Low	High	SEM	3 dpm	13 dpm	SEM	Breed	RFI	Ageing	B*A[5]
Cooking loss (mg/g)	253.84	248.84	246.13	15.03	248.69	250.52	11.49	251.66	247.55	11.59	0.91	0.82	0.69	NS[6]
Cooking time (sec/g)	4.81	4.79	4.61	0.70	16.00	16.95	2.51	4.67	4.77	0.41	0.97	0.44	0.72	<0.001
Shear force (WBSF)[3] (kg)	3.70	3.18	3.52	0.13	6.87	6.87	0.57	3.72[a]	3.21[b]	0.08	0.18	0.81	<0.001	NS
Core Std dev[4] (kg)	0.66	0.61	0.64	0.04	0.14	0.15	0.03	0.69[a]	0.59[b]	0.03	0.76	0.46	0.03	NS

[a, b] Least square means with different letters within a row within a treatment differ at $P \leq 0.05$.
[1]ANC: Angus crossbred.
[2]P value: Significance level of main effects for breed and RFI.
[3]WBSF = Warner-Bratzler shear force
[4]Core std deviation = Shear force core standard deviation.
[5]B*A = Interaction between breed and post mortem ageing.
[6]NS = Not significant.

Table 2.5 Effects of breed type, RFI ranking and days post-mortem ageing (dpm[3]) on least squares means for heat soluble collagen, heat insoluble collagen and collagen heat solubility of the m. *triceps brachii.*

Measurements	Breed				RFI			Ageing			P value[2]		
	Angus	Charolais	ANC[1]	SEM	Low	High	SEM	3 dpm	13 dpm	SEM	Breed	RFI	Ageing
Soluble collagen (mg/g)	1.43	1.64	1.46	0.31	1.57	1.45	0.19	1.26[b]	1.75[a]	0.18	0.87	0.57	<0.001
Insoluble collagen (mg/g)	5.83	5.61	4.65	0.71	5.36	5.37	0.61	5.61[a]	5.12[b]	0.60	0.34	0.91	0.01
Collagen solubility (%)	20.09	21.22	24.04	5.09	22.64	20.93	4.44	17.86[b]	25.71[a]	4.41	0.68	0.46	<0.001

[a, b] Least square means with different letters within a row within a treatment differ at $P \leq 0.05$.

[1]ANC: Angus crossbred.

[2]P value: Significance level of main effects for breed, RFI.

Table 2.6a Pearson correlation coefficients for correlations between carcass and meat quality attributes.

Measurements (units)	Moisture	Fat	Drip Loss	Cooking Loss (3dpm)	Cooking Loss (13dpm)	Cooking Time (3dpm)	Cooking Time (13dpm)	WBSF [1] (3dpm)	WBSF (13dpm)
Temperature	0.39*	-0.28	-0.18	-0.13	-0.12	-0.03	-0.14	0.33	0.29
Moisture (%)	1.00	-0.81*	0.15	-0.01	0.09	-0.02	0.02	0.15	0.07
Fat (%)		1.00	-0.19	0.09	-0.16	-0.02	-0.15	-0.05	-0.05
Drip Loss (mg/g)			1.00	0.39*	0.13	-0.24	0.08	-0.13	-0.26
Cook Loss (mg/g) (3dpm)				1.00	-0.17	0.61*	-0.08	0.39*	0.00
Cook Loss (mg/g) (13dpm)					1.00	-0.06	0.84*	0.00	0.44*
Cook time (sec/g) (3dpm)						1.00	0.09	0.42*	0.24
Cook time (sec/g) (13dpm)							1.00	-0.06	0.44*
WBSF (3dpm) (kg)								1.00	0.21
WBSF (13dpm) (kg)									1.00

Measurements (units)	Temperature (°C)	Moisture (%)	Fat (%)	Protein (%)	Drip Loss (mg/g muscle)	WBSF (3dpm)
Final weight(kg)	-0.43*	-0.40*	0.21·	0.32	0.47*	-0.26
Shrink	-0.23	-0.34	0.19	0.46*	0.07	-0.27
Dressing (%)	-0.47*	-0.40*	-0.21·	0.25	-0.40*	-0.27
Marbling	-0.47*	-0.49*	-0.57*	0.14	-0.10	-0.03
Rib eye area (cm^2)	-0.26	-0.19	0.11	0.22	0.39*	-0.39*

*Pearson's correlations are significant based on $p < 0.0016$ corrected by Bonferroni correction

[1] Warner-Bratzler shear force.

2.6b Pearson correlation coefficients for correlations between meat quality (top) and carcass quality (bottom) and intramuscular connective tissue and collagen characteristics.

Measurements (units)	Dry perimysium (g)	Wet perimysium (g)	Insoluble collagen (3 dpm) (mg/g)	Pyridinoline (nmol/g raw meat)	Pyridinoline (mol/mol collagen)
Cook Loss (mg/g) (13dpm)	0.28	0.37*	0.28	-0.13	-0.10
Cook time (sec/g) (3dpm)	0.39*	0.46*	0.30	-0.08	-0.10
WBSF (kg) (3dpm)	-0.10	-0.05	0.18	0.40*	0.36*
WBSF (kg) (13dpm)	0.04	0.15	0.60*	0.27	0.10

Measurements (units)	Dry perimysium (g)	Wet perimysium (g)	Pyridinoline (nmol/g raw meat)	Pyridinoline (mol/mol collagen)
Final weight(kg)	0.45*	0.43*	-0.12	-0.17
Shrink	-0.07	-0.10	-0.38*	-0.12
Dressing (%)	0.47*	0.41	-0.26	-0.38*
Marbling	-0.18	-0.12	-0.07	-0.03
Rib eye area (cm^2)	0.44*	0.54*	-0.13	-0.28

*Pearson's correlations are significant at $P < 0.0016$ as corrected by Bonferroni correction.

2.6c Pearson correlation among IMCT, collagen and collagen cross-links characteristics

Measurements (units)	Total Collagen (mg/g)	Soluble collagen (3dpm)	Soluble collagen (13dpm)	Insoluble collagen (3dpm)	Insoluble collagen (13dpm)	Solubility (3dpm) (%)	Solubility (13dpm) (%)	Ehrlich chromogen (nmol/g)	Ehrlich chromogen (mol/mol)
Wet Perimysium (%)	-0.48*	-0.21	-0.34	0.08	0.53*	-0.21	-0.34	0.42*	-0.26
Total collagen (mg/g)	1.00	0.17	0.16	-0.01	0.85*	-0.36*	-0.41*	0.76*	-0.63*
Soluble collagen (mg/g) (3dpm)		1.00	0.31	-0.78*	0.05	0.82*	0.05	-0.04	0.15
Soluble collagen (mg/g) (13dpm)			1.00	-0.21	-0.38*	0.11	0.76*	0.11	-0.06
Insoluble collagen (mg/g) (3dpm)				1.00	0.10	-0.68*	-0.13	0.08	0.05
Insoluble collagen (mg/g) (13dpm)					1.00	-0.40*	-0.78*	0.66*	-0.56*
Solubility (%) (3dpm)						1.00	0.31	0.28	-0.31
Solubility (%) (13dpm)							1.00	-0.38*	0.28
Ehrlich chromogen (nmol/g raw meat)								1.00	-0.08
Ehrlich chromogen (mol/mol collagen)									1.00

*Pearson's correlations are significant based on $p < 0.0016$ corrected by Bonferroni correction

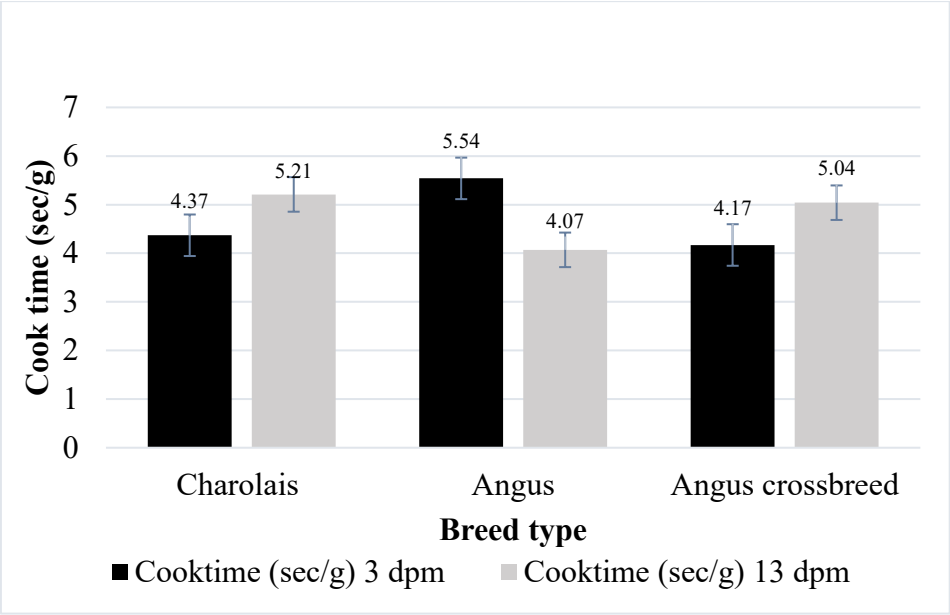

Figure 2.1 Cooking time (sec/g) of m. *triceps brachii* as affected by an interaction between breed type and post-mortem ageing. Error bars indicate the standard errors of the means.

Chapter 3: Gene expression of the intramuscular collagen characteristics biochemical mechanisms affecting the eating quality of m. *triceps brachii*.

3.1 Introduction

Identifying and understanding the biological molecules and genes that can serve as indicators to identify live animals and carcasses or meat cuts with desirable meat quality attributes especially tenderness may provide a solution to the major challenge of controlling beef quality faced by the beef industry. In particular, the industry is interested in being able to increase the eating quality and value of the overall carcass by adding value to muscles that contain high levels of intramuscular connective tissue (IMCT) (BCRC, 2018).

The application of expressional genomics with the use of quantitative real time PCR (qrt-PCR) has discovered potential genes and mechanisms related to myofibrillar proteins and the tenderness of beef (Page et al., 2002). The importance of calpain enzymes to myofibrillar protein degradation during post-mortem ageing has been well documented and the expression of calpain genes and that of calpastatin, their inhibitor, are known to be associated with beef tenderness (Smith et al., 2000; Casas et al., 2006; Page et al., 2002). Also, the expressions of genes for leptin, thyroglobulin and Dyacylglycerol acyltransferase (DGAT) in relation to marbling have been identified (Buchanan et al., 2003; Thaller et al., 2003). However, to date, there is limited application of expressional genomics to high intramuscular collagen content muscles such as the m. *triceps brachii* in relation to beef toughness.

In skeletal muscles, several genes of enzymes and proteins such as *lysyl oxidase (LOX), lysyl hydroxylase (LH), collagen types I, II, III, IV, V and VI,* insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), transforming growth factors (TGF), *SMAD*-

related proteins*, Prolyl-4-hydroxylase* (*P4HA1), integrins* and *pituitary homeobox 2 (PITX2),* are involved in the formation and regulation of collagen and collagen cross-linking (Guo and Dalrymple 2017). *Matrix metalloproteinase (MMPs)*, key enzymes responsible for the degradation of extracellular matrix such as collagen and their activities are regulated by the *tissue inhibitor matrix metalloproteinase (TIMPs)* proteins, which regulate the activities of *MMPs* (Christensen and Purslow, 2016). However, it is not known the extent to which the expression of the genes for these enzymes and proteins is associated with the synthesis and degradation of collagen or the formation of collagen cross-links in the m. *triceps brachii.* It is also not known as to whether the expression of these genes is affected by breeds and post-mortem ageing solubility rate.

The objective of this study is therefore to investigate the effect of breed type and collagen heat solubility on the expression levels of genes related to the synthesis and degradation of collagen. Another objective of this study was to relate the expression of genes related to collagen synthesis and degradation to the toughness and collagen characteristics of beef. For the purpose of this study, the m. *triceps brachii,* a muscle from the beef forequarter considered a high connective tissue muscle, was used. This study tested the hypothesis that total collagen content, collagen solubility, and meat quality attributes including Warner Bratzler shear force (WBSF), are related to the expression of genes involved in the synthesis and degradation of collagen.

3.2 Materials and Methods

3.2.1 Animal management, slaughtering and physio-chemical determination

Animal management and slaughtering, and measurement of physio-chemical properties such as proximate analysis, carcass and meat quality traits, intramuscular

connective characteristics were all performed as described in Chapter 2 Sampling of muscle for gene expression analysis occurred within 30 minutes post mortem from the carcasses of all steers. Approximately 20 g of the *triceps brachii* muscle from the base of the scapula (chuck) was removed during sampling for RNA extraction following sterile procedures and were immediately placed in a labeled Whirlpak^{TM} bag, frozen in liquid nitrogen, and stored at -80ºC for subsequent gene expression analysis. Animals to be examined further using gene expression analysis (n= 24) were selected based on breed type and the difference in collagen heat solubility between day 3 and day 13 post-mortem ageing (highest, lowest) after the completion of analyses performed in Chapter 2. Four animals with the highest and lowest collagen heat solubility were selected within each of the breed types (purebred Angus and Charolais, and Angus crossbred) (n = 8 each).

3.2.2 Ribonucleic acid (RNA) extraction and isolation from *Triceps brachii* muscle

Samples stored in -80ºC were removed and immediately kept in dry ice, and labeled tubes were also kept in liquid nitrogen. Frozen samples were ground using a mortar, pestle and spatula previously cleaned and sterilized by being wrapped with aluminium foil and baked in an oven at 250ºC for three hours. Liquid nitrogen was added to each sample during grinding and 0.1 g was put into a bead tube (Precellys, The Science of Lysing, France) and kept at -80ºC until further analysis. Extraction and isolation of RNA from ground samples were performed as described by Chomczynski and Sacchi (1987). The total RNA sample was extracted and isolated using the guanidinium thiocynanate-phenol-chloroform extraction method known as Trizol reagent by adding 1 mL to each sample (Ambion, Life Technology, USA) Samples were homogenized using a PRECEllys 24 Homogenizer (Bertin, France) at 6200 rpm for 30 seconds. Homogenization was repeated twice with 10 seconds between each

homogenization, and then incubated at room temperature (RT) for 20 minutes. Centrifugation (Eppendorf, Germany) of incubated samples was performed at 12000g at 4ºC for 10 minutes and the supernatant was transferred into a new, labeled 1.5 mL plastic tube. Chloroform (200 µL) (Fisher Scientific, Ottawa, Ontario) was added to the supernatant, mixed by vortex, incubated at RT for 2 minutes and then centrifuged at 12000g at 4ºC for 15 minutes. The top clear layer of supernatant was collected into a new, labeled tube and precipitation was performed by adding 250 µL isopropanol (Fisher Scientific, Ottawa, Ontario) and 250 µL of 1.2M sodium acetate in 0.8M sodium chloride (NaCl). The supernatant was incubated for 10 minutes and centrifuged at 12000g for 10min at 4ºC. The supernatant was removed, the pellet was washed with 1 mL of 75% ethanol to remove residue and centrifuged at 8300 rpm for 3 minutes. The pellet was left to dry after decanting the ethanol, and it was then solubilized in 100 µL of nuclease free water. Ten (10) µL of 3M sodium acetate and 250 µL of 100% ethanol were then added for re-precipitation and the extracted RNA incubated at -20ºC overnight. For washing of the isolated RNA, centrifugation was performed at 16000g for 30 minutes at 4ºC, the ethanol was discarded, 500 µL of 75% ethanol was added and centrifugation was performed at 12000g for 5 minutes at 4ºC. Washing was repeated one more time, all ethanol was then discarded, and the precipitated RNA was dried. RNA was dissolved in 50 µL of molecular grade water.

3.2.3 Evaluation of isolated RNA

The concentration, purity and quality of the extracted RNA was evaluated using a UV spectrophotometer (Nanodrop Technologies, USA) and RNA integrity was confirmed using the Agilent Tapestation (2100 Bioanalyzer, Agilent Tapestation, UK). The amount and purity of RNA were obtained based on absorbance at 260 nm and 230 nm in the ND-1000

spectrophotometer (Nanodrop Technologies, USA). This was performed by loading 1 µL of sample without dilution into the Nanodrop. After the quantification, RNA integrity number (RINe) was obtained by diluting the concentration of isolated RNA to less than 500 µL to determine RNA quality and integrity. Sample buffer (5 µL) was added to 1 µL of RNA, and mixed by vortex at 2000 rpm for 1min. The RNA was denatured by heating to 72ºC for 3 minutes, then placed on ice for 2 minutes, centrifuged and finally loaded into an Agilent Tapestation (2100 Bioanalyzer, Agilent Tapestation, UK). Bands corresponding to 18S and 28S ribosomal RNAs were obtained after capillary electrophoresis. RNA integrity was deemed adequate if a number above 7 was obtained and the range obtained in this analysis was between 6.6 and 8.4.

3.2.4 Reverse transcription and complementary DNA (cDNA) synthesis

A reaction mixture total volume of 20 µL for each of the 24 samples was prepared for reverse transcription of cDNA using iScript RT Supermix (RNase H+ Moloney murine leukemia virus (MMLV) reverse transcriptase, RNase inhibitor, dNTPs, oligo (dT), random primers, buffer, $MgCl_2$ and stabilizers) (Bio-Rad, USA). The reaction included a range of 1.7 µL to 5.6 µL template RNA, 4µl iScript RT super mix (Bio-Rad, USA) and a range of 7 - 14µL nuclease-free water. A negative control (NO-RT) was prepared using 4µl iScript NO-RT control super mix (Bio-Rad, USA) to ensure cDNA samples were not contaminated with genomic DNA. The complete reaction mix was put in ice and incubated in a thermocycler (AB Applied Biosystems, USA). In the thermocycler, priming was at 25°C for 5 minutes, reverse transcription was at 46°C for 20 minutes, reverse transcription inactivation which stops the reaction process was at 95°C for 1 minute. The cDNA was diluted 2:40 for use in quantitative real-time PCR.

3.2.5 Housekeeping/ Reference Gene for *Triceps brachii* muscle

Glyceraldehyde 3-phosphate dehydrogenase (GADPH), B-Actin and 18S ribosomal RNA were selected as reference or housekeeping genes (HKG) for normalization based on the literature.

3.2.6 Primers design for candidate genes or target genes

Primers (both forward and reverse) were designed for the twenty seven candidate genes selected to be associated with IMCT, collagen and cross-links formation and degradation, using Primer Express software (AB Applied Biosystems, USA; Table 3.1). The candidate or target genes were *COL1A1, COL3A1, COL5A1, COL6A1, LOX, LH1, FN1, IGF1, TGFB1, FGFR1, FGF2, ITGA1, ITGA11, ITTGB1, P4HA1, PITX2, MMP1, MMP2, MMP8, MMP13, SMAD2, SMAD3, SMAD4, SMAD6, SMAD7, TIMP1, and TIMP3* (Table 3.2). The coding sequences of some of the genes were obtained from the literature while those of the rest and that of *18S ribosomal RNA* (the endogenous/reference gene for normalization) were obtained from the NCBI database. The default setting of the primer express software used included 20-24 base primer length and 20-30 base probe length. The %GC was 30-80, amplicon size was set to 50-150 and melting temperature (^{TM}) for primers was 58-60°C.

3.2.7 Polymerase chain reaction (PCR) for designed primers of target genes

The cDNA for three of the samples was used as a template to test primers and each sample was diluted to 5ng/µL and 50ng/µL concentrations and a negative control (NC). For the amplification of each target gene, a master mix was made for each PCR reaction by adding 2 µL of 10XPCR buffer, followed by 0.5µL of 10mM dNTP, 1 µL of $MgCl_2$, 1µL of forward and reverse primer, and finally, the addition of 0.25 µL of the polymerase enzyme (Taq polymerase). In the PCR plate for a single reaction, 5.75 µL of master mix were

aliquoted, 14.25 µL of molecular water was added and 1 µL of template were added to each of the 5ng/µL, 50ng/µL concentration tubes and 1µL of molecular grade water was added to the NC PCR tube. The mixture was mixed by vortex and spinned. The program used in the thermocycler (Thermofisher, Applied Biosystems, USA) was as follows: preheating at 94ºC for 5mins; denaturation at 94ºC for 30 seconds; annealing at 60ºc for 30secs; extension at 72ºC for 30secs; final extension was for 7minutes at 72ºC; and cooling at 4ºC. The number of cycles programmed was 30.

3.2.7.1 Detection of amplified target genes using agarose gel electrophoresis

For 1.5% agarose gel electrophoresis, 2.7 g of agarose gel powder (Invitrogen, USA) was added to 180 mL of 1XTBE buffer (Thermofisher, Invitrogen, USA). The solution was boiled for 4 minutes and dissolved completely in a microwave for 2 minutes, cooled to 50^0C - 60^0C and 1.8 µL of SyBR green dye (Thermofisher, USA) was added. The mixed gel solution was poured into an electrophoresis assembled tray and the comb (Thermofisher, USA) was inserted to create wells and left to solidify. To monitor the migration of the samples on the gel, a 100bp ladder consisting of 4 µL of 6X loading dye (3ml of glycerol (30%), 25mg of Bromophenol blue (0.25%), 25mg of xylene cyanol (0.25%) were vortexed. Firstly, 7 µL for each sample at 5ng/µl, 50ng/µl concentration and NC were loaded into the wells of the electrophoresis chamber (Thermofisher, USA) and the 100bp ladder (Invitrogen, USA) was also loaded in the first and last well to serve as the molecular grade markers. Gel electrophoresis was run for 64 minutes at 150 volts. After the run, each gel was visualized in a 2D gel electrophoresis scanner (GE Healthcare Bios-Sciences AB, Japan).

3.2.8 Quantitative real-time PCR (qPCR) for gene expression of target genes

The qPCR was performed to determine the expression level of the target genes relative to the 18S ribosomal RNA HKG used. Three technical replicates for each sample for both target and reference gene were performed, generating an average expression to minimize technical errors. For dilution, 2 µL cDNA was added to 78 µL nuclease-free water as the template. The reaction master mix for each sample in triplicate included 5 µL Fast SYBR® Green Master Mix (Applied Biosystems), 0.5 µL each for forward and reverse primer, and 2 µL nuclease free water. Reactions were performed in 10 µL total volume for each sample, containing 2 µL cDNA and 8 µL master mix added to MicroAmp fast optical 384 well plates (Applied Biosystems, Singapore) for each of the replicates and sealed with optical adhesive film (Applied Biosystems, Singapore. The reactions were run in a Step One Plus™ Real-Time PCR System (AB Applied Biosystems, USA) where initial denaturation was at 95°C for 20 seconds, annealing at 95°C for 30 seconds, and extension and collection of data at 60°C for 30 seconds. The number of cycles for amplification was set at 40. The melting curve stage was set at 95°C for 15 seconds, 60° for 1 minute and 95°C for 15 seconds, respectively.

3.3. Statistical analysis

The general linear model (GLM) procedure of R software (Version 3.5.1) was used to fit the appropriate mixed models and data analysis was performed using a split plot design. The slaughter group within each breed type was included as a random effect. Least square means of each treatment with their residual errors were compared, with the differences significant at $P<0.05$ using Tukey's Honestly Significant Difference (HSD) test. Non-significance level was set at $P > 0.05$ for the model and interactions at $P > 0.05$ were removed.

The relative expression levels for each of the 27 candidate or target genes were calculated as the mean differences of the delta CT values (ΔCT) of the target gene and the HKG (*18S ribosomal RNA*). The general linear (GLM) procedure of R software (Version 3.5.1) was used to fit the appropriate model and data analysis was performed as a two-way ANOVA, with the effects of breed type and collagen heat solubility tested on the expression level of all the twenty seven target genes. Least squares mean of each treatment with their residual error were compared for differences using Tukey's Honestly Significant Difference (HSD) test with significance set at $P < 0.05$ for the model. Interactions at $P > 0.05$ were also removed from the model. Pearson correlation analysis was performed to calculate the correlation coefficients and determine linear relationships between carcass, meat quality, and intramuscular connective tissue characteristics and the differences in the expression level of genes involved in the synthesis and degradation of collagen. Pearson correlation analysis was performed using R software (Version 3.5.1) with the Hmisc package (Version 4.0-2), and a Bonferroni correction was used to compensate for the likelihood of Type I error. Fifty two comparisons were made, and the correlation coefficients were deemed significant at an adjusted P value of 0.001 as calculated by α/number of comparisons (0.05/52) (Mahmood et al., 2016).

3.4 Results

The primers (both forward and reverse) designed for the twenty seven candidate genes and their functions are presented in Table 3.1 and 3.2 respectively. The level of expression for each of the HKG for each breed type showed *18S ribosomal RNA* as the most stable reference gene as detected by Fast SYBR® Green Master Mix (Applied Biosystems) because it was not affected by breed type or collagen heat solubility rate.

3.4.1 Effects of breed type and collagen solubility on carcass and meat quality attributes

As presented in Table 3.3, there were no significant differences due to breed type for sarcomere length, crude protein, intramuscular fat, moisture, drip loss, ultimate pH, and WBSF ($P > 0.05$). There was an effect of breed on muscle temperature at carcass fabrication ($P < 0.05$), where Angus and Angus crossbred steer muscles had the highest mean value for temperature at fabrication compared to those of the Charolais steers ($P = 0.02$). The selection for collagen heat solubility rate post-mortem ageing had a significant effect on ultimate pH, crude protein, intramuscular fat and drip loss ($P < 0.05$). Muscles with low collagen heat solubility had a lower mean amount of crude protein compared to those with high collagen heat solubility ($P = 0.02$), and the amount of intramuscular fat present in meat was greater in high collagen solubility muscles that in low ($P = 0.03$). The mean value for ultimate pH was lower in low collagen solubility muscles compared to those that were high ($P = 0.03$). For drip loss, the mean value was greater in high collagen solubility muscles than those with low solubility ($P < 0.0001$). There were no significant differences between collagen heat solubility levels on sarcomere length, temperature at fabrication, moisture content and WBSF ($P > 0.05$).

3.4.2 Effects of breed type and collagen solubility levels on IMCT, collagen and collagen cross-link characteristics

The results as presented in Table 3.4 showed there were no significant differences between Angus, Charolais and Angus crossbred muscles for all the measurements ($P > 0.05$). Muscles with high and low collagen solubility differed with respect to soluble collagen at 3 dpm and 13 dpm, and for pyridinoline cross-link concentration in collagen ($P < 0.05$). For

soluble collagen content at 3 dpm, low solubility muscles had a greater mean value compared to high solubility muscles ($P < 0.0001$) while soluble collagen at 13 dpm was greater in high solubility muscles compared to low ($P < 0.0001$). The concentration of pyridinoline in collagen was greatest in muscles selected for low collagen solubility compared to those selected for high collagen solubility ($P = 0.01$). There were no significant differences between high and low collagen solubility muscles for dry perimysium, wet perimysium, and total collagen content, Ehrlich chromogen in raw meat and collagen, and the concentration of pyridinoline cross-links in raw meat ($P > 0.05$).

3.4.3 Effects of breed type and collagen solubility levels on gene expression

As shown in Table 3.5 , breed type had significant effects ($P < 0.05$) on the mean ΔC_T values of genes related to toughness such as collagen type I, V, and VI (*COL1A1, COL5A1* and *COL6A1*), fibroblast growth factor *(FGF*), fibroblast growth factor receptor 1 (*FGFR1*) and 2 (*FGF2*), lysyl hydroxylase (*LH1*) and lysyl oxidase (*LOX*), matrix metalloproteinases (*MMP*) 1, 8 and 13, prolyl 4-hydroxylase alpha 1 (*P4HA1*), sons of mothers against decapentaplegic (*SMAD* 2, 3, and 7), transforming growth factor (*TGFB1*), and tissue inhibitor of *MMP*s (*TIMP 1*).

The Angus muscles had greater expression levels of *COL1A1, COL5A1, FGF2, LOX,* and *MMP 1* than did the Charolais and Angus crossbred ($P < 0.05$) beef steers. The ΔC_T mean for *COL6A1, LH, MMP8, MMP13, P4HA1, SMAD2, SMAD6,* and *SMAD7* indicated greater expression of these genes in Angus and Charolais than in the Angus crossbred ($P < 0.05$) beef steers. The Angus had lower expression level of *FGFR1* and *TIMP1* than did the Charolais and Angus crossbreed ($P < 0.05$) beef steers. The *TFGB1* expression level was greater in Charolais and Angus crossbred than Angus ($P < 0.05$) beef steers. The expression level of

FN1, IGF1, TGFB1, FGFR1, FGF2, ITGA1, ITGA11, ITTGB1, PITX2, MMP2, SMAD4, and *TIMP3* did not differ among the three breeds of beef steer (P > 0.05).

Analysis of variance indicated that the selection for low and high collagen solubility after post-mortem ageing had no effect on expression levels of the target genes (P > 0.05) (Table 3.6). However, there was tendency for low collagen solubility muscles to have greater expression of *FN1* (P = 0.07)*, LH1* (P = 0.09)*, P4HA1* (P =0.08) and *MMP1,* with the p value for this comparison approaching significance (P = 0.08).

3.4.4 Interaction effects of breed type and collagen solubility on expression of *COL3A1, MMP13* and *SMAD6*

In Table 3.7, there is shown a significant interaction between breed type and collagen solubility (P < 0.05) for *COL3A1, MMP13* and *SMAD6* expressions (Figure 3.1, 3.2 and 3.2, respectively). Angus and Angus cross steer carcass *triceps brachii* muscles having low collagen heat solubility had greater expression of *COL3A1* than those with high collagen solubility, while Charolais muscles with high collagen heat solubility had greater expression of *COL3A1* (P < 0.001) (Figure 3.1). The expression of *MMP13* was high in low collagen solubility muscles compared to those with high solubility for the three breed types (P = 0.03) (Figure 3.2). The Angus steers had lower expression of *SMAD6* for low solubility muscles than high solubility muscles while Charolais and Angus crossbred steers had higher expression of *SMAD6* in low solubility muscles than in high collagen solubility muscles (P = 0.03) (Figure 3.3).

3.4.5 Pearson correlation coefficients between candidate genes

Pearson correlation among candidate genes were performed to determined co-expression among genes and relationships between gene expression and beef toughness as

measured by shear force. The coefficient results are shown in Tables 3.8.1, 3.8.2, and 3.8.3. The ΔC_T mean for candidate genes related to collagen types showed that strong positive correlations existed between expression levels for *COL1A1* and *COL5A1, COL1A1* and *FGF2, COL1A1* and *MMP 1, COL1A1* and *SMAD 6, COL1A1* and *SMAD 7* ($P < 0.0001$). The expression level of *COL5A1* was positively correlated with *MMP1* and *SMAD 7* ($P < 0.0001$). A strong positive correlation existed between *COL6A1* and *FGFR1*, *COL6A1* and *FGF2, COL6A1* and *SMAD 2*, *COL6A1* and *SMAD 6* ($P < 0.0001$).

For genes related to collagen synthesis and determination of collagen cross-link type, specifically lysyl oxidase and lysyl hydroxylase, respectively, a strong positive correlation existed between *LH1* and *MMP8, LH1* and *MMP13, LH1* and *P4HA1 LH1* and *SMAD2, LH1* and *SMAD3* ($P < 0.0001$). *LOX* had a strong positive correlation with *MMP13, SMAD2, SMAD3, MMP1, P4HA1, SMAD7* and *SMAD6* ($P < 0.05$). *LOX* had a strong positive correlation with *MMP1*, *MMP13, SMAD2, SMAD3,* and *SMAD7* ($P < 0.0001$). *P4HA1* had strong positive correlations with *FGF2, FIN1, ITGA1, LH1, SMAD2, SMAD3,* and *SMAD6* ($P < 0.0001$).

For *FGF2*, a growth factor gene, strong positive correlations existed between *FGF2* and *LH1, FGF2* and *MMP1*, *FGF2* and *MMP13, FGF2* and *P4HA1, FGF2* and *SMAD 2, FGF2* and *SMAD6, FGF2* and *SMAD3*, and *FGF2* and *SMAD7* ($P < 0.0001$). *IGF-1* had strong positive correlations with *SMAD2* and *SMAD3* ($P < 0.0001$). Correlation analysis indicated that expression of *FN1* was positively correlated with expressions of *ITGA1, ITGA11*, *ITGB1, LH1*, *LOX, MMP8, MMP13,* and *SMAD2* ($P < 0.0001$).

Expression of *SMADs* showed a strong positive correlation existed between expression levels of *SMAD2* and *SMAD3*, *SMAD2* and *SMAD7* ($P < 0.0001$). A strong positive correlation

existed between *SMAD3* and *SMAD7* ($P < 0.0001$). Expression of *SMAD6* had a strong positive correlation with that of *SMAD7*, and a strong negative correlation with expression of *TIMP3* ($P < 0.0001$).

The ΔC_T mean of target genes related to integrins showed strong positive correlations existed between *ITGA1* and *ITGA11*, *ITGA1* and *ITGB1*, *ITGA1* and *LH1, ITGA1* and *MMP8, ITGA1* and *MMP13, ITGA1* and *P4HA1, ITGA1* and *SMAD2* ($P < 0.0001$). *ITGB1* had a strong positive correlation with *TIMP3* ($P < 0.0001$).

The expression levels of target genes related to collagen turnover, specifically *MMP's,* a strong positive correlation existed between *MMP1* and *SMAD3, MMP1* and *SMAD6, MMP1* and *SMAD7* ($P < 0.0001$). *MMP8* had a strong positive correlation with *MMP13*, *P4HA1, SMAD2, SMAD3*, ($P < 0.0001$). A strong positive correlation existed between *MMP13* and *FGF2, MMP13* and *FIN1, MMP13* and *ITGA1, MMP13* and *LH1, MMP13* and *LOX, MMP13* and *SMAD2, MMP13* and *SMAD3, MMP13* and *SMAD6, MMP13* and *SMAD7, MMP13* and *P4HA1* ($P < 0.0001$).

3.4.6 Pearson correlation coefficients between genes and meat quality, IMCT, and collagen characteristics

Tables 3.9 and 3.10 show the results of the Pearson correlation analysis between candidate genes for meat quality, IMCT and collagen measurements. The results obtained were not significant at $P < 0.001$; therefore, results significant at $P < 0.05$ will be discussed. For the expression levels of genes related to collagen types on all the measurements, a positive correlation existed between *COL1A1* and drip loss, *COL1A1* and cooking time at 13 dpm ($P < 0.05$). *COL3A1* had a negative correlation with Ehrlich's chromogen concentration in collagen ($P < 0.05$). *COL6A1* had a positive correlation with Ehrlich's chromogen

concentration in collagen and a negative correlation with protein content, and total collagen ($P < 0.05$).

The expression levels of target genes related to growth factors on all the measurements showed *IGF1* had a slight positive correlation with sarcomere length ($P < 0.05$). *FGFR1* was positively correlated with temperature, moisture content, WBSF at 3 dpm ($P < 0.05$), and had negative correlations with protein, and soluble collagen at 13 dpm ($P < 0.05$). *FGF2* had a positive correlation with Ehrlich's chromogen concentration in collagen ($P < 0.05$), and a negative correlation with total collagen, and Ehrlich's chromogen concentration in raw meat ($P < 0.05$).

The expression level of *LH1* was positively correlated with ultimate pH at fabrication and pyridinoline in raw meat and negatively correlated with total collagen ($P < 0.05$). Expression of *LOX* had a positive correlation with drip loss ($P < 0.05$). The expression level of *P4HA1* was positively correlated with ultimate pH at fabrication, and negatively correlated with fat and Ehrlich's chromogen concentration in raw muscle ($P < 0.05$).

The ΔC_T mean of *SMADs* genes showed *SMAD2* had a negative correlation with total collagen content, and with soluble collagen at 3 dpm ($P < 0.05$). *SMAD3* had a slight positive correlation with sarcomere length ($P < 0.05$), and a negative correlation with total collagen ($P < 0.05$). *SMAD4* was positively correlated with the amount of dry perimysium ($P < 0.05$). *SMAD6* had a negative correlation with total collagen, Ehrlich's chromogen in raw meat, and Ehrlich's chromogen in collagen ($P < 0.05$). *SMAD7* was positively correlated with cooking time at 13 dpm ($P < 0.05$).

The expression levels of target genes related to integrins on all measurement showed *ITGA1* was positively correlated with ultimate pH at fabrication, WBSF at 3 dpm, and pyridinoline in raw meat ($P < 0.05$), and had negative correlations with total collagen and Ehrlich's chromogen in raw meat ($P < 0.05$). *ITGB1* was positively correlated with pyridinoline in collagen ($P < 0.05$).

For the expression levels of genes related to matrix mettalloproteinases, a negative correlation existed between *MMP2* and moisture content ($P < 0.05$), and a positive correlation between *MMP2* and protein content ($P < 0.05$). *MMP8* had a negative correlation with Ehrlich's chromogen in raw meat ($P < 0.05$). *MMP13* was positively correlated with ultimate pH at fabrication and wet perimysium ($P < 0.05$). *MMP 13* had a negative correlation with total, and Ehrlich's chromogen in raw meat ($P < 0.05$). For expression of genes related to *MMPs* tissue inhibitor all the measurements, *TIMP1* was positively correlated with moisture ($P < 0.05$), and a negative correlation with Ehrlich's chromogen in raw meat ($P < 0.05$).

3.5 Discussion

It is well known that breeds of cattle differ in their carcass characteristics due to the differences in their physiology and genotypes; however, in this study breed had no effect on any meat quality and connective tissue characteristics except for muscle temperature at fabrication (3.3 and 3.4). The fact that the different beef steers were raised to the same intramuscular fats and physiological age could have minimized the effect of breed on meat quality and collagen measurements. Breed differences reported in several studies are often confounded with differences in fatness level (Hocquette et al., 2005). Hence, some meat scientists compare beef quality attributes from steers of different breeds raised to same intramuscular fat level. Under this condition, Chambaz et al. (2003) reported Angus and

Charolais both had pale meat with low haem iron content and similar flavour, but that Angus and Limousin produced beef that was more tender. No significant differences in eating quality were observed in another study comparing Limousin and Charolais (Hocquette et al., 2005). Golze et al. (2002) found no differences in intramuscular pH value between Limousin, Simmental and Angus cattle. Also, there were no significant differences regarding water content, water holding capacity and cooking loss in beef from Angus, Charolais, Hereford and Simmental cattle when the cattle were fed the same high energy forage and raised under the same housing condition (Jukna et al., 2017). In this thesis, Angus and Angus crossbred steers had the highest mean value for temperature at fabrication compared to Charolais. As mentioned in Chapter 2, the differences in breed type on temperature at fabrication might be a result of variation in carcass warming rate due to differences in carcass weight (Pike et al. 1993). The absence of significant differences in intramuscular connective tissue characteristics among breed types may be as a result of the type of muscle used in this study, as it is one that is heavily involved in locomotion. Several authors suggested that differences in quality traits between or among breeds may be overridden by the type of muscle or cut (Wheeler 1996; Dransfield et al., 2003).

Among the twenty seven genes expressed, sixteen genes were differentially expressed among Angus, Charolais and Angus cross breed ($P < 0.05$) (Table 3.5). The value of ΔC_T mean is inversely proportional to the amplicons, thus, higher ΔC_T value indicates reduced amplification and vice versa (Livak et al. 2001; Bieche et al. 1998). Intriguingly, *COL1A1, COL5A1, COL6A1, FGF2, LOX, LH, P4HA1, SMAD2, SMAD3, SMAD 2, SMAD 6,* and *SMAD 7, P4HA1* genes involved in the synthesis or regulation of collagen and the formation of its cross-links were significantly up-regulated in Angus beef steers, a breed known for its

superior marbling and tenderness. However, Lim et al. (2016) in their study reported that *COL1A1, COL1A2, COL5A1, COL14A1* and *COL15A1* were up-regulated in high intramuscular fat pigs. Also, in a previous study, the total amount of collagen was found to be positively correlated with intramuscular fat of the longissimus muscle (Suzuki et al., 2005). It has been suggested by several studies that collagen type 1 predominates in the perimysium (Light et al., 1984; Bailey 1979) and Wood & Fisher (1990) reported that intramuscular fat is deposited in perimysium rather than in endomysium. The expression of *COL5A1,* a fibril forming collagen, in the Angus breed was expected because this gene is associated with type I and III collagen types (Fitch 1984; Leeming and Karsdal, 2016). Gillies & Lieber (2011) and Fitch et al. (1984) suggested that *COL5A1* may be essential for type I collagen fibril formation in perimysium. Although no data existed on the effect of *COL5A1* on *COL1A1* fibril pattern or tensile strength, this study shows that *COL1A1* may be associated and up-regulated along with *COL5A1.* The mRNA expression levels of *COL1A1* and *COL5A1* might be also dependent on the age of the animal and muscle type, not only on the breed type (Liao et al., 2018; Listrat et al., 1999). Liao et al. (2018) in their study observed the expression level of *COL1A1* to be greater in the *longissimus* muscle of cattle at 3 and 24 months and lower in that of cattle below 12 and at 30 months (Liao et al., 2018). At 110 days of fetal age in different bovine muscles, *COL5A1* was not visible, but became more visible in the perimysium from 180 days fetal development and hence an increase in *COL1A1* was observed. Although muscle basement membrane primarily consists of *COL4A1*, *COL6A1* is also found to be present (Halfter et al., 1998; Myers, 1996; Marvulli et al., 1996; Nishimura et al., 1997) and this agreed with the results obtained. Hence, the expression of *COL6A1* in the Angus TB muscle may indicate the re-modelling of collagen during skeletal muscle development in the basement membrane of these steers at the time of slaughter.

There are limited studies on the effect of *FGF2, LOX, LH1, P4HA1, SMAD2,* and *SMAD3* on meat, although they may be involved in the toughness of beef because they are associated with the synthesis of collagen and the formation of collagen crosslinks (Barendse, 1997; 2002). In the development of normal skeletal muscle there is a turnover which creates a balance between enzymes responsible for collagen synthesis and its degradation (Gillies and Lieber, 2016). Essén-Gustavsson et al. (1994) concluded that increased tenderness in high intramuscular fat muscle could be caused by the presence of a weakening catalyst that degrades the collagen. Their conclusion is supported by the results of this thesis because *MMP1*, *MMP8* and *MMP13* genes were highly expressed in the Angus breed, with mean *MMP8* and *MMP13* expression being similar to that in the muscles of the Charolais. *MMP1, MMP8* and *MMP13* are collagenases associated with degradation of collagen types I, II and III, respectively (Singh et al., 2000; Wu et al., 2003 and Ohuchi et al., 1997). *MMP1* has been observed to be increased in muscles with increased fat content (Qi et al., 2016). Several studies reported that the Angus breed has increased collagen content but increased heat solubility as well; hence, a lower WBSF value is obtained when compared with other breeds (Chambaz et al., 2003; Elzo et al., 2012; Leal-Gutierrez et al., 2018; Joel et al., 2019). There was no difference in intramuscular fat level with breed type however in this thesis, and muscles with decreased development of collagen heat solubility with ageing were associated with increased intramuscular fat. Those with decreased development of collagen heat solubility with post-mortem ageing also had increased soluble collagen at day 3 post-mortem, suggesting that there may be a high level of newly synthesized collagen that is not yet firmly crosslinked as suggested by Purslow (2018) in steers with increased intramuscular fat. This was not supported by Pearson correlation coefficients however, as there was a negative correlation between *P4HA1* and intramuscular fat, suggesting that collagen synthesis is

decreased not increased in muscle with increased intramuscular fat. Further research on this relationship is necessary to understand the interaction between intramuscular fat and the contribution of collagen to beef toughness.

The expressions of *COL6A1, FGFR1, FGF2, P4HA1, SMAD 2, LH1, MMP8,* and *MMP13* were up-regulated in both Charolais and Angus beef steers. In this thesis, all steers were raised to approximately the same back fat thickness and physiological age, and this resulted in an approximately equal amount of intramuscular fat being deposited in the muscle of all the breeds at slaughter. That these genes were upregulated, particularly *COL6A1*, suggests that collagen was actively being remodelled in the endomysium of these breeds at slaughter.

Furthermore, the specific types of collagen formed may be highly dependent on the genes associated with collagen synthesis and regulation. In the Angus, results indicated that *COL1A1, COL5A1,* and *COL6A1* were up-regulated along with *FGF2, LOX, LH, P4HA1, SMAD2,* and *SMAD3,* while in the Charolais *COL6A1* appeared to be specifically up-regulated along with *FGFR1, FGF2, P4HA1, SMAD2,* and *LH*. From these results, the genes not expressed in the Charolais for the formation of *COL1A1, COL5A1* were *FGFR1, LOX* and *SMAD3,* while the genes expressed in both breeds that could be associated with synthesis of *COL6A1* were *LH, P4HA1* and *SMAD 2*. Also, *SMAD6* and *SMAD7* were both associated with the genes expressed in Angus and Charolais in this thesis. These *SMADs* have been reported to inhibit the expression of *SMAD2* and *SMAD3* which bind to *SMAD4* (Suwanabol et al., 2011) and translocate into the nucleus to activate the expression of fibrogenic genes including procollagen and enzymes catalyzing collagen cross-linking (Massague & Chen, 2000). This may explain the increase in the expression level of *MMPs* as the genes were not

affected directly or indirectly by the *SMADs* related protein inhibitor. It is therefore important to understand the relationships between all the genes and the genes associated with increased collagen cross-links and beef toughness.

In the Charolais, *TGFβ* was up-regulated but in Angus muscle it was down-regulated ($P < 0.05$). TGFβ induces fibroblasts to produce fibronectin and collagen type I (Gillies & Lieber 2011) and Montaseri et al. (2011) showed that TGFβ and IGF-1 suppress the activity of *MMP* during interleukin induction (Huang et al., 2011). TGFβ isoforms are activated by the activities of Sma and Mad related proteins (Letterio & Roberts, 1998). The expression level of *TGFβ* mRNA has been related to increased collagen content and pyridinoline cross-links in the skeletal muscle of lambs (Huang et al., 2010). With expression levels of *MMP13* and *MMP8* similar to that of the Angus, it would be expected that Charolais should produce beef as tender as that of the Angus when raised to the same intramuscular fat, and there was indeed no difference in WBSF. The Angus crossbred also known as Kinsella composite had lower expression of the candidate genes discussed than the other two breeds including *MMP's* and *TIMP's,* which indicated that this breed might have reduced background toughness compared to Charolais, and sensory evaluation would be needed to verify this as WBSF did not support this hypothesis.

As mentioned earlier, four beef steers from each of the breeds were categorized into low and high collagen heat solubility levels for the *triceps brachii muscle* based on the increase in collagen solubility with post-mortem ageing between 3 and 13 dpm. Cross et al. (1973) reported that beef tenderness was related to the percentage of collagen solubility rather than the total amount of collagen. Several studies also concluded that the quality of collagen cross-links and collagen heat solubility were important to tenderness rather than the type or

amount of IMCT or the collagen type formed (Burson et al., 1986; Bailey, 1985; Jeremiah, 2003). However, in this thesis, the target genes were not differentially expressed in the *triceps brachii* muscle due to collagen solubility levels ($P > 0.05$) (Table 3.6), but there was tendency for *triceps brachii* muscles with a low increase in collagen solubility with post mortem ageing to have greater expression of *FN1* ($P = 0.07$), *LH1* ($P = 0.09$), *P4HA1* (0.08), and *MMP1* (0.08), although not significant ($P > 0.05$). This trend might be as a result of the small sample size of animals used, and further study using large population might be necessary for proper validation.

COL3A1 in *triceps brachii* was lowly expressed in low collagen solubility Charolais muscles and greatly expressed in high solubility Charolais muscles, and this is not consistent with the result obtained for Angus and Angus crossbred. It would be expected that *COL3A1,* which is among the most abundant collagen types present in skeletal muscles would be more expressed in low collagen heat solubility muscles in Charolais breed which was the case for the Angus and Angus crossbred. However, *COL3A1* may not be associated with *triceps brachii* beef toughness compared to *COL1A1,* which is predominant in perimysium, as *COL3A1* is distributed between endomysium and epimysium (Light & Champion, 1984). The perimysium network has been found to be the major IMCT contributing to the level of difficulty in breaking cooked beef apart (Torrescano et al., 2003). Hence, *triceps brachii* muscle may consist of more perimysium containing low amounts of *COL3A1.* According to Burson & Hunt (2006), *COL3A1* was only present in *longissimus dorsi* muscle and not in muscles from the chuck such as *biceps femoris*. However, the presence of *COL3A1,* in *triceps brachii* muscle does not appear to be associated with collagen heat solubility because of the lower expression level for it observed in muscles with increased heat solubility with post-mortem ageing.

The expression level of *MMP13* in *triceps brachii* muscle was increased in low solubility muscles compared to those with high solubility in the Angus crossbred only (Figure 3.2). This suggests that *MMP13* might not be associated with increased collagen solubility and tenderness of *triceps brachii* muscle in this thesis. The results might be however, because of the presence of *TIMP1* which acts on *MMP13* and inhibits its activities. *TIMP1* is an inhibitor of matrix degradation which tightly binds to the active *MMP13* hence downregulating its activity in collagen degradation (Li et al., 2010; Dzwonek et al., 2004; Lambert et al., 2004).

The Angus steers had decreased expression of *SMAD6* for low solubility muscles compared to high solubility muscles while Charolais and Angus crossbreds had increased expression of *SMAD 6* for low solubility muscles compared to high collagen solubility muscles ($P = 0.03$) (Figure 3.3). Results suggest that *SMAD 6* might not be related to solubility of muscle collagen between the three breed types. The results from the interaction of the three genes suggest they might not be biological related to collagen heat solubility and toughness.

Pearson correlations supported the conclusion that skeletal muscle at the time of slaughter was undergoing remodelling. There was co-expression of *COL5A1* and *COL1A1*, and this is not unexpected as type V collagen is essential for interstitial type I collagen fibril formation (Leeming & Karsdal, 2016). Both genes appeared to be related to remodeling of the extracellular matrix, as their expressions were up-regulated along with that of *FGF2.* Previous studies reported that FGF2 induced the secretion of collagen types, especially type I collagen (Leung et al., 2000; Kay et al., 1982), and Leeming & Karsdal (2016) detected the secretion of type V collagen during control fibroblast secretion prompted by FGF2, supporting the

linear relationship observed between them. *COL6A1* expression was up-regulated along with that of *FGFR1* and *FGF2,* and this is not unexpected given that Type VI collagen binds with FGF and fibronectin (Carter 1982). In comparison with other collagen genes, *COL1A1* and *COL6A1* may be the most significant and important collagen types related to beef toughness because in this thesis, higher expression of *COL1A1* was related to decreased drip loss and cooking time at 13 dpm, which could positively affect cooked beef tenderness. Also, higher expression of *COL6A1* was related to decreased concentrations of Ehrlich chromogen cross-links in the m. *triceps brachii* muscle. As mentioned earlier, *COL1A1* is the most abundant collagen types and recently type VI collagen has been recognized to be the most abundant collagen in the lungs (Burgstaller et al., 2017). Specks et al. (1995) reported it was increased in fibrotic lung and Lennon et al. (2014) found that it was abundant in the renal basement membrane. Hence, increased synthesis of types I and VI collagens in skeletal muscle do not necessarily decrease the eating quality of the m. *triceps brachii.*

For genes involved in collagen turnover or degradation, collagenase enzymes; *MMP1, MMP8* and *MMP13* were co-expressed with *COL1A1, COL5A1, FIN1, FGF2, ITGA11, LH1, SMAD2, SMAD4, SMAD3, P4HA1* and *LOX* and no relationship were observed with WBSF value. However, as the amount of total amount of collagen in *triceps brachii* increased, the expressions of *MMP8*, *MMP13, LH1, P4HA1, SMAD2* and *SMAD6* were higher. Also, higher expression of *MMP8*, *MMP13, LH1, P4HA1,* and *SMAD6* was associated with high amounts of Ehrlich chromogen in raw meat. These results support the hypothesis that the formation of Ehrlich chromogen is controlled by lysyl hydroxylase 1 and, because its concentration in muscle was linearly related to that of total collagen, it may be the 'default' cross-link of type I collagen. These hypotheses are supported by the correlations indicating that pyridinoline concentrations in the *triceps brachii* decreased as the expression of *LH1* increased. Lysyl

hydroxylase 1 hydroxylates lysine residues in the triple helix of the collagen molecule, while lysyl hydroxylase 2 hydroxylates lysines in the telopeptide regions and is linked to the formation of pyridinoline (Gjaltema and Bank 2017). These results indicate then that the proportion of Ehrlich's chromogen in bovine muscle may be controlled by the relative expression of *LH1* and *LH2*. If this is truly the case, then upregulation of LH1 may decrease pyridinoline content and potentially decrease beef toughness.

Correlations also indicated that expression of *ITGA1* and *ITGB1* may also be involved in the formation of pyridinoline, but how they are involved is not clear and was not further investigated. In this thesis, up-regulation of *FGFR1* and *ITGA1* expression was related to decreased beef toughness early post-mortem. Kanematsu et al. (2004) observed an interaction between collagen type I and *FGF*, but what this meant for meat quality was not reported. The expression level of *ITGA1* in *longissimus thoracis* was reported to increase in steers compared to bulls (Seung, 2018), although the study reported that an increase in the mRNA level of *ITGA1* might suppress or reduce *COL1A1* in steers. This result, however, shows that higher expression of *FGFR1* and *ITGA1* in steers might be associated with reduced toughness of *triceps brachii* muscle that has not had prolonged post-mortem ageing. The type of muscle might also be the reason for this result; hence, further study is needed to fully understand the function of integrins in relation to beef toughness among different muscles.

3.6 Conclusion

Angus beef steers had high mean expression of genes associated with collagen synthesis, however, they also had higher expression of *MMPs* genes and lower expression of *TIMPs* genes, which could explain the increased tenderness and overall satisfactory eating quality associated with beef from this breed. Gene expression results suggest that type I and III collagen-binding integrins and fibroblast growth factors may be important genes to consider for improved beef tenderness, and that upregulation of LH1 may decrease pyridinoline content and potentially decrease beef toughness. Low expression levels of *FGFR1* and *ITGA1* were also related to increased beef toughness in the m. *triceps brachii,* with low expression level of *ITGA1* associated with increased concentrations of pyridinoline. These latter relationships warrant further research to understand the involvement of integrins with the formation of pyridinoline, which appears to be the only known mature collagen crosslink that is related to increased beef toughness in the m. *triceps brachii.* Reduced background toughness of the m. *triceps brachhii* seems to depend upon the down-regulation of *FGFR1, ITGA1,* and *TIMP* and the up-regulation of *MMPs*.

3.7 Tables and Figures

Table 3.1 Forward and reverse primers for candidate/target genes primers.

Gene name	GenBank accession	Sequence (5'- 3')	Tm ^{O}C	Annealing ^{O}C	Amplicon size bp
COL1A1	NM_001034039.2	Forward CGAGGAAATGATGGTGCGAC Reverse CTTCACCCTTAGCACCCACAG	59.4 60.6	55	100
COL3A1	NM_001076831.1	Forward ATGTTGTGCAGTTTGCCCAC Reverse AGGACCAGGATCGCCATTTC	59.9 59.8	55	127
COL5A1	XM_024999726.1	Forward CGCCTCCCACAGTGTAACG Reverse GCCTCAATTCAGTTCTTGCAAA	60.0 60.1	60	98
COL6A1	NM_001143865.2	Forward CGACTGCGCCATCAAGAAG Reverse CCGTCAGTCACCACAACCAA	59.3 60.5	60	90
LOX	NM_173932.4	Forward ACACACACAGGGCTTGAGTC Reverse TCAGGCACCAAATAGCTGGG	60.2 60.0	55	138
FN1	NM_001163778.1	Forward TCAGAGACGGGCAAGAGAGA Reverse AGTAATGTCGGGAGTGGTGC	60.0 59.8	55	146
TGFB1	NM_001166068.1	Forward CTGACCCGCAGAGAGGAAATA Reverse GGTTCATGCCGTGAATGGTG	59.2 59.8	55	142
FGFR1	NM_001110207.1	Forward GGCAGTGACACCACCTACTT Reverse AGCCACGGGGTTTGGTTTG	59.6 61.1	55	129
FGF2	NM_174056.4	Forward CCACTTCAAGGACCCCAAGC	60.9		

		Reverse GTAGTTTGATGTGTGGGTCGC	59.5	55	116
ITGA1	XM_005221521.4	Forward CACCAACCCAAAAGGAGGGT Reverse TGGGGCTGACATCAGAACAG	60.1 60.0	55	101
ITGA11	XM_002690525.6	Forward GCCTACAGCACCGTCCTAAA Reverse TCGATGCTGCCATCTGAGTC	59.8 59.9	55	89
ITGB1	NM_174368.3	Forward GCCTTGCATTGCTGCTGATT Reverse CAGTTGTCACAGCACTCTTG	60.1 57.0	55	138
MMP2	NM_174745.2	Forward TGATGGCGCCCATTTATACC Reverse GCCGGTGCCAGTATCAATGT	58.1 60.8	60	110
MMP8	XM_024975688.1	Forward TTTCCTGTTGCTGCCCATGA Reverse ATGCAGTGAGTAGCTGCTGG	60.2 60.1	60	117
MMP9	NM_174744.2	Forward CCATTAGCACGCACGACATC Reverse GAGGTCGAAGGTCACGTAGC	59.7 60.2	55	131
MMP13	NM_174389.2	Forward TTGTTGGTCTCTGCCCCTTC Reverse AATCACAGAGCTTGCTGCAG	59.9 58.8	55	148
TIMP1	NM_174471.3	Forward GATGTCGTCATCAGGGCCAA Reverse GGGTGTAGATGAACCGGATG	60.1 57.8	55	145
TIMP3	NM_174472.4	Forward TCTGGCAACGACATCTACGG Reverse	59.8	60	151

		TTCCTCCAATGTCCAGCGAG	59.8		
IGF1	NM_001077828.1	Forward CCATCTCCCTGGATTTCTTTTTG Reverse GAAGAGATGCGAGGAGGATGTG	57.7 60.6	60	177
LH1(PLOD1)	NM_174148.1	Forward TCCACTACCCCCAAAAACGG Reverse GGCATCCACGCTGAAGTAGT	59.9 60.1	60	218
P4HA1	NM_001075770.1	Forward GGACTGTTTTGAGTTGGGCAAA Reverse CGGTAGAAACCTCGCCTTCA	59.8 59.8	60	110
PITX2	NM_001097991	Forward CCGAAGACCCGTCCAAGAA Reverse GCTGCATAAGCCCGTTGAAC	59.3 60.2	60	270
SMAD2	NM_001046218.1	Forward GGAACTGCCCCCTCTGGAT Reverse ATCCAGGAGGTGGCGTTTCT	61.0 61.2	60	110
SMAD3	NM_001205805.1	Forward TGAAGCGAAGTTTGGGCGG Reverse GCAGGATGGACGACATGGTT	61.3 61.4	60	136
SMAD4	NM_001076209.1	Forward CCCCATCCCGGACATTACT Reverse CGATCTCCTCCAGAAGGGTCTA	58.5 60.2	60	200
SMAD6	NM_001206145.1	Forward CCTGGGACCTGAGACAGAGTTG Reverse CTTCCTTCTTACTCCCTGCAAAAA	61.99 59.17	60	130
SMAD7	NM_001192865.1	Forward GGCATTCCTCGGAAGTCAAGA Reverse CATCTGGACAGTCTGCTGTGGATT	60.1 60.3	55	189

Table 3.2 Collagen characteristics candidate genes and their functions.

Gene	Gene name	Gene product functions	References
COL1A1, COL3A1, COL5A1, COL6A1	Collagen type I, III, V, V1 (Alpha I chain)	Provide strength and support in the muscle	Ricard-Blum & Ruggiero (2005).
LH1(PLOD1)	Lysyl hydrolyse also known as procollagen lysine-2-oxoglurate 5 dioxygenase	For collagen and cross-links synthesis inside of the cell during post translational modification	Myllyharju & Kivirikko (2004).
LOX	Lysyl oxidase	Initiating and regulating covalent cross-linking of collagen	Huang et al. (2012).
P4HA1	Prolyl-4-hydroxylase	Controls hydoxyproline formation and proper folding of newly synthesized procollagen chains	Myllyharju (2003); Pajunen et al. (1990).
IGF1, FGFR1, FGF2, TGFB1	Insulin growth factor, fibroblast growth factors and Transforming growth factor	Controls fibrogenic proliferation and differentiation. Aid stimulation of connective tissue synthesis	Seung (2018).
FN1	Fibronectin	Binding of extracellular matrix like collagens	Frantz et al. (2010).
ITGA1, ITGA11, ITGB1	Integrins	Receptor for cell adhesion to collagen types	Hynes (2002); Popova (2007).
PITX2	Paired-like homeodomain transcription factor 2	Acts as transcription factor and regulator of procollagen lysyl hydroxylase gene	Logan et al. (1998).
SMAD2, SMAD3,SMAD4	*SMAD* related proteins	Activates the expression of procollagen and enzymes catalyzing collagen cross-linking	Massague & Chen (2000); Suwanabol et al. (2011).
SMAD6, SMAD7	*SMAD* related proteins	Acts as inhibitors of *SMAD* related proteins	Moustakas et al. (2001).
MMP2, MMP8, MMP9, MMP13	Matrix metalloproteinases	Responsible for degradation of collagen	Christensen & Purslow (2016).
TIMP1, TIMP2, TIMP3	Tissue inhibitor metalloproteinases	Inhibits and controls the activities of *MMP*s	Visse & Nagase (2003).

Table 3.3 Effects of breed type and collagen heat solubility level on least squares means for meat quality characteristics of the m. *triceps brachii.*

Measurement	Breed				Solubility			P value[3]	
	Angus	Charolais	ANC[1]	SEM[2]	Low	High	SEM[2]	Breed	Solubility
Sarcomere length (μm)	2.30	2.15	2.40	0.14	2.25	2.34	0.09	0.47	0.92
Ultimate pH	5.55	5.53	5.58	0.01	5.52[d]	5.58[c]	0.01	0.25	0.03
Temperature (^{O}C)	2.92[a]	1.37[b]	3.16[a]	0.54	2.64	2.32	0.54	0.02	0.07
Moisture (%)	74.89	73.97	75.08	0.31	74.46	74.83	0.21	0.21	0.10
Crude protein (cm^2)	19.47	20.31	19.57	0.12	19.60[b]	19.96[a]	0.10	0.07	0.02
Intramuscular Fat (%)	3.87	4.15	2.90	0.38	4.00[a]	3.28[b]	0.26	0.28	0.03
Drip loss (mg/g muscle)	29.32	45.33	37.39	2.54	34.71[b]	39.98[a]	1.96	0.07	<0.0001
WBSF[4] 3dpm[5] (kg)	4.29	3.42	3.97	0.20	3.29	3.88	3.90	0.16	0.90
WBSF[4] 13dpm[5] (kg)	3.46	3.04	3.33	0.17	3.29	3.29	3.27	0.37	0.92

[a, b] Least squares means with different letters within a row and treatment differ at $P \leq 0.05$.
[1]ANC: Angus crossbred.
[2]SEM: Standard error of the mean.
[3]P value: Probability of the F test, with significance at $P < 0.05$.
[4]WBSF: Warner-Bratzler shear force.
[5]dpm: Days post-mortem ageing.

Table 3.4 Effects of breed type and collagen heat solubility level on least squares means for intramuscular connective tissue and collagen characteristics of the m. *triceps brachii.*

Measurement	Breed				Solubility			P value[3]	
	Angus	Charolais	ANC[1]	SEM[2]	Low	High	SEM[2]	Breed	Solubility
Perimysium dry weight (%)	3.55	2.24	8.50	1.09	4.16	5.36	0.90	0.10	0.35
Perimysium wet weight (%)	15.57	19.52	12.78	3.35	17.36[c]	14.55[d]	1.97	0.55	0.01
Total collagen (mg/g)	7.33	7.16	5.02	0.60	6.86	6.12	0.49	0.18	0.32
Soluble collagen 3 dpm[4] (mg/g muscle)	1.34	1.88	1.44	0.27	2.05 [c]	1.01 [d]	0.19	0.48	<0.0001
Soluble collagen 13 dpm[4] (mg/g muscle)	1.77	2.72	1.65	0.49	1.23 [d]	2.87 [c]	0.44	0.30	<0.0001
Ehrlich's chromogen (mol/mol collagen)	0.32	0.37	0.37	0.04	0.34	0.36	0.03	0.55	0.55
Ehrlich's chromogen (nmol/g muscle)	7.39	7.80	5.85	0.60	7.27	6.76	0.43	0.27	0.38
Pyridinoline (mol/mol collagen)	0.16	0.12	0.15	0.32	0.71[c]	0.12[d]	0.01	0.07	0.01
Pyridinoline (nmol/g muscle)	1.86	1.31	1.25	0.28	1.31	1.64	0.26	0.21	0.14

[c, d] Least squares means with different letters within a row within a treatment differ at $P \leq 0.05$.
[1]ANC: Angus crossbred
[2]SEM: Standard error of the mean.
[3]P Value: Probability of the F test, with significance at $P < 0.05$.
[4]dpm: days post-mortem ageing.

Table 3.5 Effects of breed type on least squares means (± standard error of the mean) of expression levels of 24 genes (ΔCT) related to collagen characteristics synthesis and turnover.

Gene ($\Delta CT\ mean$)	Angus (n = 8)	Charolais (n = 8)	Angus crossbred (n = 8)	P value[1]
Collagen synthesis				
COL1A1	4.50[a]	8.50[b]	11.28[c]	<0.0001
	(0.66)	(0.66)	(0.66)	
COL5A1	9.76[a]	12.62[b]	13.33[b]	<0.0001
	(0.54)	(0.54)	(0.54)	
COL6A1	8.21[ab]	7.61[a]	9.38[b]	0.02
	(0.42)	(0.42)	(0.42)	
FGFR1	12.97[ab]	11.39[a]	13.16[b]	0.03
	(0.50)	(0.50)	(0.50)	
FGF2	7.77[a]	8.74[a]	11.85[b]	<0.0001
	(0.55)	(0.55)	(0.55)	
FN1	6.66	6.39	7.02	0.57
	(0.42)	(0.42)	(0.42)	
IGF1	9.00	10.58	11.08	0.33
	(1.01)	(0.93)	(0.93)	
ITGA1	8.99	8.81	9.75	0.16
	(0.38)	(0.35)	(0.35)	
ITGA11	11.66	11.40	12.17	0.37
	(0.38)	(0.38)	(0.38)	
ITGB1	6.42	5.35	5.94	0.57
	(0.70)	(0.70)	(0.70)	
LH1	5.64[a]	5.84[a]	8.10[b]	0.01
	(0.57)	(0.57)	(0.57)	
LOX	7.18[a]	8.58[ab]	8.85[b]	0.02
	(0.41)	(0.41)	(0.41)	
P4HA1	7.03[a]	7.86[a]	10.27[b]	<0.0001
	(0.54)	(0.54)	(0.54)	

PITX2	14.53	13.55	15.41	0.24
	(0.88)	(0.78)	(0.88)	
SMAD2	3.92[a]	3.97[ab]	6.07[b]	0.03
	(0.58)	(0.58)	(0.58)	
SMAD3	9.66[a]	10.24[ab]	12.39[b]	0.03
	(0.68)	(0.68)	(0.68)	
SMAD4	9.15	9.14	9.58	0.61
	(0.41)	(0.38)	(0.38)	
SMAD7	9.95[a]	14.03[b]	17.81[c]	<0.0001
	(0.66)	(0.67)	(0.67)	
TFGB1	13.34[b]	10.90[a]	12.29[ab]	0.01
	(0.50)	(0.50)	(0.50)	
Collagen degradation				
MMP 1	2.87[a]	7.98[b]	10.94[c]	<0.0001
	(0.78)	(0.78)	(0.78)	
MMP 2	9.26	10.43	8.86	0.30
	(0.72)	(0.72)	(0.72)	
MMP 8	8.03[a]	8.55[ab]	10.58[b]	0.04
	(0.68)	(0.68)	(0.68)	
TIMP1	10.43[ab]	9.33[a]	11.12[b]	0.01
	(0.33)	(0.33)	(0.33)	
TIMP3	8.91	7.83	7.22	0.12
	(0.56)	(0.56)	(0.56)	

Low ΔCT mean values indicate higher expression levels while high ΔCT mean value indicate low expression levels

[a, b] Least squares means with different letters within a row differ at $P \leq 0.05$.

[1]P value: Probability of the F test, with significance at $P < 0.05$.

Table 3.6 Effects of solubility level on least squares means (± standard error of the mean) of expression levels of 24 genes (ΔCT) related to collagen characteristics synthesis and turnover.

Gene ($\Delta CT\ mean$)	Low (n = 12)	High (n = 12)	P value[1]
Collagen synthesis			
COL1A1	8.19	8.31	0.88
	(0.54)	(0.54)	
COL5A1	12.20	11.60	0.15
	(0.45)	(0.45)	
COL6A1	8.28	8.52	0.77
	(0.34)	(0.34)	
FGFR1	12.65	12.37	0.48
	(0.40)	(0.40)	
FGF2	9.26	9.65	0.16
	(0.45)	(0.45)	
FN1	6.23	7.16	0.07
	(0.34)	(0.34)	
IGF1	9.81	10.63	0.42
	(0.76)	(0.80)	
ITGA1	9.43	8.94	0.25
	(0.30)	(0.29)	
ITGA11	8.94	9.43	0.72
	(0.29)	(0.30)	
ITGB1	11.65	11.82	0.67
	(0.31)	(0.31)	
LH1	5.18	6.63	0.09
	(0.57)	(0.57)	
LOX	5.91	7.14	0.80
	(0.47)	(0.47)	
P4HA1	7.81	8.96	0.08
	(0.44)	(0.44)	

SMAD 2	14.84	14.15	0.37
	(0.70)	(0.68)	
SMAD 3	4.06	5.24	0.10
	(0.48)	(0.48)	
SMAD 4	10.17	11.36	0.15
	(0.55)	(0.55)	
SMAD 7	9.55	9.36	0.67
	(0.31)	(0.33)	
PITX 2	13.64	14.21	0.47
	(0.54)	(0.54)	
TFGB 1	12.16	12.19	0.96
	(0.41)	(0.41)	
Collagen degradation			
MMP 1	7.76	8.64	0.08
	(0.33)	(0.33)	
MMP 2	7.18	7.34	0.85
	(0.64)	(0.64)	
MMP 8	9.20	9.83	0.46
	(0.58)	(0.58)	
TIMP 1	10.12	10.46	0.39
	(0.28)	(0.28)	
TIMP 3	7.79	8.18	0.55
	(0.45)	(0.45)	

Low ΔCT mean values indicate higher expression levels while high ΔCT mean value indicate low expression levels

[c, d] Least squares means with different letters within a row differ at $P \leq 0.05$.

[1]P value: Probability of the F test, with significance at $P < 0.05$.

Table 3.7 Interaction effects of breed type and collagen heat solubility rates (high or low) on least squares means (± standard error of the mean) of *COLA3A1, MMP13* & *SMAD 6* expression level.

Genes ($\Delta CT\ mean$)	Breed type				Solubility			Interaction
	Angus (n = 8)	Charolais (n = 8)	ANC[1] (n = 8)	P Value[2]	Low (n = 12)	High (n = 12)	P value[2]	Breed * Solubility P value
COL3A1	10.38	10.53	10.10	0.08	10.47	10.21	0.14	<0.0001
	(0.40)	(0.40)	(0.40)		(0.33)	(0.33)		
MMP 13	5.02[a]	6.81[ab]	8.96[b]	<0.001	6.12[b]	7.74[a]	0.02	0.03
	(0.57)	(0.57)	(0.57)		(0.46)	(0.46)		
SMAD 6	7.74[a]	9.49[a]	12.06[b]	<0.001	9.55	9.98	0.56	0.03
	(0.62)	(0.62)	(0.62)		(0.50)	(0.50)		

Low ΔCT mean values indicate higher expression levels while high ΔCT mean value indicate low expression levels

[a, b] Least squares means with different letters within a row within a treatment differ at $P \leq 0.05$.

[1]ANC: Angus crossbred.

[2]P Value: Probability of the F test, with significance at $P < 0.05$.

Table 3.8 Pearson correlation coefficients between candidate genes.

3.8.1

Genes ($\Delta CT\ mean$)	*COL1A1*	*COL5A1*	*COL6A1*	*FGFR1*	*FGF2*	*FN1*	*ITGA1*	*ITGA11*	*ITGB1*	*LH1*	*LOX*	*MMP1*
COL1A1	1.00	0.78*	0.34	0.09	0.72*	0.19	0.29	0.18	-0.09	0.45	0.64	0.092*
COL5A1		1.00	0.37	0.01	0.60	0.07	0.13	0.24	-0.01	0.26	0.44	0.86*
COL6A1			1.00	0.69*	0.78*	0.51	0.50	0.50	0.02	0.59	0.44	0.47
FGFR1				1.00	0.56	0.45	0.54	0.58	0.40	0.31	0.18	0.16
FGF2					1.00	0.37	0.58	0.40	-0.05	0.65*	0.62	0.81*
FN1						1.00	0.77*	0.70*	0.68*	0.77*	0.69*	0.25
ITGA1							1.00	0.72*	0.79*	0.76*	0.44	0.34
ITGA11								1.00	0.09	0.42	0.24	0.34
ITGB1									1.00	-0.06	0.44	-0.04
LH1										1.00	0.21	0.59
LOX											1.00	0.69*
MMP1												1.00

*Pearson correlations are significant a $P < 0.001$ based upon a Bonferroni correction.

3.8.2

Genes (Δ CT $mean$)	*COL1A1*	*COL5A1*	*COL6A1*	*FGF2*	*FN1*	*IGF1*	*ITGA1*	*ITGB1*	*LH1*	*LOX*	*MMP1*
MMP8	0.43	0.23	0.4	0.56	0.76*	0.51	0.72*	0.29	0.96*	0.64	0.44
MMP13	0.58	0.38·	0.54	0.73*	0.67*	0.43	0.76*	0.20	0.81*	0.81*	0.64
P4HA1	0.52	0.28	0.58	0.73*	0.62	0.47	0.73*	0.11	0.91*	0.65	0.57
SMAD2	0.56	0.45	0.69*	0.73*	0.77*	0.65*	0.70*	0.35	0.86*	0.72*	0.60
SMAD3	0.64	0.54	0.54	0.72*	0.66*	0.71*	0.64	0.32	0.76*	0.72*	0.67*
SMAD6	0.65	0.56	0.69*	0.93*	0.28	0.42	0.63	-0.23	0.58	0.58	0.74*
SMAD7	0.92*	0.78*	0.51	0.86*	0.25	0.61	0.35	-0.05	0.56	0.66	0.95*
TIMP3	-0.43	-0.31	-0.25	-0.55	0.29	-0.14	0.20	0.80*	-0.14	-0.21	-0.43

*Pearson correlations are significant at $P < 0.001$ based upon a Bonferroni correction

3.8.3

Genes ($\Delta CT\ mean$)	*MMP8*	*MMP13*	*P4HA1*	*SMAD2*	*SMAD3*	*SMAD6*	*SMAD7*	*TIMP3*
MMP8	1.00	0.82*	0.92*	0.82*	0.76*	0.52	0.50	-0.13
MMP13		1.00	0.87*	0.76*	0.75*	0.75*	0.66*	-0.25
P4HA1			1.00	0.77*	0.73*	0.73*	0.64	-0.32
SMAD2				1.00	0.93*	0.63	0.67*	-0.13
SMAD3					1.00	0.61	0.73*	-0.14
SMAD6						1.00	0.76*	-0.67*
SMAD7							1.00	-0.47
TIMP3								1.00

*Pearson correlations are significant a $P < 0.001$ based upon a Bonferroni correction

Table 3.9 Pearson correlation coefficients between candidate genes associated with meat quality attributes.

Genes ($\Delta CT\ mean$)	Sarcomere Length	Ultimate pH	Temperature	Moisture	Fat	Protein	Drip Loss	Cook time (13dpm)	WBSF (3dpm)
COL1A1	0.34	0.32	0.15	0.14	-0.26	-0.02	0.42*	0.46*	-0.33
COL6A1	0.22	0.28	0.33	0.00	-0.28	-0.46*	-0.12	0.24	0.10
FGFR1	0.38	0.22	0.56*	0.46*	-0.33	-0.43*	-0.35	0.00	0.44*
IGF1	0.46	0.12	0.11	0.28	-0.36	0.07	0.23	0.25	-0.13
ITGA1	0.38	0.45*	0.33	0.14	-0.20	-0.11	-0.01	0.19	0.42*
LH1	0.23	0.43*	0.29	0.17	-0.37	0.00	0.11	0.33	0.18
LOX	0.29	0.36	0.04	-0.03	-0.12	0.13	0.44*	0.12	-0.23
MMP1	0.28	0.23	0.16	-0.03	-0.16	-0.09	0.31	0.41*	-0.23
MMP2	-0.28	0.07	-0.25	-0.48*	0.34	0.44*	0.20	0.00	-0.05
MMP13	0.26	0.51*	0.08	0.01	-0.25	0.08	0.32	0.18	-0.03
P4HA1	0.26	0.43*	0.23	0.23	-0.46*	0.05	0.27	0.30	0.06
SMAD2	0.41*	0.37	0.24	0.23	-0.31	-0.19	0.10	0.24	0.04
SMAD3	0.51*	0.34	0.18	0.27	-0.35	-0.02	0.16	0.13	0.00
SMAD7	0.32	0.23	0.15	0.10	-0.30	-0.07	0.31	0.45*	-0.28
TIMP1	0.41	0.26	0.60*	0.46*	-0.38	-0.35	-0.06	0.24	0.29

*Pearson correlations are significant at $P < 0.05$.

Table 3.10 Pearson correlation coefficients between candidate genes associated with IMCT and collagen characteristics.

Genes ($\Delta CT\ mean$)	Dry perimysium	Wet perimysium	Total collagen	Soluble collagen (3dpm)	Soluble collagen (13dpm)	Ehrlich (nmol/g raw meat)	Ehrlich (mol/mol collagen)	Pyridinoline (nmol/g raw meat)	Pyridinoline (mol/mol collagen)
COL3A1	-0.05	0.16	0.19	0.21	0.01	0.00	-0.42*	-0.10	-0.07
COL6A1	0.25	-0.17	-0.54*	-0.22	-0.40·	-0.41	0.43*	0.19	-0.06
FGFR1	0.15	-0.02	-0.35	-0.10	-0.44*	-0.38	0.08	0.18	0.04
FGF2	0.11	-0.31	-0.57*	-0.19	-0.18	-0.44*	0.43*	0.09	-0.21
ITGA1	0.09	-0.24	-0.55*	-0.32	-0.13	-0.48*	0.27	0.55*	0.25
ITGA11	0.39	0.02	-0.49*	-0.25	-0.20	-0.52*	0.16	0.27	0.26
ITGB1	0.07	0.04	-0.11	-0.12	0.18	0.24	0.06	0.41	0.52*
LH1	0.07	-0.35	-0.56*	-0.40	-0.05	-0.48*	0.29	0.42*	0.06
MMP8	0.13	-0.32	-0.55*	-0.35	-0.08	-0.52*	0.21	0.36	0.03
MMP13	-0.05	-0.46*	-0.59*	-0.26	-0.02	-0.51*	0.32	0.27	-0.15
P4HA1	0.20	-0.37	-0.70*	-0.45*	-0.17	-0.62*	0.35	0.29	-0.14
SMAD2	0.02	-0.32	-0.45*	-0.27	-0.02	-0.38	0.32	0.28	0.06
SMAD4	-0.54*	-0.37	0.13	0.16	-0.10	0.12	-0.15	0.16	0.11
SMAD6	0.10	-0.38	-0.64*	-0.25	-0.22	-0.43*	0.48*	0.10	-0.29
PITX2	0.08	-0.22	-0.32	0.07	-0.20	-0.42*	-0.22	-0.13	-0.32
TIMP1	0.01	-0.15	-0.31	-0.17	-0.24	-0.53*	-0.07	0.24	0.04

*Pearson correlations are significant at $P < 0.05$.

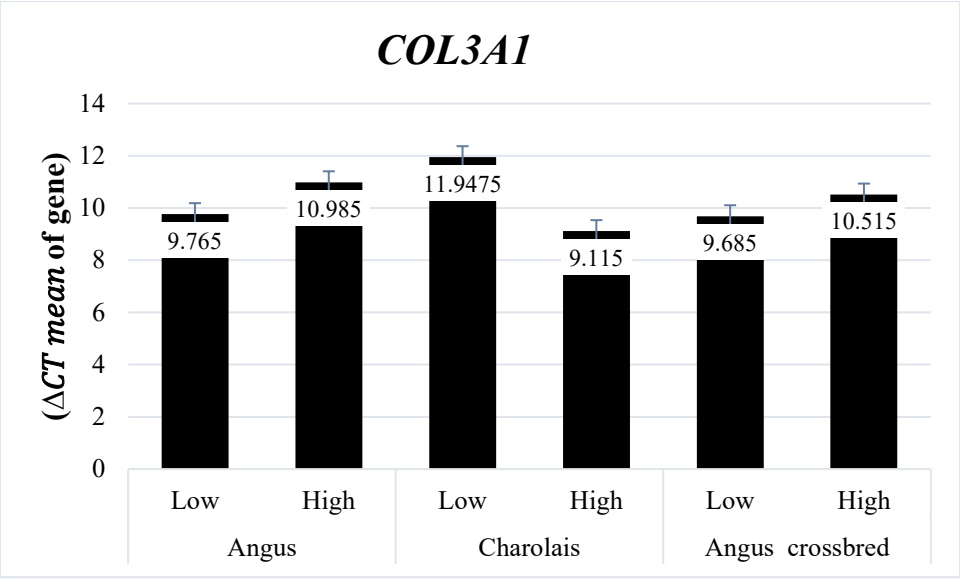

Figure 3.1 Interaction effects between breed type and collagen solubility levels on expression level of *COL3A1*
Lower values indicate high expression level while higher value indicates low expression level

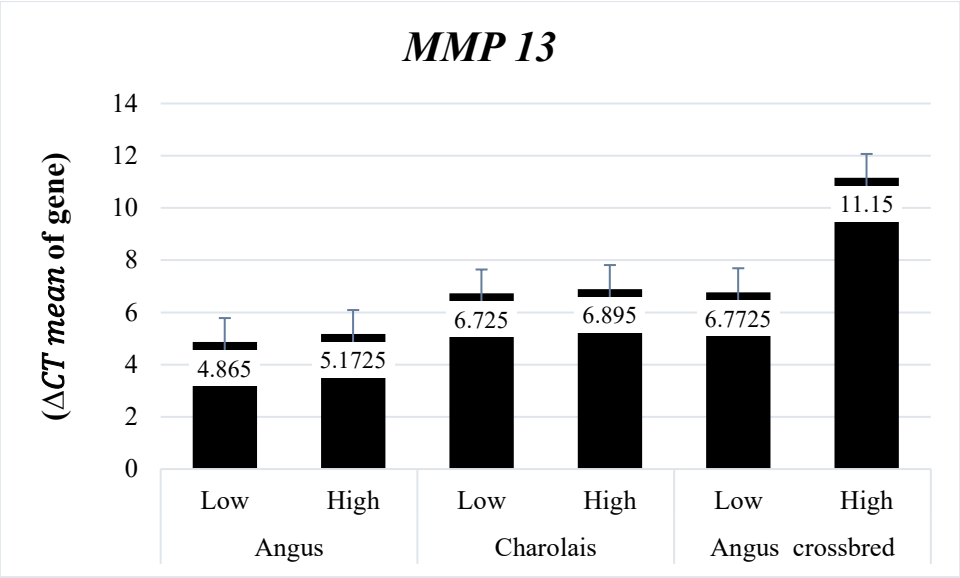

Figure 3.2 Interaction effects between breed type and collagen solubility levels on expression level of *MMP 13*
Lower values indicate high expression level while higher value indicates low expression level

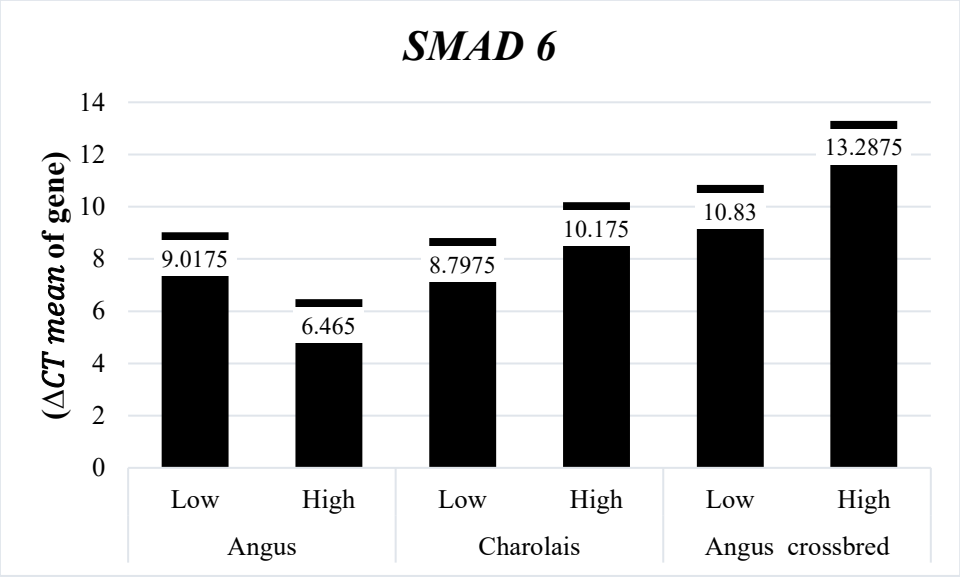

Figure 3.3 Interaction effects between breed type and collagen solubility levels on expression level of *SMAD 6.*
Lower values indicate high expression level while higher value indicates low expression level

4.1 General Summary

Triceps brachii, a muscle from the chuck on the beef carcass, is an underutilized cut characterized by high amount of intramuscular connective tissue (IMCT) with abundant of collagen and collagen cross-links, and therefore perceived to be tough. Production factors such as cattle breed, genetics, age, feed, muscle activity and processing factor such post-mortem ageing may affect the contribution of collagen and collagen cross-links to beef toughness. The genetic selection for animals that efficiently utilize less feed than expected (low RFI) rather than for animals that consume an expected amount of feed (high RFI) has recently been adopted to reduce meat production cost. Hence, this research examined the influence of breed type, selection for residual feed intake (RFI) and post-mortem ageing on meat and carcass quality attributes and intramuscular connective tissue characteristics in the bovine m. *triceps brachii*. Expression of genes for enzymes and proteins involved in the synthesis and turnover of collagen and its cross-links may provide an insight to genes that are specifically associated with the eating quality of m. *triceps brachii.* Thus, the second objective involved the use of quantitative real-time polymerase chain reaction (RT-qPCR) to examine the effect of breed type and collagen heat solubility on collagen candidate genes, and also to test the hypothesis that phenotypic measurements of meat quality attributes and intramuscular connective tissue characteristics will be related to the differences in the expression level of genes involved in the synthesis and degradation of collagen.

Overall, the results of the first study (Chapter 2), suggested the effects of breed type and the genetic selection of RFI animals on *triceps brachii muscle* were limited. Post-mortem ageing however increased collagen solubility at 13 dpm, and there was a concomitant decrease in shear force value. Clear differences were observed between the final weight and

dressing percentage in the Charolais breed which was greater than in the Angus, and Angus crossbred steers (also known as Kinsella composite). Also, the perceived toughness of m. *triceps brachii* may be restricted to the presence of myofibrillar proteins and pyridinoline cross-links in cooked beef. The most important outcome in the comparison of high versus low RFI steers was that the high RFI steers produced carcasses with a greater mean grade marbling score than low RFI steers; however, no differences in the amount of intramuscular fat were observed with near-infrared spectroscopy. For the second study (Chapter 3), breed type had the most significant effects on expression levels of the target genes while whether muscle had a low or high increase in intramuscular collagen solubility with post-mortem ageing had no effect except through the interaction with breed type for *COL3A1, MMP13* and *SMAD6*. Collagen type genes *COL1A1, COL5A1,* and *COL6A1,* and collagenase genes *MMP 1*, *MMP 8* and *MMP 13* were up-regulated in Angus, a breed known for its superior marbling and tenderness compared to the other breeds. The result suggested that the collagen types genes may be up-regulated in breeds that are pre-disposed to increased intramuscular fat muscle and this agreed with previous studies. However, *COL6A1, MMP8,* and *MMP13* were also up-regulated in the Charolais, a large-muscled breed known for lean meat production. The possible reason may be because the steers were raised to approximately the same back fat thickness and physiological age, and this may have resulted in an approximately equal amount of intramuscular fat being deposited in the muscle of all the breeds. The Pearson correlation results suggested *COL6A1, FGFR1*, *ITGA1*, *ITGB1, MMP 8,* and *MMP13,* may be important genes that influence beef tenderness.

In conclusion, the toughness of the *triceps brachii* can be decreased with post-mortem ageing. Raising animals to the same physiological age, with the same feed and back fat

thickness minimized the effect of the different breed steers on carcass and meat characteristics. With limited effects of RFI on meat quality measurements, production costs can be reduced by selecting for low RFI animals without sacrificing product quality, however, sensory study is required to validate consumer acceptance. For both studies, the amount of intramuscular fats reduced the variation among breeds and also influenced the expression of collagen types genes and *MMP's* among breed and muscles type; therefore, future research exploring the genes associated with intramuscular fat should be considered for increased tenderization.

This research thesis is the first to investigate the effects of breed (Angus, Charolais and Angus crossbred) and RFI levels beef steers raised to approximately the same maturity level or physiological age and what it means for the overall eating quality of triceps brachii steaks. It is also the first work to explore the genes related to collagen synthesis and degradation in beef and its relationship with beef toughness. Hence, there is need for further research to be done.

4.2 Future works and study limitation

Chapter 2 of this thesis showed that proper post-mortem ageing practice can be used to decrease beef toughness of high intramuscular connective tissue muscle. However, further study may be beneficial by increasing the ageing days to validate the best and economical ageing days for the beef industry practice. Raising animals to the same maturity level, intramuscular fat and under the same feeding and housing condition minimized the effect of breeds on carcass and meat quality measurements; however, further study increasing the sample size might be needed to verify observed results as only 23 to 24 steers represented each breed and were all from one farm. In the future, sensory evaluation study should be

performed to confirm consumer acceptability of the *triceps brachii* muscle after post-mortem ageing especially for additional ageing days.

For the gene expression studies in Chapter 3, there were limited studies on the target genes in beef to which to compare the results observed. Future work should examine how the proportion of Ehrlich chromogen in bovine muscle may be controlled by the relative expression of *LH1* and *LH2*. Ehrlich chromogen has not been related to beef toughness, and if it could be the cross-link preferentially formed in muscle, then upregulation of LH1 may decrease pyridinoline content and potentially decrease beef toughness. Other gene and meat quality relationships should also be investigated to understand the biological mechanisms behind them. Hence, further study is needed to explore collagen genes and their relationship to beef toughness. Future work should also consider the differences in the expression level of collagen genes among various muscles, and the relationship between collagen solubility and meat quality and gene expression should also be pursued as most correlations approached significance ($P < 0.1$), which suggests that significance might have been achieved with an increase in sample size.

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