UNLOCKING THE ROLE OF SMALL HEAT SHOCK PROTEINS AND APOPTOSIS IN POSTMORTEM PROTEOLYSIS AND MEAT QUALITY CHARACTERISTICS OF SKELETAL MUSCLES UNDER DIFFERENT CONDITIONS

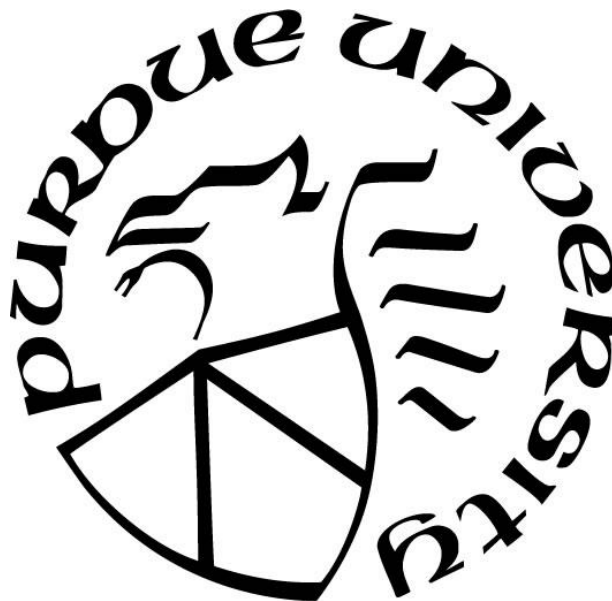by

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To meat lovers

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ABSTRACT

Postmortem aging has been extensively practiced as value-adding process due to the beneficial impacts on meat palatability. Meat tenderization occurred through proteolytic fragmentation of myofibrillar structural proteins via endogenous protease systems, which is considered as the primary drive to enhance major palatability attributes including tenderness, juiciness, and flavor. Recent theoretical framework proposes apoptosis, or programmed cell death, as the preceding step that initiates postmortem proteolysis. Whereas small heat shock proteins have been consistently recognized as meat quality biomarkers, probably due to their protective activities against proteolysis through anti-stress, anti-apoptotic, and chaperoning functionalities. To shed light on detailed mechanisms controlling postmortem proteolysis and consequential impacts on the development of fresh meat quality characteristics, postmortem proteolytic changes of small heat shock proteins, apoptotic factors, and myofibrillar structural proteins were profiled in postmortem skeletal muscles under different metabolic backgrounds and across species.

In beef, three muscles, *longissimus lumborum* (LL), *semimembranosus* (SM), and *psoas major* (PM), have been selected to represent glycolytic, intermediate, and oxidative muscle types. Tenderness and water - holding capacity were determined, and proteolysis, apoptotic features, and small heat shock proteins were measured in 8 beef carcasses at 1, 2, 9, 16, and 23 days of aging. PM exhibited limited aging potential in quality developments shown by lower extents of shear force, water-holding capacity, and proteolytic changes, including calpain 1 autolysis, troponin T, and HSP27 compared to LL and SM. Conversely, LL had an increase in tenderization and water-holding capacity, which was accompanied with more extended calpain 1 autolysis, proteolysis and HSP27 degradation, compared with other muscles. The results of this study suggest that postmortem proteolytic changes of myofibrillar proteins, small HSPs and apoptotic factors occur in a muscle-specific manner, which is likely attributed to different rate and extent of meat quality developments of each muscle during aging.

Callipyge lambs are a unique genetic background showing calpastatin over-expression, muscle hypertrophy in loin and hindquarter area, substantially compromised meat tenderization potential, and a shift of muscle fiber composition towards fast-glycolytic directions. Proteome and metabolome changes in muscles from callipyge mutation (+/C) and non-callipyge phenotype (+/+, C/+, and C/C) lambs were profiled to provide insight into the biochemical changes affecting meat

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quality attributes. M. longissimus thoracis from lambs with all four possible callipyge genotype (n = 4, C/+, C/C, +/C, and +/+) were collected after 3d aging and analyzed using mass-spectrometry based platforms. Among identified proteomes, cytochrome c (pro-apoptotic protein) was detected with significantly lower abundances in +/C. Anti-apoptotic HSP70, BAG3, and PARK7 were over-abundant in +/C, which could result in delayed apoptosis and possibly attributed to tougher meat in callipyge lambs. Eight glycolysis enzymes were overabundant in +/C lambs, whereas 3 enzymes involved in TCA cycle were overabundant in non-callipyge ones (C/C and/or C/+). Twenty-five metabolites were affected by genotypes (P < 0.05), including metabolic co-factors, polyphenols, and AA/short peptides.

Pig production is facing increased public pressure regarding antibiotic usage restriction. Recently, dietary L-glutamine at cost effective level (0.2%) was identified as an effective antibiotic alternative in post-transport nursery pig diets. To evaluate carcass and meat quality characteristics in market-ready pigs when 0.2% dietary L-glutamine was applied as for early-life post-weaning and transport recovery, pigs (N=480) were weaned and transported in two replication trials in SPRING (April of 2017) vs. SUMMER (July of 2016), fed 3 different diets (Non: no antibiotic, Anti: 441 ppm chlortetracycline and + 38.6 ppm tiamulin, Gln: 0.20% L-glutamine) for 14 days after transport, and fed basal diet until reaching market weight. Pairs of *longissimus dorsi* (LD) and *psoas major* (PM) muscles from each carcass (n=10/diet/trial) were separated at 1 d and 7 d postmortem, respectively. Carcass yield and meat physical and quality attributes were evaluated. Overall impacts of Gln on physical attributes of carcasses and porcine muscles were minimal. No dietary effects were found in carcass, proximate composition, water-holding capacity, or shear force. Significant difference between trials were found in terms of productivity and pork/carcass qualities, where SPRING replicates showed increased body weight, faster pH decline, paler surface color, higher intra-muscular fat deposition, and improved tenderness and water-holding capacity as indicated by lower shear force values, thaw-purge loss, and cooking loss (P < 0.05).

The pork and carcass quality results give rise to a postulation that different metabolism and animal growth might have been occured between the two production trials, consequentially differentiated meat quality development. In this regard, myofibrillar proteolysis, small heat shock proteins, and apoptotic factors were characterized during 7 d postmortem aging in porcine LD and PM muscles from both seasonal trials, combined with metabolomics profiles of 1d samples using the GC-TOF-MS/MS platform. Compared to SUMMER counterparts, SPRING muscles showed

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concurrence of more extended apoptosis, further calpain 1 autolysis, and increased structural protein degradation (P<0.05). SPRING muscles showed more ATP catabolism compounds and increase in carbohydrates, branched-chain amino acids, and 16-18 carbon fatty acids, which could be chemistry fingerprints of increased cellular oxidative stress, consequentially favoring onset of apoptosis and proteolysis. Meanwhile, SUMMER pigs showed increased stress-defending metabolites, such as ascorbic acid, antioxidant amino acids, and decreased inhibitory neuro-transmitter GABA, which may indicate elevated stress-defending activity in SUMMER pigs that possibly inhibited apoptosis and proteolysis.

CHAPTER 1.   LITERATURE REVIEW

*Starts on page 16 (Postmortem aging impacts on meat quality attributes) and ends on page 21 (end of Impact on water-holding capacity and other processing attributes)

The two terms, *muscle to meat conversion* and *postmortem aging,* refer to the two consecutive periods after animal slaughter, during which a collection of biological/physiochemical changes occur in skeletal muscles under extreme conditions due to cession of oxygen and macronutrient supply (Devine & Klinth, 2004; Honikel & Werner Klinth, 2004). ATP depletion and on-set of rigor mortis defined the boundary of the two processes. Muscle to meat conversion (biochemical changes that are generally recognized a s ATP-dependent) establishes a large portion of potentials to which extent the desirable quality traits can be developed and undesirable traits could be eliminated, whereas ideal management and processing practice during postmortem aging can ensure that the potentials are fully reached (Brewer & Novakofski, 2008; Huff-Lonergan, Zhang, & Lonergan, 2010; Kim, Warner, & Rosenvold, 2014; Koohmaraie, 1996). The literature review aims to articulate the importance of postmortem conditioning to the development of meat quality attributes, which will be presented in three sections: I. A brief overview on impacts of postmortem conditioning on meat quality development; II. Recent progress regarding detailed early biochemical activities in mediating postmortem proteolysis; and III. A concise discussion on how pre-slaughter stressors may influence quality characteristics development of postmortem skeletal muscles, with an emphasis on weaning and transport stress.

1.1   Postmortem aging impacts on meat quality attributes

1.1.1   Impact on palatability

During the conversion process of muscle into meat, substantial biochemical/biophysical changes occur in muscle, and these changes directly influence meat quality attributes (Brewer &

Novakofski, 2008; Huff-Lonergan, Zhang, and Lonergan, 2010; Kim, Warner, & Rosenvold, 2014; Koohmaraie, 1996; Kristensen & Purslow, 2001). Significant improvements in meat palatability attributes occur through cytoskeletal myofibrillar protein degradation by endogenous proteases during aging process (Huff-Lonergan & Lonergan, 2005; Kemp, Sensky, Bardsley, Buttery, & Parr, 2010; Kim et al., 2014; Kristensen & Purslow, 2001; Lepper-Blilie, Berg, Buchanan, & Berg, 2016; Spanier, Flores, McMillin, & Bidner, 1997). Tenderness, juiciness and flavor are the currently-established major beef palatability attributes (Robbins et al., 2003) with tenderness considered to be the most influential beef palatability attribute (Miller, 2001; Miller et al., 1997; Savell et al., 1987). In this regard, therefore, significant improvement in tenderness is the primary driver for extended postmortem aging of meat, and thus, has been the most extensively studied.

Proteolysis is the predominant factor influencing impacts of postmortem aging on meat tenderization. The rate and extent of the aging response and subsequent meat tenderization are dependent upon various factors such as species, animal age, diet, breed, individual muscle, marbling content, and/or aging condition (Bratcher, Johnson, Littell, & Gwartney, 2005; Gruber et al., 2006; Smith, Tatum, and Belk, 2008). Although it varies, most rapid and substantial changes in meat tenderness occurs between 3 and 7 days postmortem, and then the rate of beef tenderization declines with time (Koohmaraie & Geesink, 2006). However, a substantial increase in meat tenderness of beef from mature animals or muscles with high background toughness was also reported for extended aging up to 28 days (Colle et al., 2016; Hutchison, 2007; Phelps et al., 2016; Santos et al., 2016; Stelzleni, 2006). This could be due to the decrease in mechanical strength of the intramuscular connective tissue through the proteolytic action of endogenous enzymes, resulting in increased collagen solubility, decreased breaking strength, and dissociated structural integrity of muscle connective tissue with the extended aging (Dutson, Smith, & Carpenter, 1980; Lewis, Purslow, & Rice, 1991; Nishimura, 2015; Nishimura, Fang, Ito, Wakamatsu, & Takahashi, 2008; Nishimura, Liu, Hattori, & Takahashi, 1998; Stanton & Light, 1990; Wu, Dutson, & Carpenter, 1981). As proposed by Nishimura (2015), the strength and structural integrity of collagen fibrils is stabilized by proteoglycan, which can be degraded during postmortem aging, exposing more active sites for potential degradation enzymes (e.g. lysosomal glycosidase or β-glucuronidase) to further weaken the structural integrity and thus tenderize meat. Another proposed theory involves two populations of connective tissue: the weak population of connective tissue that responds to postmortem aging (and cooking process) through proteolysis of enzymes,

partially corresponding to meat tenderization, while the strong population of connective tissue would not be affected by proteolysis and thus would remain to define background toughness (Purslow, 2014).

Considerable increases in savoury/beef flavor occurs during the process of aging (particularly dry-aging) as a result of liberation of flavor-related compounds, these include nucleotide compounds; Maillard reaction-related sugar fragments, such as glucose; other flavor related volatile compounds such as n-aldehydes (e.g. pentanal and hexanal) and ketones, which also include lipid oxidation-related products (Maga, 1994; Martins, Jongen, & Van Boekel, 2000; Yaylayan, Keyhani, & Wnorowski, 2000). The umami/beefy taste and flavor characteristic of aged meat results from a complex interaction between sulfur containing amino acids, aspartic acid and glutamic acid, nucleotide compounds, and β-histidyl dipeptides (Dashdorj, Amna, & Hwang, 2015). Postmortem energy metabolism also results in an increase in sugar fragments through degradation of glycogen content, which in turn increases substrates that are responsible for the Maillard reaction (Martins et al., 2000; Yaylayan et al., 2000). Further, it has been reported that a prolonged aging regime (over 28 days) tremendously increased volatile compounds that are important to aroma development (Ba, Park, Dashmaa, & Hwang, 2014; Watanabe et al., 2015).

While it is generally agreed that aging improves meat flavor, not all studies reported that noticeable positive role of extended aging has on meat flavor development (Brewer & Novakofski, 2008; Lepper-Blilie et al., 2016). Spanier et al. (1997) reported that 4 days of aging at 4 °C improved desirable flavor traits, such as sweetness and beefy flavor, but with longer aging, increasing undesirable traits like bitterness and sourness were detected. Campo, Sañudo, Panea, Alberti, and Santolaria (1999) also found prolonged aging (up to 21 days) to increase global flavor intensity coupled with livery odor development in loins from different breeds. Aging decreases the concentrations of glycogen and glucose 6-phosphate significantly from day 4 to day 15 (Meinert, Schäfer, Bjergegaard, Aaslyng, & Bredie, 2009), while prolonged aging increases the ribose content of meat (Koutsidis et al., 2008). Prolonged aging also releases free fatty acids, which then react with proteins and other flavor precursors to affect the aroma and/or flavor of aged meat (Wang et al., 2013). Since free fatty acids are more prone to oxidation and can accelerate the loss of product due to off-odor and off-flavor, aging regimes should be controlled to maximize desirable flavor and minimize off-odor and flavor.

A positive impact of aging on juiciness of beef steaks has been reported (Campo et al., 1999; Teye & Okutu, 2009). Given there is a general positive correlation between sensory tenderness and juiciness (de Lima Silva, Contreras-Castillo, & Ortega, 2007; Guzek, Glabska, Wierzbicki, Wierzbicki, & Cierach, 2012; Otremba et al., 2000; Shackelford, Wheeler, & Koohmaraie, 1995), improvements in tenderness with aging may have synergistic effects on other perceived palatability, such as juiciness. In fact, some studies proposed a "halo effect," where improved tenderness could boost the perceived juiciness of meat, and vice versa (Hughes, Oiseth, Purslow, & Warner, 2014; Jenkins et al., 2011). Most recently, an improvement of juiciness coinciding with the early activation of calpain-2 was found, which suggests postmortem proteolysis may play a role in juiciness development (Colle et al., 2018). Juiciness is highly dependent on the moisture retention ability of cooked meat, but interestingly, lack of strong correlation was found between perceived juiciness and water-holding capacity of meat (Hughes et al., 2014). As a theoretical explanation of cooked meat to retain water has not been fully established, further studies elucidating the underlying mechanisms and/or possible inter-relationships between muscle protein thermal denaturation behavior, functionality and juiciness of aged meat are needed.

1.1.2   Impact on color and oxidation

Postmortem aging has a considerable influence on initial color and color stability of meat during retail display (Vitale, Pérez-Juan, Lloret, Arnau, & Realini, 2014). Initial improvement in surface redness of aged meat has been reported when compared with non-aged or relatively short-term aged meat (Abdullah & Qudsieh, 2009; Teixeira, Pereira, & Rodrigues, 2011). Such improvements (although transient) could be attributed to a decrease in oxygen consumption of respiratory enzymes within mitochondria of aged meat. Decreased oxygen consumption results in allowing more oxygen to be available to bind with myoglobin forming a thicker layer of oxymyoglobin, resulting in meat with increased bloomed color and/or color intensity compared to early postmortem muscles with high oxygen consumption (Seideman, Cross, Smith, & Durland, 1984).

However, extended aging may negatively impact product color and/or lipid oxidative stability. Extended aging periods reduced "blooming potential" and accelerated surface discoloration and oxidized off-flavor development in meat, when repackaged and displayed under

retail light conditions (Kim, Frandsen, & Rosenvold, 2011; Kim, Stuart, Black, & Rosenvold, 2012; King, Shackelford, Kalchayanand, & Wheeler, 2012; Pouzo, Descalzo, Zaritzky, Rossetti, & Pavan, 2016). Discoloration due to metmyoglobin and darkening due to surface dehydration may result in economic losses, regardless of the extent to which aging enhances eating quality (Kim & Hunt, 2011; Kim, Stuart, Rosenvold, & Maclennan, 2013).

The exact mechanisms by which aging influences color and oxidative stability of muscles have not been fully determined. There could be a number of intrinsic factors associated with prolonged aging, such as the accumulation of pro–oxidants (e.g. heme and non–heme iron), and/or the depletion of endogenous reducing compounds or antioxidants (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013; Kim et al., 2012; Kim, Frandsen, & Rosenvold, 2011). Madhavi and Carpenter (1993) reported decreased NAD+ concentration and metmyoglobin reductase activity in beef psoas major and longissimus lumborum muscles over 21 days of aging. A decrease in reducing compounds such as α-Tocopherol and β-Carotene in Argentina buffalo meat was observed during a 25-day aging period (Descalzo et al., 2008). Recently, Ma et al. (2017) found that oxidative stability could be differently influenced by muscle type and aging duration. In particular, aged psoas major steaks had greater lipid oxidation coupled with more rapid discoloration, lower free radical scavenging activity, and higher non-heme iron content, when compared with steaks from aged longissimus lumborum and semimembranosus muscles. Further, the authors found metabolites with anti-oxidant properties, such as NAD/NADH, acyl carnitines, nucleotides, nucleosides, and glucuronides varied in quantity among specific muscle types with aging (Ma et al., 2017).

1.1.3   Impact on water-holding capacity and other processing attributes

Water-holding capacity is considered one of the main factors affecting both the quality and value of meat (Huff-Lonergan & Lonergan, 2005). An increase in moisture loss during the muscle-to-meat conversion process is inevitable. After exsanguination, muscle undergoes rigor processes, in which crosslinks are made between the muscle filaments, pushing intracellular water out of the myofibril and ultimately out of the muscle cell due to the stiffening of the muscle fibers (Huff-Lonergan & Lonergan, 2005). After the resolution of rigor, continued water migration of meat can be observed in the form of purge or drip during aging. However, an improvement in water-holding

capacity is often observed in long-term aged meat, which is considered to be due to postmortem proteolysis of structural/cytoskeleton proteins including desmin, titin, nebulin and integrin (Kristensen & Purslow, 2001; Lawson, 2004; Melody et al., 2004; Zhang, Lonergan, Gardner, & Huff-Lonergan, 2006). Several models have been proposed to explain how changes of muscle structure during aging would affect moisture retaining properties of meat. According to Huff-Lonergan and Lonergan (2005), degradation of costamere linkages during postmortem aging will reduce myofibril shrinkage, leaving more space within muscle fibers to retain water. The "sponge effect", in addition, has been proposed, where myofibrillar proteins are broken down to disturb the drip channel and hence increasing the ability of the muscle to hold the water within the cell (Farouk, Mustafa, Wu, & Krsinic, 2012).

With the increase of postmortem aging period, proteolysis increases, but also susceptibility of fresh meat to develop protein oxidation rises upon being exposed to oxidative conditions (e.g. retail display). Carbonyls and sulfhydryl groups are often produced through the oxidation during aging, leading to loss of functional groups and thus forming intra and/or inter protein disulphide cross-linking. This significantly impairs muscle protein functionality and negatively influences water-holding capacity (Li, Wei, Wang, Zhang, & Mehmood, 2018; Utrera & Estévez, 2012; Xiong, 2000). Further, protein denaturation, as indicated by loss of tertiary structure, exposes the previously folded hydrophobic amino acid residuals, adversely affecting the water-binding ability of the protein. Moreover, several studies have found that oxidative conditions result in negative impacts on μ-calpain (or calpain 1) and subsequently a decrease in protein degradation in postmortem muscles, which in turn affects a decrease in water-holding capacity and/or tenderness of meat (Kim, Huff-Lonergan, Sebranek, & Lonergan, 2010; Rowe et al., 2004).

1.2   Major biochemical changes in postmortem skeletal muscles

As previously stated, postmortem aging, the term primarily used to refer to post-rigor proteolytic events, substantially impacts meat quality. It is well acknowledged that the underlying regulatory mechanisms of postmortem aging are heavily relying on pre-rigor biochemical activities, or the muscle to meat conversion process. Key factors mediating postmortem proteolysis should be further mapped (Ouali et al., 2013). In this section, recent progress elucidating pre-rigor metabolism and cell fate-determining (survival/death) activities and their possible roles in regulating postmortem calpain system will be discussed.

1.2.1   Energy metabolism

The major biochemical changes over the muscle to meat conversion are focusing on carbohydrate metabolism processes, which include *glycogenolysis* and *glycolysis*, and for a very short antemortem period, *aerobic respiration* (England, Matarneh, Scheffler, & Gerrard, 2017). Upon slaughter, oxygen supply is stopped due to cessation of heart pulse and blood circulation. However, at the cellular level, life activities are still going on to maintain overall homeostasis. Without oxygen, aerobic respiration can only be sustained for a very short period time, then the major form of ATP generation switches to anerobic glycolysis pathway (Honikel & Werner Klinth, 2004). Anerobic respiration can only release a small amount of energy in glucose to form ATP. Moreover, cessation of blood prevented continuous supply of macro nutrients, making ATP consumption quickly exceeds ATP generation, ultimately lead to ATP depletion and onset of rigor mortis (Honikel & Werner Klinth, 2004). At the point of rigor mortis on-set, which is also recognized as the completion of muscle to meat conversion, muscle reaches the maximum stiffness.

1.2.1.1 The pH drop

The shifted energy metabolism homeostasis in early postmortem muscles will lead to two major chemical and/or physiological changes that are essential to meat quality development: pH drop, and irreversible actomyosin crosslink formation. Right after animal death, glucose supply from blood ceases immediately, making muscle glycogen storage the only source of glucose supply. Glucose units are removed from glycogen in the form of glucose-1-phosphate and enter glycolysis cycle (Honikel & Werner Klinth, 2004). Although oxygen residuals carried by myoglobin are still available for a very short period of time, excessive pyruvate cannot be processed through TCA cycle in a timely manner (Honikel & Werner Klinth, 2004) and therefore must be converted to less toxic lactate (Honikel & Werner Klinth, 2004; Matarneh, England, Scheffler, & Gerrard, 2017). Lactate accumulation gradually drags down muscle pH from 7.2 to an endpoint near 5.6 within 24 hours (time can be ranged from 12 hours to 48 hours depending on species, individual and genetic variation, and muscle characteristics). The ultimate pH will be reached upon on-set of rigor mortis (Matarneh et al., 2017).

pH decline could be the single most important factor affecting color, water-holding capacity (WHC), and protein functionality (Aberle, Forrest, Gerrard, & Mills, 2012a; Bowker, Grant, Forrest, & Gerrard, 2000). This is because muscle proteins have an isoelectric point (pI) of

5.1, at which point proteins carry the minimum surface net charge and have least affinity to water molecules (Aberle et al., 2012a). As pH shift away from pI (either higher or lower than pI), side chains of the AA residues (particularly myosin) will be charged again to gain water affinity. Therefore, an ideal ultimate pH (pH_u) of fresh meat should be fall into 5.4-5.8. Low meat pH_u (5.3-5.4) results in inferior WHC, poor protein functionality, and pale in color (Aberle et al., 2012a; Bowker et al., 2000).

1.2.1.2 Actomyosin crosslink formation

During muscle contraction, binding of calcium ion to the troponin complex initiates a formation change of tropomyosin, exposing the binding site on actin that specific to myosin binding (Aberle, Forrest, Gerrard, & Mills, 2012b). Myosin then binds to actin, hydrolyzes ATP and releases ADP and Pi. Myosin heavy chain tilts the head (power stroke) to pull the thin filament towards z-line (Aberle et al., 2012b). In the following step, relaxation of actin-myosin binding hydrolyzes another ATP molecule that binds to the myosin heavy chain. In this regard, when ATP pool is depleted, the actin-myosin crosslink is irreversibly formed and therefore muscles reach the rigor mortis (Matarneh et al., 2017).

Actin-myosin crosslink formation is crucial to meat quality attributes. When excessive contraction occurs while the muscle reaches rigor mortis, it forms a tight actin-myosin complex while shorten the sarcomere length. This is a detrimental situation to meat quality development, because shortening eliminates the "working space" of protease system to degrade muscle structural proteins. Ultimately, reduced extent of proteolysis impairs meat tenderness (Aberle et al., 2012a). Cold shortening is a result of improper chilling protocols. if postmortem glycolysis goes slow, ATP is more scarce than normal chilling condition (Aberle et al., 2012a). Excessive temperature decline during early ante-mortal stage slows down the rate of glycolysis, decreasing the level of calcium ion uptake of sarcoplasmic reticulum from cytosol. ATP supply is also restricted, less actomyosin crosslink can be relaxed, ultimately resulting in excessive muscle contraction (Aberle et al., 2012a).

1.2.1.3 Recent progress on postmortem energy metabolism regulation

It was generally accepted that pH_u is determined when glycogen is depleted. However, early postmortem energy metabolism was described in an oversimplified theoretical framework (Matarneh, 2017). Recent studies pointed out that residual glycogen and glycolytic intermediates

are consistently observed in postmortem muscles after the onset of rigor mortis, suggesting that the endpoint of muscle to meat conversion may be controlled by more complex processes. (England et al., 2017; England, Matarneh, Scheffler, Wachet, & Gerrard, 2015). To explain the endpoint of postmortem glycolysis, there are two hypotheses made from early literatures: I. inactivation of glycolytic enzymes due to pH decline and II. loss of adenine nucleotides through the phosphagen system (Bendall, 1960; Kastenschmidt, Hoekstra, & Briskey, 1968; Matarneh, 2017). For the first hypothesis, England, Matarneh, Scheffler, Wachet, and Gerrard (2014) indicated that Phosphofructokinase 1 (PFK-1), the enzyme catalyzes the conversion of fructose 6-phosphate and ATP to fructose 1,6-bisphosphate and ADP, loses enzymatic activity completely at pH 5.5. Given that the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate is the committed step of glycolysis, pH decline induced inactivation of PFK-1 could eventually lead glycolysis to stop. More recently, studies indicate that ATP retention plays a critical role in modulating the endpoint of postmortem metabolism (Matarneh, Beline, E Silva, Shi, & Gerrard, 2018; Ramos et al., 2020). According to England et al. (2015), loss of adenosine nucleotides species is crucial to halt glycolysis. The loss of adenine nucleotides via phosphagen system is mainly through the irreversible conversion of AMP to IMP, which is presented in the following reactions:

$$2\text{ADP} \xleftrightarrow{\ ADK\ } \text{ATP} + \text{AMP} \ (1)$$

$$\text{AMP} \xrightarrow{\ AMPD\ } \text{IMP} \ (2)$$
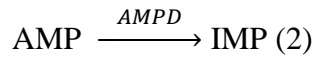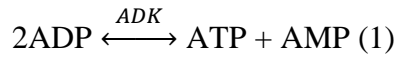
Reaction (1) is catalyzed by adenylate kinase, which is responsible for reversible interconversions between different forms of adenine nucleotides ATD, ADP and AMP. Reaction (2), the conversion of AMP to IMP, is catalyzed by AMP deaminase (AMPD), which facilitates reaction (1) to move forward. The two reactions are an important compensatory mechanism to maintain ATP pool under acute conditions (England et al., 2017; England et al., 2015). Reduced AMPD activity extends glycolysis and pH decline; AMPD activity helps to explain variations in ultimate pH (pH 5.4–5.8) in the same muscle between pig individuals of same RN- genetic background. This leads to the postulation that exhaustion of adenine nucleotides, primarily via AMPD catalytic activity to convert AMP to IMP, defined the end point of postmortem glycolysis (England et al., 2015).

1.2.2   The battle between cell death vs. survival

Antemortal cellular stress, primarily hypoxia, disrupts cellular structural and functional homeostasis quickly. Receptors that located on the cell membrane and organelles - mitochondria, lysosome, endoplasmic reticulum (or sarcoplasmic reticulum) - receive death stimuli and initiate signaling pathways that determine cell fate, which involve in activation of stress defending system and programmed cell death (apoptosis), and anti-apoptotic processes (Strasser, O'connor, & Dixit, 2000). Recently published models propose to include apoptotic and anti-apoptotic processes into muscle to meat conversion framework (Ouali et al., 2013). These events are considered to have long-term implications in postmortem aging and meat quality development (Longo, Lana, Bottero, & Zolla, 2015).

1.2.2.1 Apoptosis

It has been recently proposed that apoptosis is the proceeding step activates μ-calpain system, which refers to one of the programmed cell death mechanisms that universally triggered by mitochondria dysfunction (Sierra & Oliván, 2013). Oxygen depletion during slaughter and exsanguination disrupts energy metabolism homeostasis (Herrera-Mendez, Becila, Boudjellal, & Ouali, 2006). Cellular lipid bilayer membrane systems lose structural integrity, including mitochondria inner membrane. Leaky, malfunctioning mitochondria release pro-apoptotic proteins from inner membrane space into the cytosol (Sierra & Oliván, 2013), activating different caspase family members and enabling enzyme systems that responsible for degrading cellular structural and functional compartments (Sierra & Oliván, 2013).

The role of oxidative stress in this fate determining event should not be overlooked, because mitochondria activities generates both reactive oxygen species (ROS) production and antioxidant molecules of the cells (Ott, Gogvadze, Orrenius, & Zhivotovsky, 2007). With normal respiration process being disrupted, ROS generation cannot be effectively eliminated by antioxidant system (Ott et al., 2007). This situation leads to oxidative stress and/or oxidative damage, conditions in which cellular constituents, including protein, DNA, and lipids are oxidized and damaged, altering and disrupting normal function of cell organelles (Sierra & Oliván, 2013). The oxidized molecules also directly modulate the onset of apoptosis via participating in cell signaling transduction and facilitating cytochrome c release (Ott et al., 2007). Further, excessive

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ROS accumulation depolarize mitochondrial inner membrane, reinforcing the release of pro-apoptotic signals, leading apoptotic process to move forward (Ott et al., 2007).

A number of studies pointed out the importance of taking apoptosis into consideration to further understand aging related meat quality development (Huang, Huang, Xu, & Zhou, 2009; Kemp, Bardsley, & Parr, 2006; Laville et al., 2009). Kemp et al. (2006) reported a negative correlation between shear force and caspase 3, 7, and 9 activities in pork *longissimus* muscles. Further, Huang et al. (2009) found that inhibition of caspase 3 decreases myofibrillar protein degradation in broilers. Laville et al. (2009) demonstrated that in "high quality", tender beef muscles, a more extended degradation of mitochondrial membrane proteins was found at early postmortem stage compared to tough meat samples, suggesting a linkage between meat tenderization and pre-rigor mitochondria dysfunction. These studies strongly indicate that apoptosis should be the upstream event that further affect proteolysis.

1.2.2.2 Anti-stress molecules

Heat shock proteins (HSPs) are a family of ubiquitous and highly conserved chaperone proteins. As stress induced molecules, small HSPs belong to a family of molecular chaperones with molecular weight ranging from 15 to 43 kDa (Liu & Steinacker, 2001). Members of this family share a conserved alpha-crystallin domain (Liu & Steinacker, 2001). In postmortem muscles, small HSPs expressions are up-regulated by pre-slaughter thermal challenges among other stressors (Deng et al., 2013; Tang, Bao, Sultan, Nowak, & Hartung, 2013). Small HSPs basal expression level is generally higher in cattle with dominant *Bos indicus* background, which are characterized as producing meat with inferior tenderness compared to cattle with dominant *Bos taurus* background (Mullins, Zerby, Fitzpatrick, & Parker, 2016). In addition, the role of small HSPs in protecting cytoskeletal proteins such as actin, titin, and troponin T has been reported in live cardiac and skeletal muscles and in situ models (Kötter et al., 2014; Lomiwes, Hurst, et al., 2014; Wójtowicz et al., 2015).

A number of HSPs were identified as possible meat quality biomarker by omics researches with initial validations (Di Luca, Elia, Mullen, & Hamill, 2013; Di Luca, Hamill, Mullen, Slavov, & Elia, 2016; Picard & Gagaoua, 2017). Lomiwes, Farouk, Wu, and Young (2014) found that the modulation of several small HSPs including αB‑crystallin, HSP20, and HSP27 during the meat

aging is partially related to beef tenderness. Chaperone proteins HSP27 and αβ-crystallin were among the substrates of postmortem proteolysis (Lametsch, Roepstorff, & Bendixen, 2002; Morzel et al., 2004). The level of small HSPs can be changed up to the first 22 h postmortem (Jia, Hollung, Therkildsen, Hildrum, & Bendixen, 2006). In addition, meat quality variation among different muscle types and breeds may be related to their different abundance of small HPSs (Picard et al., 2014). Yu et al. (2009) found that in PSE-like LD muscle induced by 2 hours preslaughter transport of pigs, the expression of HSP90, HSP70, HSP27 and αB‑crystallin tended to decrease. In broilers, Xing, Xu, Jiang, and Deng (2016) found lower expression of HSP70 in the PSE-like muscles. Lomiwes, Farouk, Wiklund, and Young (2014) proposed that the effect of small HSPs on regulating proteolysis was likely related to their ATP-independent chaperone activity, such as protective binding to myofibrils and mediating apoptosis progress. However, ATP-dependent chaperone activities of the HSP families involves in stabilization and correction of unstable, misfolded and denatured proteins (Hendrick & Hartl, 1993) are less investigated in postmortem muscles (Xing, Gao, Tume, Zhou, & Xu, 2019). In fact, previous studies reported that expression of different isoforms of HSP27 to vary according to metabolic difference and muscle fibre type (Neufer & Benjamin, 1996). Also, Xu et al. (2009) reported that the Large White pig, a breed characterised by more glycolytic metabolism properties, has higher HSP27 and HSP70 expressions compared to Meishan, a more oxidative breed. According to a muscle kinetics study (Thompson, Maynard, Morales, & Scordilis, 2003), HSP27 expression in responding to acute oxidation stress is faster and higher in *biceps brachii* muscles, a muscle type with more glycolytic Type IIA fibers, in comparison with *vastus lateralis* muscles, a type I (red) muscle. All these evidences suggested possible linkages between energy metabolism in modulating small HSPs activity. Overall, the causation effect between the biological and protective roles of small HSPs and the meat quality development are still ambiguate. Since ATP level decreases rapidly during muscle to meat conversion, the pre-rigor behavior (special and temporal) of how small HSPs responding to post-slaughter stress warrants further research to understand consequential impacts on apoptosis and proteolysis events and meat quality development.

1.2.3 Calpain 1 in meat quality development

Although muscles retain several endogenous protease systems, including calpain system, lysosomal proteases, cathepsins, and ubiquitin-dependent proteasome (Koohmaraie & Geesink, 2006), meat tenderization is mainly recognized as depending on proteolytic dissociation of myofibrillar and structural integrities via calpain system, especially calpain 1 (Bhat, Morton, Mason & Bekhit, 2018). Among different isoforms of calpain, such as calpain 1, 2 and 3, the role of calpain 1 received most attention during postmortem proteolysis due to its reasonable requirement of Ca2+ concentration for half-maximal proteolytic activities (Bhat et al., 2018, Cong, Goll, Peterson, & Kapprell, 1989). The enzymatic activity escalation of calpain 1 depends on its autolysis of 80 kDa subunit to 78 kDa intermediate and 76 kDa final product, and the cleavage of 28 kDa small subunit into 18 kDa fragments (Melody et al., 2004). Direct molecular evidence in calpain 1 knockout mice demonstrates that inhibition of calpain 1 expression prevents postmortem proteolytic degradation of myofibrillar and cytoskeletal proteins (Geesink, Kuchay, Chishti, & Koohmaraie, 2006). Several studies have shown that extent of calpain 1 autolysis and structural/myofibrillar protein fragmentation is closely associated to meat quality development such as tenderness and WHC (Huff-Lonergan & Lonergan, 2005; Huff-Lonergan et al., 2010). For example, a positive relationship was observed between WHC enhancement and calpain-autolysis dependent desmin degradation, implying a possibility that disruption of proteinaceous linkages via calpain 1 increased intra-myofiber space to retain free myo-water (Huff-Lonergan & Lonergan, 2005). Similarly, the proteolysis of the myofibrillar proteins might be related to the differences in the tenderness and WHC of pork (Melody et al. 2004). Less desmin degradation was found to increase muscle fiber shrinkage and consequentially greater purge losses (Davis, Sebranek, Huff-Lonergan, & Lonergan, 2004).

Factors hampering postmortem muscles to activate calpain 1 include allosteric inhibitors (calpastatin), pH, temperature, and ionic strength. Titration of these factors have been shown to affect calpain 1 autolysis and postmortem proteolysis of myofibrillar proteins, consequentially impact meat quality development (Hopkins & Thompson, 2001; Koohmaraie & Geesink, 2006). For instance, pH decline and ionic strength increase can alter hydrophilic properties of calpain and induce calpain aggregation. resulting in decreased proteolytic activity against myofibrillar proteins (Huff-Lonergan & Lonergan, 2005). Tender postmortem meat can be induced by the pre-rigor high

temperature incubation period due to an early activation of calpain 1 (Hwang & Thompson, 2001; Liu, Ruusunen, Puolanne, & Ertbjerg, 2014). High expression of calpastatin greatly inhibited proteolysis activity in mice (Kent, Spencer, & Koohmaraie, 2004) and correlated with limited postmortem proteolysis and high shear force in cattle and lamb (Geesink & Koohmaraie, 1999; Lonergan, Huff-Lonergan, Wiegand, & Kriese-Anderson, 2001). Moreover, recent studies suggest novel biochemical factors to regulate calpain autolysis and activation during muscle-meat conversion and postmortem aging, including and energy metabolism paradigm and ATP availability (Dang et al., 2020; Ramos et al., 2020), oxidation and/or nitrosylation (Liu, Li, Wang, Zhou, & Zhang, 2016; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004), and phosphorylation (Chen et al., 2016; Li et al., 2017). According to Ouali and Talmant (1990), calpastatin level was negatively associated with glycolytic features among oxidative red, glycolytic red, and glycolytic white muscles. Also, calpain activity and calpain-mediated proteolysis have been reported to vary depend upon muscle types (Delgado, Geesink, Koohmaraie, Marchello, & Goll, 2001; Veiseth, Shackelford, Wheeler, & Koohmaraie, 2001), suggesting that different metabolism background may lead calpain activity to change. It was also established that an increased oxidation level could eliminate activation of calpain 1 in muscles (Carlin, Huff-Lonergan, Rowe, & Lonergan, 2006; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004). This was probably due to that the Cys residues of calpain 1 catalytic center are susceptible targets of oxidative attack. Under oxidative stress conditions, formation of a disulfide bond between Cys115 and Cys108 were documented to down-regulate proteolytic activity (Lametsch, Lonergan, & Huff-Lonergan, 2008).

1.2.4 Muscle plasticity

Given that apoptosis and stress defending activities are likely pre-rigor, ATP consuming activities (Ouali et al., 2013), whether shifted metabolism properties would lead to alternations in (anti- or pro-) apoptosis response was under-explored in literatures. It is well established that ATP retention in fast glycolytic vs. slow oxidative muscles could be different (Watanabe, Tsuneishi, & Takimoto, 1991). Note that the pre-rigor time window before depletion of ATP is actually very short, a small delay or promote of the up-stream events could possibly make a noticeable difference in activating down-stream events. Therefore, further studies exploring pre-rigor apoptotic response should be at least focusing on two directions, 1. Whether energy availability would show any

promotion or blockage effects on apoptotic and/or anti-apoptotic systems activation; and 2. Bridging a direct causation evidence (or lack thereof) between the pre-rigor apoptotic response and metabolism and postmortem aging process.

In this regard, muscle fiber plasticity shows potential to serve as a research platform to address the aforementioned research questions. Metabolism, apoptosis, and proteolysis events can be investigated from muscles with gradually shifted metabolic backgrounds while other confounding variations can be controlled. In brief, muscle plasticity refers to the ability of muscles to modify fiber type compositions to accommodate biological, physical, or environmental alternations, usually concurrent with structural and functional changes (Lefaucheur & Gerrard, 2000). There are four types of muscle fibers: Type I → Type IIA → Type IIX → Type IIB (from left to right the fibers are getting more glycolytic fast twitch characteristics), featured by different myosin heavy chain isoforms (Aberle, Forrest, Gerrard, & Mills, 2012c). Type I is typical red muscle fiber characterized with higher oxidative metabolism properties, where type IIB is the representative white fiber type with more glycolytic energy metabolism features (Aberle et al., 2012c). Type IIA and IIX are intermediate types (Aberle et al., 2012c). The oxidative nature of red type I muscle fibers determines its higher concentration in mitochondria, myoglobin content, lipid content, and lower glycogen storage (Aberle et al., 2012c). This is the major fiber type found in muscles require long-lasting slow contractions and/or supportive muscles such as psoas major, diaphragm, and soleus (Sutherland et al., 2006), which are more resistant to fatigue. In contrary, Type IIB muscle fibers have more efficient glycolytic apparatus, lower mitochondria content, and higher glycogen storage. Muscles rich in IIB fibers have higher strength and capable of intense/fast contractions, however, are less resistant to fatigue (Aberle et al., 2012c).

Factors inducing muscle plasticity shifts can be generally classified as internal, such as muscle type, species, and genetics/breed, and external factors such as nutrition and environmental temperature (Lefaucheur & Gerrard, 2000). Some genetic mutations can switch muscle fiber composition towards the directions of either more white-glycolytic or red-oxidative. For example, callipyge gene in sheep can increase expression of calpastatin and cause muscle hypertrophy around hind quarter of the animals (Koohmaraie, Doumit, Shackelford, Lonergan, & Wheeler, 1995). It also increases type IIB fibers compared to non callipyge genotype (Fleming‑Waddell et al., 2007). In pigs, Meishan pigs are characterized with more red/oxidative fiber composition (Lefaucheur, Milan, Ecolan, & Le Callennec, 2004), whereas Halothane and RN- genes induce

more glycolytic or fast oxido-glycolytic fibers (Depreux, Grant, & Gerrard, 2002). In terms of external, on-farm management related factors, it is well documented that long-term exposure to cold temperature induce more red-oxidative fibers and enhance overall oxidative enzymes activity, whereas high ambient temperature inhibits both glycolytic and oxidative metabolism, suggesting a general reduction of energy consumption of the muscles (Lefaucheur & Gerrard, 2000).

1.2.5   Stress response impacts on animal production and meat quality

When animals are exposed to stressors, whether intrinsic or extrinsic, stress occurs, posing changes to the normal physiological equilibrium and threaten homeostatic states (Wiepkema & Koolhaas, 1993). Stress can be classified by their sources into fear, dehydration, hunger, increased fatigue, and physical injury (Xing et al., 2019), which acts as multivariate and complex influence to animal stress responses (Bianchi, Petracci, & Cavani, 2006; Dokmanović et al., 2014). From a meat quality perspective, postmortem biochemical changes are affected by preslaughter stress response that impacts animals' physiological and metabolic functions (Aberle et al., 2012a). These stress responses could affect many physiological activities including energy homeostasis, intracellular ion dynamics, protease system, and protein integrity in skeletal muscle (Ouali et al., 2006; Scheffler & Gerrard, 2007), all of which affect muscle-meat conversion. The effects of various stressors affecting meat quality characteristics have been depicted by a number of reviews for poultry, swine, and ruminants (Ali, Kang, & Joo, 2008; Ferguson & Warner, 2008; Schwartzkopf-Genswein et al., 2012), concluding that animal welfare and meat quality development are greatly affected by the type, duration, and intensity of the stressors and the susceptibility of the animal to the stresses. It also has been shown that severe preslaughter stress can cause significant meat quality defects such as bruises, ecchymosis (blood splash and spotting), and increased pathogen transfection (Barbut et al., 2008; Bianchi et al., 2006), leading economic losses to the meat producers. However, more details remain to be clarified in terms of specific stressors impacts on biochemical changes and consequential variations in meat quality. Especially, it is reasonable to postulate that as animals get more towards finishing period, acute stress is more likely to influence final meat quality attributes. Hence, most studies focused on long-term stress or sensitive events that occurred during finishing or marketing period, whereas little information is available regarding how early-life stress would have long term impact on meat quality and

productivity attributes. Due to the specific research interest of the present study, the following sections will be primarily focusing on weaning and transport stress. Impact of weaning and transporting on animal growth and production outcomes and the possibility of using L-glutamine as mitigation strategy will be discussed.

1.3   Weaning and transporting stress effects on pig production and meat quality

1.3.1   Weaning

Multi-site production system is widely applied due to the potential benefits of infectious pathogen reduction and growth performance enhancement, necessitating the need of transporting weaning pigs from their mothers to separated growing facilities (Harris, 2008). As such, concurrent stressors from weaning and transporting could have cumulative effects on piglets, leading to harmful consequences in pig production and welfare (Lewis & Berry, 2006).

Both weaning and transporting are recognized as complexed stress situation for farm animals. Weaning comprises mixed stressors such as withdraw of maternal care, abrupt social environmental changes, mixing with unfamiliar cohorts, and most apparently, a drastic alternation from a whole milk liquid diet to low quality solid feed (Niekamp, Sutherland, Dahl, & Salak-Johnson, 2007). These stressors alter physiological response and reduce feed and liquid intake of pigs (Colson, Orgeur, Foury, & Mormède, 2006; Hay et al., 2001). Weaning changes intestinal morphology, such as reducing villus height and increasing villus width and crypt depth (Moeser et al., 2007; Wijtten, van der Meulen, & Verstegen, 2011), along with increased intestinal permeability and mucosal inflammation (Bomba et al., 2014; Mccracken, Spurlock, Roos, Zuckermann, & Gaskins, 1999). Meanwhile, cessation of mother milk supply stopped weaned pigs from receiving passive immunity from the sow, whereas immature immune system alone is facing elevated pathogen challenge due to compromised gut barrier function (Campbell et al., 2013; Lallès et al., 2004), leading nursery pigs to be more susceptible to microbial challenges with a higher incidence of diarrhea and diseases (Pluske, Hampson, & Williams, 1997; Wijtten et al., 2011). As a result, piglet inflammatory cytokines are increased (Pié et al., 2004). Given that clinical and subclinical immune activation consumes a large portion of absorbed glucose (Huntley,

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Nyachoti, & Patience, 2017), nutrient absorption and utilization can be markedly decreased, eliminating available resources to support growth and maintenance.

Weaning pigs can loss 100g to 250g BW after the first day of weaning and such loss generally takes 4 days to recover(Campbell, Crenshaw, & Polo, 2013). Compared to pigs maintained on the sow, weaning at 21-day reduced feed intake (−36%), BW gain (−47%) and small intestine weight (−26%) after 7 days post-weaning (Wang et al., 2008). Suppressed growth in early life can have long term effect on lean/fat deposition patterns: Wolter and Ellis (2001) found that pigs weaned to a conventional starter diets show a slower rate of weight gain, compared to pigs weaned to a milk-based easy digestible diet. Conventional diets also decrease final BW and lean deposition and increase BF when pigs are market ready (Wolter & Ellis, 2001). Therefore, optimizing weaning management can, to a certain extent, improve future production outcomes. In terms of meat quality development, effects of different weaning management conditions are mostly studied in ruminants, but it seems that weaning management is not a significant source of meat quality variation (Blanco, Villalba, Ripoll, Sauerwein, & Casasús, 2009; Cañeque et al., 2001; Dalle Zotte, Rémignon, & Ouhayoun, 2005; Oliete et al., 2006; Sañudo et al., 1998).

1.3.2 Transport

Transport is the most stressful event in the operation chain of livestock production, lead to significantly compromised animal welfare and huge production losses (Chambers, Grandin, Heinz, & Srisuvan, 2001). Pigs can be transported several times through their lifespan, mainly for reasons such as re-locating near feed sources, re-stocking, marketing, and slaughter (Chambers et al., 2001). Several studies have argued that the same (or similar) set of factors contribute to the stress in transporting piglets and adult pigs to the slaughterhouse (for review, see Roldan-Santiago et al., 2013). To cope with vehicle vibration, muscle contraction is a must to maintain balance and posture. From this perspective, transporting durations, trailer design, and floor setting among other factors such as road construction and driver skills level could directly related to the chance for a pig to develop fatigue and exhaustion (Pérez et al., 2002).

Transport effects on meat quality development was mostly implied during pre-slaughter stage. To cope with vehicle vibration, excessive muscle contraction could lead to increased cortisol, excessive glycolysis, fatigue and in severe case non-ambulatory pigs (Nyberg, Lundström, Edfors-

Lilja, & Rundgren, 1988; Ritter et al., 2009; Zhang et al., 2009). Non-ambulatory pigs, though have no food safety issue, are facing pressures from public and legislative entities to be banned from entering food supply chain (Dalla Costa et al., 2019; Repphun, 2011). Moreover, energy expenditure from muscle contraction escalates glycolysis, release acidic product - lactate and pyruvate - into plasma, leading to disrupted homeostasis. Upon slaughter, disrupted peri-mortal energy metabolism interferes with carcass temperature and pH drop, increasing the possibility to develop PSE-like quality defects (Guàrdia et al., 2004). Improper trailer density (too high or too low), slippery floor, and lack of bedding may lead to fight or falling on the ground (Ritter et al., 2006), causing quality defects such as broken bones and skin bruise (Dalla Costa et al., 2007).

1.3.3   L-glutamine

Usually, management protocols during post – transport stage of weaning pigs utilize dietary antibiotics to help with stress recovery. However, with increasing concerns of antibiotic abuse and resistance, public voices and legislative drives are calling for nutraceutical alternatives to reduce antibiotic use (Landers, Cohen, Wittum, & Larson, 2012). Currently, the technical strategies in developing antibiotic replacements includes but not limited to strengthening gut health, reducing pathogen load, and enhancing competitor microbes for pathogens (Mingmongkolchai & Panbangred, 2018; Papatsiros et al., 2013; Wen, 2018). L-glutamine as a promising example, have been identified as a metabolism modulator to mitigate catabolic status (Wu & Thompson, 1990) and an efficient energy source of intestinal epithelium cells (Wu, Knabe, Yan, & Flynn, 1995). Therefore, L-glutamine use might be effective in alleviating post weaning and transport damage by enhancing gut barrier health and function and strengthening immune system.

1.3.3.1 L-glutamine metabolism in skeletal muscles

As an l-α-amino acid, R group of L-glutamine is denoted as $NH_2\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}$. It is considered as the most abundant AA in the body because it counts for 40%-60% of the AA pool in skeletal muscle (Cruzat, 2019). Under non-stressed conditions, L-glutamine is counted as non-essential amino acid (Cruzat, 2019). Under catabolic situations such as disease, stress, surgery or extended exercise, cellular requirements for L-glutamine could exceeds innate synthesis capacity, and L-glutamine supplementation becomes necessary (Cruzat, 2019; B. Wang et al., 2015). Dietary L-glutamine requirement can increase when individuals are facing stress conditions (Cruzat,

Pantaleão, Donato Jr, De Bittencourt Jr, & Tirapegui, 2014; Rogero et al., 2008). Therefore, it is nowadays classified as conditionally essential amino acid (Cruzat, 2019).

Although many enzymes and biochemical pathways are involved in L-glutamine metabolism, its bio-synthesis and hydrolysis are mainly occurred through the following two reactions:

$$\text{Glutamate} + NH_3 + \text{ATP} \xleftrightarrow{GS} \text{Glutamine} + \text{ADP} + \text{Pi} \quad (3)$$

$$\text{Glutamine} + H_2O \xrightarrow{GLS} \text{Glutamate} + NH_4^+ \quad (4)$$
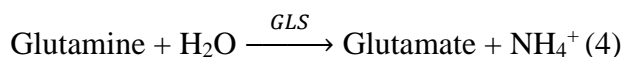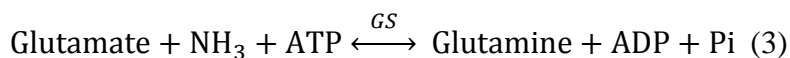
In reaction (3), glutamine synthetase (GS) catalyzed the condensation of glutamate and ammonia to form glutamine through an acyl-phosphate intermediate. Hydrolysis of glutamine, which is catalyzed by glutaminase (GLS), generates glutamine and ammonium ion. Both enzymes are ubiquitously presented in most tissues, whereas one of the enzymes, either GS or GLS, is higher in activity than the other to avoid futile cycle (Cruzat, 2019).To some extent, skeletal muscles play a pivot role in modulating whole body glutamine availability. In skeletal muscles, GS is predominant in activity than GLS (Cruzat, 2019). Glutamine synthesis in skeletal muscle cells takes the following steps: first, glutamate is produced via branched chain amino acid (BCAA) transamination, along which α-ketoglutarate is consumed and branched chain keto acids are generated. Then glutamine incorporate free ammonia to synthesize glutamine via catalytic activity of GS.

The synthesized L-glutamine and be transferred to several organs and tissues for different metabolic purpose. Especially, glutamine hydrolysis generates glutamate, which is further converted to intermediate metabolites of TCA cycle, including α-ketoglutarate, succinate, fumarate, malate or even pyruvate. These metabolites can be used to produce a wide range of nucleotides, lipids, and amino acids to support cellular structure and activities, especially in those predominantly glutamine consuming tissues such as small intestine, immune system, and kidney (Cruzat, 2019). L-glutamine can serve as a nitrogen carrier, transfers non-toxic form of ammonia, and helps maintaining acid-base balance by participating in urea production and HCO_3^- reabsorption (Cruzat, 2019). The 5-carbon skeleton derived from glutamine hydrolysis can be utilized as for energy supply, especially in small intestine enterocytes and immune system. In catabolic situations, whole body demand of glutamine increases markedly. To support glutamine supply, excessive proteolysis is expected in skeletal muscles.

1.3.3.2 Use L-glutamine as a growth promoter

In previous research, L-glutamine supplementation as a means of nutritional support are commonly beneficial, shown as promoted growth rate (Yi et al., 2005), improved stress defending system (Wischmeyer et al., 2001), and strengthened gut barrier health (Wang et al., 2015; H. Wang et al., 2015). Researchers found that L-glutamine can be effective in improving growth performance as a 1% food supplement (Wu, Meier, & Knabe, 1996) in nursery diets. The effects of L-glutamine following a health challenge was evaluated by Yi et al., (2005). The experiment design is to feed pigs with 2% added L-glutamine for 12 d postweaning, then orally challenge the pigs with E. coli K88+ (Yi et al., 2005). The ADG of L-glutamine pigs was equal to non-challenged pig at 48 h after the challenge and showed better growth performance than pigs without L-glutamine (Yi et al., 2005). Improvements of feed efficiency and intestinal morphology in pigs supplemented with L-glutamine were also found compared to non-glutamine supplemented animals. Recent researches investigated the possibility to use L-glutamine as an antibiotic alternative in nursery diets (Johnson & Lay Jr, 2017). The trials supplemented L-glutamine at 0.20% for the first 14 d post-weaning and found improved growth performance compared to pigs without supplements or with dietary antibiotics (Duttlinger, Kpodo, Lay Jr, Richert, & Johnson, 2019; Johnson & Lay Jr, 2017).

1.3.3.3 L-glutamine physiological functions and benefits

Below listed several perspectives of L-glutamine to support immune system and enhance cellular anti-stress activities. In brief, the major protective function of L-glutamine includes being the precursor to support antioxidant defense system, modulating anti-stress proteins, and supporting immune and intestinal systems (Cruzat et al., 2014).

Support antioxidative system: There are two major way for L-glutamine to support antioxidant defense system of the cells: facilitate generation of NADPH and facilitate generation of glutathione. Generation of NADPH is mainly through conversion between malate and pyruvate, whereas glutamic acid is the most important precursor for malate replenish (Newsholme et al., 2003). A high L-glutamine/glutamic acid ratio in cell favors glutamic acid generation to support this biochemical reaction. Similarly, generation of glutathione is depending on glutamic acid (glutamic acid), cysteine, glycine, and NADPH availability (Newsholme et al., 2003). Maintaining

a high L-glutamine/glutamic acid ratio will favor glutathione generation therefore non-enzymatic antioxidant power of the cells (Cruzat et al., 2014).

Modulate heat shock protein expression: L-glutamine supplementation enhance HSP70 and HSP27 expression (Wischmeyer et al., 2001). Recent evidence indicate that metabolism of L-glutamine can enhance expression of heat shock factor 1 (HSF1) to mediate HSP70 expression, whereas induction of HSP70 expression showed protective activity in chemical challenged colon epithelial cells (Akagi et al., 2013). As previously discussed, HSP70 play vital role in cell protection under stress condition include inhibit apoptotic signaling pathway (Jäättelä, Wissing, Kokholm, Kallunki, & Egeblad, 1998), protect protein from misfolding or unfolding (Duncan, Cheetham, Chapple, & Van Der Spuy, 2015), protect cytoskeletal structures, and maintain antioxidant defense system (Li et al., 2011).

Support immune system: Overall the activated immune system/cells are of high oxidative status, whereas L-glutamine supplementation can help to maintain several activated immune cell functions include neutrophils, macrophage, and lymphocytes (Cruzat, 2019). In neutrophils, recognizing and killing pathogens mainly depends on "oxidative burst". Once this immune cell is activated, L-glutamine requirement is high because "oxidative burst" require large amount of NADPH to support NADPH oxidase enzyme activity to maintain the high oxidative environment, while L-glutamine is the major source for NADPH generation (Garcia et al., 1999; Pithon-Curi, Levada, Lopes, Doi, & Curi, 2002). In activated immune system, lymphocytes B and T cells can utilize L-glutamine as energy source (Ardawi & Newsholme, 1983; Newsholme et al., 1999). Compared to glucose, of which energy metabolism route starts from glycolysis, L-glutamine can be converted to multiple TCA intermediates, providing multiple sites for L-glutamine to enter the cycle directly, sustain a rapid ATP generation to support a surge of cell communication and proliferation activities (Cruzat, 2019).

Gut health: Gastrointestinal system contains a major reservoir of immune cells. Supportive function of L-glutamine supplementation has been discussed. It is also evident that L-glutamine is the major energy source for intestinal epithelial cells to support cell proliferation (Wu et al., 1995). L-glutamine enhance tight junction protein expression to maintain stronger gut barrier (Wang et al., 2015).

1.4 Objective

As discussed by aforementioned sections, it is necessary to update the concept of meat quality development by establishing linkages between apoptosis, energy metabolism, stress defending system and articulate in-between interactions on postmortem proteolysis. Therefore, the primary study objective of this dissertation is to evaluate postmortem changes of small heat shock proteins, apoptosis features, myofibrillar proteolysis, and metabolic characteristics in postmortem skeletal muscles under different metabolic, genetic, or management conditions across species.

1.5 Literatures cited

Abdullah, A. Y., & Qudsieh, R. I. (2009). Effect of slaughter weight and aging time on the quality of meat from Awassi ram lambs. *Meat Science, 82* (3), 309-316.

Aberle, E. D., Forrest, J. C., Gerrard, D. E., & Mills, E. W. (2012a). Conversion of Muscle to Meat: Biochemistry of Meat Quality *Principles of meat science* (pp. 97-129). Iowa: Kendall Hunt Publishing.

Aberle, E. D., Forrest, J. C., Gerrard, D. E., & Mills, E. W. (2012b). Muscle contraction and energy metabolism *Principles of meat science* (pp. 61-74). Iowa: Kendall Hunt Publishing.

Aberle, E. D., Forrest, J. C., Gerrard, D. E., & Mills, E. W. (2012c). Structure, composition, and development of animal tissues *Principles of meat science* (pp. 7-60). Iowa: Kendall Hunt Publishing.

Akagi, R., Ohno, M., Matsubara, K., Fujimoto, M., Nakai, A., & Inouye, S. (2013). L-glutamine protects intestinal barrier function of colon epithelial cells from ethanol by modulating Hsp70 expression. *Pharmacology, 91* (1-2), 104-111.

Ali, M., Kang, G.-H., & Joo, S. T. (2008). A review: Influences of pre-slaughter stress on poultry meat quality. *Asian-Australasian Journal of Animal Sciences, 21* (6), 912-916.

Ardawi, M. S. M., & Newsholme, E. A. (1983). L-glutamine metabolism in lymphocytes of the rat. *Biochemical Journal, 212* (3), 835-842.

Ba, H. V., Park, K., Dashmaa, D., & Hwang, I. (2014). Effect of muscle type and vacuum chiller ageing period on the chemical compositions, meat quality, sensory attributes and volatile compounds of Korean native cattle beef. *Animal science journal, 85* (2), 164-173.

Barbut, S., Sosnicki, A., Lonergan, S., Knapp, T., Ciobanu, D. C., Gatcliffe, L.,... Wilson, E. (2008). Progress in reducing the pale, soft and exudative (PSE) problem in pork and poultry meat. *Meat science, 79* (1), 46-63.

Bendall, J. (1960). Postmortem changes in muscle. *The structure and function of muscle, 2* (Part 1), 243-309.

Berry, R., & Lewis, N. (2001). The effect of duration and temperature of simulated transport on the performance of early-weaned piglets. *Canadian Journal of Animal Science, 81* (2), 199-204.

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2018). Role of calpain system in meat tenderness: A review. *Food Science and Human Wellness*, *7* (3), 196-204.

Bianchi, M., Petracci, M., & Cavani, C. (2006). The influence of genotype, market live weight, transportation, and holding conditions prior to slaughter on broiler breast meat color. *Poultry science, 85* (1), 123-128.

Blanco, M., Villalba, D., Ripoll, G., Sauerwein, H., & Casasús, I. (2009). Effects of early weaning and breed on calf performance and carcass and meat quality in autumn-born bull calves. Livestock Science, 120(1-2), 103-115.

Bomba, L., Minuti, A., Moisá, S. J., Trevisi, E., Eufemi, E., Lizier, M.,... Prandini, A. (2014). Gut response induced by weaning in piglet features marked changes in immune and inflammatory response. *Functional & integrative genomics, 14* (4), 657-671.

Bowker, B., Grant, A., Forrest, J., & Gerrard, D. (2000). Muscle metabolism and PSE pork. *Journal of Animal Science, 79* (1), 1-8.

Bratcher, C. L., Johnson, D. D., Littell, R. C., & Gwartney, B. L. (2005). The effects of quality grade, aging, and location within muscle on Warner–Bratzler shear force in beef muscles of locomotion. *Meat Science, 70* (2), 279-284.

Brewer, S., & Novakofski, J. (2008). Consumer sensory evaluations of aging effects on beef quality. *Journal of food science, 73* (1).

Campbell, J. M., Crenshaw, J. D., & Polo, J. (2013). The biological stress of early weaned piglets. *Journal of animal science and biotechnology, 4* (1), 19.

Campo, M., Sañudo, C., Panea, B., Alberti, P., & Santolaria, P. (1999). Breed type and ageing time effects on sensory characteristics of beef strip loin steaks. *Meat science, 51* (4), 383-390.

Cañeque, V., Velasco, S., Diaz, M., Pérez, C., Huidobro, F., Lauzurica, S., . . . Gonzalez, J. (2001). Effect of weaning age and slaughter weight on carcass and meat quality of Talaverana breed lambs raised at pasture. Animal Science, 73(1), 85-95.

Carlin, K. M., Huff-Lonergan, E., Rowe, L., & Lonergan, S. M. (2006). Effect of oxidation, pH, and ionic strength on calpastatin inhibition of μ-and calpain 2. *Journal of Animal Science, 84* (4), 925-937.

Chambers, P., Grandin, T., Heinz, G., & Srisuvan, T. (2001). Guidelines for humane handling, transport and slaughter of livestock. *RAP Publication (FAO).*

Chen, L., Li, X., Ni, N., Liu, Y., Chen, L., Wang, Z., . . . Zhang, D. (2016). Phosphorylation of myofibrillar proteins in post-mortem ovine muscle with different tenderness. Journal of the Science of Food and Agriculture, 96(5), 1474-1483.

Colle, M., Nasados, J., Rogers, J., Kerby, D., Colle, M., Van Buren, J.,... Doumit, M. (2018). Strategies to improve beef tenderness by activating calpain-2 earlier postmortem. *Meat science, 135*, 36-41.

Colle, M., Richard, R., Killinger, K., Bohlscheid, J., Gray, A., Loucks, W.,... Doumit, M. (2016). Influence of extended aging on beef quality characteristics and sensory perception of steaks from the biceps femoris and semimembranosus. *Meat science, 119*, 110-117.

Cong, J., Goll, D., Peterson, A., & Kapprell, H. (1989). The role of autolysis in activity of the Ca2+-dependent proteinases (mu-calpain and calpain 2). *Journal of Biological Chemistry, 264* (17), 10096-10103.

Cruzat, V. F. (2019). L-glutamine and Skeletal Muscle *Nutrition and Skeletal Muscle* (pp. 299-313): Elsevier.

Cruzat, V. F., Pantaleão, L. C., Donato Jr, J., de Bittencourt Jr, P. I. H., & Tirapegui, J. (2014). Oral supplementations with free and dipeptide forms of L-glutamine in endotoxemic mice: effects on muscle L-glutamine -glutathione axis and heat shock proteins. *The Journal of nutritional biochemistry, 25* (3), 345-352.

Dalla Costa, O., Faucitano, L., Coldebella, A., Ludke, J., Peloso, J., Dalla Roza, D., & da Costa, M. P. (2007). Effects of the season of the year, truck type and location on truck on skin bruises and meat quality in pigs. *Livestock science, 107* (1), 29-36.

Dalla Costa, O. A., Dalla Costa, F. A., Feddern, V., dos Santos Lopes, L., Coldebella, A., Gregory, N. G., & de Lima, G. J. M. M. (2019). Risk factors associated with pig pre-slaughtering losses. *Meat science, 155*, 61-68.

Dalle Zotte, A., Rémignon, H., & Ouhayoun, J. (2005). Effect of feed rationing during post-weaning growth on meat quality, muscle energy metabolism and fibre properties of Biceps femoris muscle in the rabbit. Meat Science, 70(2), 301-306.

Dang, D. S., Buhler, J. F., Davis, H. T., Thornton, K. J., Scheffler, T. L., & Matarneh, S. K. (2020). Inhibition of mitochondrial calcium uniporter enhances postmortem proteolysis and tenderness in beef cattle. Meat Science, 162, 108039.

Dashdorj, D., Tripathi, V. K., Cho, S., Kim, Y., & Hwang, I. (2016). Dry aging of beef; Review. *Journal of animal science and technology, 58* (1), 20.

Davis, K. J., Sebranek, J. G., Huff-Lonergan, E., & Lonergan, S. M. (2004). The effects of aging on moisture-enhanced pork loins. *Meat science, 66* (3), 519-524.

de Lima Silva, M., Contreras-Castillo, C. J., & Ortega, E. M. M. (2007). Efeito do cozimento na qualidade do músculo Semitendinosus. *Ciência e Tecnologia de Alimentos , 27* (3).

Delgado, E. F., Geesink, G. H., Koohmaraie, M., Marchello, J. A., & Goll, D. E. (2001). The calpain system in three muscles of normal and callipyge sheep. *Journal of Animal Science, 79* (2), 398-412. doi: 10.2527/2001.792398x

Deng, L., Yang, G., Zhang, C., Zhang, Z., Xu, L., & He, C. (2013). Variation in expression of HSPs and their corresponding mRNA transcripts in the peripheral blood lymphocytes of beef cattle by transportation durations. *Journal of Animal and Veterinary Advances, 12* (5), 612-617.

Depreux, F., Grant, A., & Gerrard, D. (2002). Influence of halothane genotype and body-weight on myosin heavy chain composition in pig muscle as related to meat quality. *Livestock Production Science, 73* (2-3), 265-273.

Descalzo, A. M., Rossetti, L., Sancho, A. M., García, P., Biolatto, A., Carduza, F., & Grigioni, G. (2008). Antioxidant consumption and development of oxidation during ageing of buffalo meat produced in Argentina. *Meat Science, 79* (3), 582-588.

Devine, C. E., & Klinth, W. J. (2004). CONVERSION OF MUSCLE TO MEAT | Ageing *Encyclopedia of Meat Sciences* (pp. 330-338). Oxford: Elsevier.

Di Luca, A., Elia, G., Mullen, A. M., & Hamill, R. M. (2013). Monitoring post mortem changes in porcine muscle through 2-D DIGE proteome analysis of Longissimus muscle exudate. *Proteome science, 11* (1), 1.

Di Luca, A., Hamill, R. M., Mullen, A. M., Slavov, N., & Elia, G. (2016). Comparative proteomic profiling of divergent phenotypes for water holding capacity across the post mortem ageing period in porcine muscle exudate. *PloS one, 11* (3), e0150605.

Dokmanović, M., Velarde, A., Tomović, V., Glamočlija, N., Marković, R., Janjić, J., & Baltić, M. (2014). The effects of lairage time and handling procedure prior to slaughter on stress and meat quality parameters in pigs. *Meat science, 98* (2), 220-226.

Duncan, E. J., Cheetham, M. E., Chapple, J. P., & van der Spuy, J. (2015). The role of HSP70 and its co-chaperones in protein misfolding, aggregation and disease *The networking of chaperones by co-chaperones* (pp. 243-273): Springer.

Dutson, T. R., Smith, G. C., & Carpenter, Z. L. (1980). LYSOSOMAL ENZYME DISTRIBUTION IN ELECTRICALLY STIMULATED OVINE MUSCLE. *Journal of Food Science, 45* (4), 1097-1098. doi: 10.1111/j.1365-2621.1980.tb07533.x

Duttlinger, A. W., Kpodo, K. R., Lay Jr, D. C., Richert, B. T., & Johnson, J. S. (2019). Replacing dietary antibiotics with 0.20% l-glutamine in swine nursery diets: impact on health and productivity of pigs following weaning and transport. *Journal of animal science, 97* (5), 2035-2052.

England, E., Matarneh, S., Scheffler, T., & Gerrard, D. (2017). Perimortal muscle metabolism and its effects on meat quality *New aspects of meat quality* (pp. 63-89): Elsevier.

England, E. M., Matarneh, S., Scheffler, T., Wachet, C., & Gerrard, D. (2015). Altered AMP deaminase activity may extend postmortem glycolysis. *Meat science, 102*, 8-14.

England, E. M., Matarneh, S. K., Scheffler, T. L., Wachet, C., & Gerrard, D. E. (2014). pH inactivation of phosphofructokinase arrests postmortem glycolysis. *Meat science, 98* (4), 850-857.

Farouk, M. M., Mustafa, N. M., Wu, G., & Krsinic, G. (2012). The "sponge effect" hypothesis: An alternative explanation of the improvement in the waterholding capacity of meat with ageing. *Meat Science, 90* (3), 670-677. doi: 10.1016/j.meatsci.2011.10.012

Ferguson, D., & Warner, R. D. (2008). Have we underestimated the impact of pre-slaughter stress on meat quality in ruminants? *Meat science, 80* (1), 12-19.

Fleming‐Waddell, J. N., Wilson, L. M., Olbricht, G. R., Vuocolo, T., Byrne, K., Craig, B. A.,... Bidwell, C. A. (2007). Analysis of gene expression during the onset of muscle hypertrophy in callipyge lambs. *Animal genetics, 38* (1), 28-36.

Garcia, C., Pithon-Curi, T. C., de Loudes Firmano, M., Pires de Melo, M., Newsholme, P., & Curi, R. (1999). Effects of adrenaline on glucose and L-glutamine metabolism and superoxide production by rat neutrophils. *Clinical science, 96* (6), 549-555.

Gareau, M. G., Jury, J., & Perdue, M. H. (2007). Neonatal maternal separation of rat pups results in abnormal cholinergic regulation of epithelial permeability. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 293* (1), G198-G203.

Geesink, G. H., & Koohmaraie, M. (1999). Postmortem proteolysis and calpain/calpastatin activity in callipyge and normal lamb biceps femoris during extended postmortem storage. *Journal of Animal Science, 77* (6), 1490-1501.

Geesink, G. H., Kuchay, S., Chishti, A. H., & Koohmaraie, M. (2006). μ-Calpain is essential for postmortem proteolysis of muscle proteins. *J. Anim Sci., 84* (10), 2834-2840. doi: 10.2527/jas.2006-122

Goll, D. E., Thompson, V. F., Li, H., Wei, W., & Cong, J. (2003). The Calpain System. *Physiological Reviews, 83* (3), 731-801. doi: 10.1152/physrev.00029.2002

Gruber, S. L., Tatum, J. D., Scanga, J. A., Chapman, P. L., Smith, G. C., & Belk, K. E. (2006). Effects of postmortem aging and USDA quality grade on Warner-Bratzler shear force values of seventeen individual beef muscles. *Journal of Animal Science, 84* (12), 3387-3396. doi: 10.2527/jas.2006-194

Guàrdia, M., Estany, J., Balasch, S., Oliver, M., Gispert, M., & Diestre, A. (2004). Risk assessment of PSE condition due to pre-slaughter conditions and RYR1 gene in pigs. *Meat Science, 67* (3), 471-478.

Guzek, D., Glabska, D., Wierzbicki, A., Wierzbicki, J., & Cierach, M. (2012). Relationship between basic beef texture attributes and their perception by Polish consumers. *Italian Journal of Food Science, 24* (3), 231.

Harris, D. L. (2008). *Multi-site pig production*: John Wiley & Sons.

Hendrick, J. P., & Hartl, F.-U. (1993). Molecular chaperone functions of heat-shock proteins. *Annual review of biochemistry, 62* (1), 349-384.

Herrera-Mendez, C. H., Becila, S., Boudjellal, A., & Ouali, A. (2006). Meat ageing: Reconsideration of the current concept. *Trends in Food Science & Technology, 17* (8), 394-405. doi: http://dx.doi.org/10.1016/j.tifs.2006.01.011

Honikel, K. O., & Werner Klinth, J. (2004). CONVERSION OF MUSCLE TO MEAT | Glycolysis *Encyclopedia of Meat Sciences* (pp. 314-318). Oxford: Elsevier.

Hopkins, D. L., & Thompson, J. M. (2001). The relationship between tenderness, proteolysis, muscle contraction and dissociation of actomyosin. *Meat Science, 57* (1), 1-12. doi: 10.1016/s0309-1740 (00)00065-6

Huang, M., Huang, F., Xu, X., & Zhou, G. (2009). Influence of caspase3 selective inhibitor on proteolysis of chicken skeletal muscle proteins during post mortem aging. *Food Chemistry, 115* (1), 181-186.

Huff-Lonergan, E., & Lonergan, S. M. (2005). Mechanisms of water-holding capacity of meat: The role of postmortem biochemical and structural changes. *Meat Science, 71* (1), 194-204.

Huff-Lonergan, E., Mitsuhashi, T., Beekman, D. D., Parrish, F. C., Jr., Olson, D. G., & Robson, R. M. (1996). Proteolysis of specific muscle structural proteins by mu-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. *Journal of Animal Science, 74* (5), 993-1008.

Huff-Lonergan, E., Zhang, W., & Lonergan, S. M. (2010). Biochemistry of postmortem muscle — Lessons on mechanisms of meat tenderization. *Meat Science, 86* (1), 184-195. doi: 10.1016/j.meatsci.2010.05.004

Hughes, J., Oiseth, S., Purslow, P., & Warner, R. (2014). A structural approach to understanding the interactions between colour, water-holding capacity and tenderness. *Meat science, 98* (3), 520-532.

Huntley, N. F., Nyachoti, C. M., & Patience, J. F. (2017). 145 Immune system stimulation increases nursery pig maintenance energy requirements. Journal of Animal Science, 95(suppl_2), 68-69.

Hutchison, S. (2007). *Improving the Value of Cull Cows Through Antemortem Management Practices and Postmortem Enhancement Technologies.* PhD Dissertation, Kansas State University, Manhattan, Kansas.

Hwang, I. H., & Thompson, J. M. (2001). The interaction between pH and temperature decline early postmortem on the calpain system and objective tenderness in electrically stimulated beef longissimus dorsi muscle. *Meat Science, 58* (2), 167-174. doi: 10.1016/s0309-1740 (00)00147-9

Jäättelä, M., Wissing, D., Kokholm, K., Kallunki, T., & Egeblad, M. (1998). Hsp70 exerts its anti‐apoptotic function downstream of caspase‐3‐like proteases. *The EMBO journal, 17* (21), 6124-6134.

Jenkins, K., Vasconcelos, J., Hinkle, J., Furman, S., de Mello, A., Senaratne, L.,... Calkins, C. (2011). Evaluation of performance, carcass characteristics, and sensory attributes of beef from finishing steers fed field peas 1. *Journal of animal science, 89* (4), 1167-1172.

Jia, X., Hollung, K., Therkildsen, M., Hildrum, K. I., & Bendixen, E. (2006). Proteome analysis of early post‐mortem changes in two bovine muscle types: M. longissimus dorsi and M. semitendinosis. *Proteomics, 6* (3), 936-944.

Johnson, J., & Lay Jr, D. (2017). Evaluating the behavior, growth performance, immune parameters, and intestinal morphology of weaned piglets after simulated transport and heat stress when antibiotics are eliminated from the diet or replaced with L-glutamine. *Journal of animal science, 95* (1), 91-102.

Kastenschmidt, L., Hoekstra, W., & Briskey, E. (1968). Glycolytic intermediates and co‐factors in ʺfast‐ʺ and ʺslow‐glycolyzingʺ muscles of the pig. *Journal of Food Science, 33* (2), 151-158.

Kemp, C., Bardsley, R., & Parr, T. (2006). Changes in caspase activity during the postmortem conditioning period and its relationship to shear force in porcine longissimus muscle. *Journal of animal science, 84* (10), 2841-2846.

Kemp, C. M., Sensky, P. L., Bardsley, R. G., Buttery, P. J., & Parr, T. (2010). Tenderness – An enzymatic view. *Meat Science, 84* (2), 248-256. doi: https://doi.org/10.1016/j.meatsci.2009.06.008

Kent, M., Spencer, M., & Koohmaraie, M. (2004). Postmortem proteolysis is reduced in transgenic mice overexpressing calpastatin. *Journal of Animal Science, 82* (3), 794-801.

Kim, Y. H., Lonergan, S. M., & Huff-Lonergan, E. (2010). Protein denaturing conditions in beef deep semimembranosus muscle results in limited calpain 1 activation and protein degradation. *Meat Science, 86* (3), 883-887.

Kim, Y. H. B., Frandsen, M., & Rosenvold, K. (2011). Effect of ageing prior to freezing on colour stability of ovine longissimus muscle. *Meat Science, 88* (3), 332-337. doi: DOI: 10.1016/j.meatsci.2010.12.020

Kim, Y. H. B., Warner, R. D., & Rosenvold, K. (2014). Influence of high pre-rigor temperature and fast pH fall on muscle proteins and meat quality: a review. *Animal Production Science, 54*, 375-395. doi: http://dx.doi.org/10.1071/AN13329

King, D., Shackelford, S., Kalchayanand, N., & Wheeler, T. (2012). Sampling and aging effects on beef longissimus color stability measurements. *Journal of Animal Science, 90* (10), 3596-3605.

Koohmaraie, M. (1992). Ovine skeletal muscle multicatalytic proteinase complex (proteasome): purification, characterization, and comparison of its effects on myofibrils with μ-calpains. *Journal of animal science, 70* (12), 3697-3708.

Koohmaraie, M. (1996). Biochemical factors regulating the toughening and tenderization processes of meat. *Meat Science, 43* (Supplement 1), 193-201.

Koohmaraie, M., Doumit, M. E., Shackelford, S. D., Lonergan, S. M., & Wheeler, T. L. (1995). A muscle hypertrophy condition in lamb (callipyge): characterization of effects on muscle growth and meat quality traits1. *Journal of Animal Science, 73* (12), 3596-3607. doi: 10.2527/1995.73123596x

Koohmaraie, M., & Geesink, G. H. (2006). Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. *Meat Science, 74* (1), 34-43. doi: http://dx.doi.org/10.1016/j.meatsci.2006.04.025

Kötter, S., Unger, A., Hamdani, N., Lang, P., Vorgerd, M., Nagel-Steger, L., & Linke, W. A. (2014). Human myocytes are protected from titin aggregation-induced stiffening by small heat shock proteins. *The Journal of Cell Biology, 204* (2), 187-202. doi: 10.1083/jcb.201306077

Koutsidis, G., Elmore, J., Oruna-Concha, M. J., Campo, M. M., Wood, J. D., & Mottram, D. (2008). Water-soluble precursors of beef flavour. Part II: Effect of post-mortem conditioning. *Meat science, 79* (2), 270-277.

Kristensen, L., & Purslow, P. P. (2001). The effect of ageing on the water-holding capacity of pork: role of cytoskeletal proteins. *Meat science, 58* (1), 17-23.

Lallès, J.-P., Boudry, G., Favier, C., Le Floc'h, N., Luron, I., Montagne, L.,... Sève, B. (2004). Gut function and dysfunction in young pigs: physiology. *Animal Research, 53* (4), 301-316.

Lametsch, R., Lonergan, S., & Huff-Lonergan, E. (2008). Disulfide bond within calpain 1 active site inhibits activity and autolysis. *Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics, 1784* (9), 1215-1221.

Lametsch, R., Roepstorff, P., & Bendixen, E. (2002). Identification of Protein Degradation during Post-mortem Storage of Pig Meat. *Journal of Agricultural and Food Chemistry, 50* (20), 5508-5512. doi: 10.1021/jf025555n

Landers, T. F., Cohen, B., Wittum, T. E., & Larson, E. L. (2012). A review of antibiotic use in food animals: perspective, policy, and potential. *Public health reports, 127* (1), 4-22.

Laville, E., Sayd, T., Morzel, M., Blinet, S., Chambon, C., Lepetit, J.,... Hocquette, J. F. (2009). Proteome changes during meat aging in tough and tender beef suggest the importance of apoptosis and protein solubility for beef aging and tenderization. *Journal of agricultural and food chemistry, 57* (22), 10755-10764.

Lawson, M. A. (2004). The role of integrin degradation in post-mortem drip loss in pork. *Meat science, 68* (4), 559-566.

Lefaucheur, L., & Gerrard, D. (2000). Muscle fiber plasticity in farm mammals. *J. Anim. Sci, 77*, 1-19.

Lefaucheur, L., Milan, D., Ecolan, P., & Le Callennec, C. (2004). Myosin heavy chain composition of different skeletal muscles in Large White and Meishan pigs. *Journal of animal science, 82* (7), 1931-1941.

Leibbrandt, V., Ewan, R., Speer, V., & Zimmerman, D. R. (1975). Effect of weaning and age at weaning on baby pig performance. *Journal of Animal Science, 40* (6), 1077-1080.

Lepper-Blilie, A., Berg, E., Buchanan, D., & Berg, P. (2016). Effects of post-mortem aging time and type of aging on palatability of low marbled beef loins. *Meat science, 112*, 63-68.

Lewis, G. J., Purslow, P. P., & Rice, A. E. (1991). The effect of conditioning on the strength of perimysial connective tissue dissected from cooked meat. *Meat science, 30* (1), 1-12.

Lewis, N. J., & Berry, R. J. (2006). Effects of season on the behaviour of early-weaned piglets during and immediately following transport. *Applied Animal Behaviour Science, 100* (3-4), 182-192.

Li, Q., Zhang, M., Chen, Y.-J., Wang, Y.-J., Huang, F., & Liu, J. (2011). Oxidative damage and HSP70 expression in masseter muscle induced by psychological stress in rats. *Physiology & behavior, 104* (3), 365-372.

Li, X., Wei, X., Wang, H., Zhang, C.-h., & Mehmood, W. (2017). Relationship Between Protein Denaturation and Water Holding Capacity of Pork During Postmortem Ageing. *Food Biophysics*, 1-7.

Li, Z., Li, X., Gao, X., Shen, Q. W., Du, M., & Zhang, D. (2017). Phosphorylation prevents in vitro myofibrillar proteins degradation by μ-calpain. Food chemistry, 218, 455-462.

Lindahl, G., Lagerstedt, Å., Ertbjerg, P., Sampels, S., & Lundström, K. (2010). Ageing of large cuts of beef loin in vacuum or high oxygen modified atmosphere–Effect on shear force, calpain activity, desmin degradation and protein oxidation. *Meat Science, 85* (1), 160-166.

Liu, J., Ruusunen, M., Puolanne, E., & Ertbjerg, P. (2014). Effect of pre-rigor temperature incubation on sarcoplasmic protein solubility, calpain activity and meat properties in porcine muscle. *LWT-Food Science and Technology, 55* (2), 483-489.

Liu, R., Li, Y., Wang, M., Zhou, G., & Zhang, W. (2016). Effect of protein S-nitrosylation on autolysis and catalytic ability of µ-calpain. *Food chemistry*, 213, 470-477.

Liu, Y., & Steinacker, J. M. (2001). Changes in skeletal muscle heat shock proteins: pathological significance. *Front Biosci, 6* (D12-25), D12-D25.

Lomiwes, D., Farouk, M., Wiklund, E., & Young, O. (2014). Small heat shock proteins and their role in meat tenderness: A review. *Meat science, 96* (1), 26-40.

Lomiwes, D., Farouk, M., Wu, G., & Young, O. (2014). The development of meat tenderness is likely to be compartmentalised by ultimate pH. *Meat Science, 96* (1), 646-651.

Lomiwes, D., Hurst, S., Dobbie, P., Frost, D., Hurst, R., Young, O., & Farouk, M. (2014). The protection of bovine skeletal myofibrils from proteolytic damage post mortem by small heat shock proteins. *Meat Science, 97* (4), 548-557.

Lonergan, S. M., Huff-Lonergan, E., Wiegand, B. R., & Kriese-Anderson, L. A. (2001). Postmortem proteolysis and tenderization of top loin steaks from Brangus cattle. *Journal of Muscle Foods, 12* (2), 121-136.

Longo, V., Lana, A., Bottero, M. T., & Zolla, L. (2015). Apoptosis in muscle-to-meat aging process: The omic witness. *Journal of proteomics*.

Ma, D., Kim, Y. H. B., Cooper, B., Oh, J.-H., Chun, H., Choe, J.-H.,... Min, B. (2017). Metabolomics Profiling to Determine the Effect of Postmortem Aging on Color and Lipid Oxidative Stabilities of Different Bovine Muscles. *Journal of Agricultural and Food Chemistry, 65* (31), 6708-6716. doi: 10.1021/acs.jafc.7b02175

Madhavi, D. L., & Carpenter, C. E. (1993). Aging and processing affect color, metmyoglobin reductase and oxygen consumption of beef muscles. *Journal of Food Science, 58* (5), 939-942. doi: doi:10.1111/j.1365-2621.1993.tb06083.x

Maenz, D., Patience, J., & Wolynetz, M. (1994). The influence of the mineral level in drinking water and the thermal environment on the performance and intestinal fluid flux of newly-weaned pigs. *Journal of animal science, 72* (2), 300-308.

Maga, J. A. (1994). Umami flavour of meat. In F. Shahidi (Ed.), *Flavour of Meat and Meat Products* (pp. 98-115): Chapman and Hall.

Martins, S. I., Jongen, W. M., & Van Boekel, M. A. (2000). A review of Maillard reaction in food and implications to kinetic modelling. *Trends in food science & technology, 11* (9-10), 364-373.

Matarneh, S. K. (2017). *Defining the role of mitochondria in fresh meat quality development.* PhD, Virginia Tech.

Matarneh, S. K., Beline, M., e Silva, S. d. L., Shi, H., & Gerrard, D. E. (2018). Mitochondrial F1-ATPase extends glycolysis and pH decline in an in vitro model. *Meat science, 137*, 85-91.

Matarneh, S. K., England, E. M., Scheffler, T. L., & Gerrard, D. E. (2017). The conversion of muscle to meat *Lawrie´s Meat Science* (pp. 159-185): Elsevier.

McCracken, B. A., Spurlock, M. E., Roos, M. A., Zuckermann, F. A., & Gaskins, H. R. (1999). Weaning anorexia may contribute to local inflammation in the piglet small intestine. *The Journal of nutrition, 129* (3), 613-619.

Meinert, L., Schäfer, A., Bjergegaard, C., Aaslyng, M. D., & Bredie, W. L. (2009). Comparison of glucose, glucose 6-phosphate, ribose, and mannose as flavour precursors in pork; the effect of monosaccharide addition on flavour generation. *Meat science, 81* (3), 419-425.

Melody, J., Lonergan, S. M., Rowe, L., Huiatt, T. W., Mayes, M. S., & Huff-Lonergan, E. (2004). Early postmortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles1. *Journal of animal science, 82* (4), 1195-1205.

Melody, J. L., Lonergan, S. M., Rowe, L. J., Huiatt, T. W., Mayes, M. S., & Huff-Lonergan, E. (2004). Early postmortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles. *Journal of Animal Science, 82* (4), 1195-1205.

Miller, R. (2001). Beef flavor: A white paper. Centennial, CO: *National Cattlemen's Beef Association*.

Mingmongkolchai, S., & Panbangred, W. (2018). Bacillus probiotics: an alternative to antibiotics for livestock production. *Journal of applied microbiology, 124* (6), 1334-1346.

Moeser, A. J., Klok, C. V., Ryan, K. A., Wooten, J. G., Little, D., Cook, V. L., & Blikslager, A. T. (2007). Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 292* (1), G173-G181.

Morzel, M., Chambon, C., Hamelin, M., Santé-Lhoutellier, V., Sayd, T., & Monin, G. (2004). Proteome changes during pork meat ageing following use of two different pre-slaughter handling procedures. *Meat science, 67* (4), 689-696.

Mullins, C., Zerby, H., Fitzpatrick, L., & Parker, A. (2016). cattle possess greater basal concentrations of HSP27, alpha B-crystallin, and HSP70 in skeletal muscle in vivo compared with cattle. *Journal of animal science, 94* (1), 424-429.

Neufer, P. D., & Benjamin, I. J. (1996). Differential Expression of αb-Crystallin and Hsp27 in Skeletal Muscle during Continuous Contractile Activity: relationship to myogenic regulatory factors. *Journal of Biological Chemistry, 271* (39), 24089-24095.

Newsholme, P., Curi, R., Curi, T. P., Murphy, C., Garcia, C., & De Melo, M. P. (1999). L-glutamine metabolism by lymphocytes, macrophages, and neutrophils: its importance in health and disease. *The Journal of nutritional biochemistry, 10* (6), 316-324.

Newsholme, P., Lima, M., Procópio, J., Pithon-Curi, T., Bazotte, R., & Curi, R. (2003). L-glutamine and glutamate as vital metabolites. *Brazilian Journal of Medical and Biological Research, 36* (2), 153-163.

Nishimura, T. (2015). Role of extracellular matrix in development of skeletal muscle and postmortem aging of meat. *Meat science, 109*, 48-55.

Nishimura, T., Fang, S., Ito, T., Wakamatsu, J. i., & Takahashi, K. (2008). Structural weakening of intramuscular connective tissue during postmortem aging of pork. *Animal science journal, 79* (6), 716-721.

Nishimura, T., Liu, A., Hattori, A., & Takahashi, K. (1998). Changes in mechanical strength of intramuscular connective tissue during postmortem aging of beef. *Journal of animal science, 76* (2), 528-532. doi: /1998.762528x

Nyberg, L., Lundström, K., Edfors-Lilja, I., & Rundgren, M. (1988). Effects of transport stress on concentrations of cortisol, corticosteroid-binding globulin and glucocorticoid receptors in pigs with different halothane genotypes. *Journal of Animal Science, 66* (5), 1201-1211.

O'Mahony, S. M., Marchesi, J. R., Scully, P., Codling, C., Ceolho, A.-M., Quigley, E. M.,... Dinan, T. G. (2009). Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biological psychiatry, 65* (3), 263-267.

Oliete, B., Carballo, J., Varela, A., Moreno, T., Monserrat, L., & Sánchez, L. (2006). Effect of weaning status and storage time under vacuum upon physical characteristics of meat of the Rubia Gallega breed. Meat science, 73(1), 102-108.

Otremba, M., Dikeman, M., Milliken, G., Stroda, S., Chambers Iv, E., & Chambers, D. (2000). Interrelationships between descriptive texture profile sensory panel and descriptive attribute sensory panel evaluations of beef Longissimus and Semitendinosus muscles. *Meat science, 54* (4), 325-332.

Ott, M., Gogvadze, V., Orrenius, S., & Zhivotovsky, B. (2007). Mitochondria, oxidative stress and cell death. *Apoptosis, 12* (5), 913-922.

Ouali, A., Gagaoua, M., Boudida, Y., Becila, S., Boudjellal, A., Herrera-Mendez, C. H., & Sentandreu, M. A. (2013). Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved. *Meat Science, 95* (4), 854-870. doi: https://doi.org/10.1016/j.meatsci.2013.05.010

Ouali, A., Herrera-Mendez, C. H., Coulis, G., Becila, S., Boudjellal, A., Aubry, L., & Sentandreu, M. A. (2006). Revisiting the conversion of muscle into meat and the underlying mechanisms. *Meat Science, 74* (1), 44-58.

Ouali, A., & Talmant, A. (1990). Calpains and calpastatin distribution in bovine, porcine and ovine skeletal muscles. *Meat Science, 28* (4), 331-348.

Papatsiros, V., Katsoulos, P.-D., Koutoulis, K., Karatzia, M., Dedousi, A., & Christodoulopoulos, G. (2013). Alternatives to antibiotics for farm animals. *CAB Rev, 8*, 1-15.

Pérez, M., Palacio, J., Santolaria, M., Aceña, M., Chacón, G., Gascón, M.,... Garcıa-Belenguer, S. (2002). Effect of transport time on welfare and meat quality in pigs. *Meat science, 61* (4), 425-433.

Phelps, K., Drouillard, J. S., Silva, M., Miranda, L., Ebarb, S., Van Bibber-Krueger, C.,... Gonzalez, J. M. (2016). Effect of extended postmortem aging and steak location on myofibrillar protein degradation and Warner-Bratzler shear force of beef M. semitendinosus steaks. *Journal of Animal Science, 94* (1), 412-423.

Picard, B., & Gagaoua, M. (2017). Proteomic investigations of beef tenderness *Proteomics in Food Science* (pp. 177-197): Elsevier.

Picard, B., Gagaoua, M., Micol, D., Cassar-Malek, I., Hocquette, J.-F. o., & Terlouw, C. E. (2014). Inverse relationships between biomarkers and beef tenderness according to contractile and metabolic properties of the muscle. *Journal of Agricultural And Food Chemistry, 62* (40), 9808-9818.

Pié, S., Lallès, J., Blazy, F., Laffitte, J., Sève, B., & Oswald, I. (2004). Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. *The Journal of nutrition*, 134(3), 641-647.

Pithon-Curi, T. C., Levada, A. C., Lopes, L. R., Doi, S. Q., & Curi, R. (2002). L-glutamine plays a role in superoxide production and the expression of p47phox, p22phox and gp91phox in rat neutrophils. *Clinical Science, 103* (4), 403-408.

Purslow, P. P. (2014). New developments on the role of intramuscular connective tissue in meat toughness. *Annual Review of Food Science and Technology, 5*, 133-153.

Ramos, P. M., Wright, S. A., Delgado, E. F., van Santen, E., Johnson, D. D., Scheffler, J. M.,... Scheffler, T. L. (2020). Resistance to pH decline and slower calpain-1 autolysis are associated with higher energy availability early postmortem in Bos taurus indicus cattle. *Meat science, 159*, 107925.

Repphun, A. M. (2011). Pigs-in-a-Blanket: How Current Meat Inspection Regulations Wrap America in False Security. *Drake J. Agric. L., 16*, 183.

Ritter, M., Ellis, M., Berry, N., Curtis, S., Anil, L., Berg, E.,... Driessen, B. (2009). Transport losses in market weight pigs: I. A review of definitions, incidence, and economic impact. *The professional animal scientist, 25* (4), 404-414.

Ritter, M., Ellis, M., Brinkmann, J., DeDecker, J., Keffaber, K., Kocher, M.,... Wolter, B. (2006). Effect of floor space during transport of market-weight pigs on the incidence of transport losses at the packing plant and the relationships between transport conditions and losses. *Journal of animal science, 84* (10), 2856-2864.

Robbins, K., Jensen, J., Ryan, K., Homco-Ryan, C., McKeith, F., & Brewer, M. (2003). Consumer attitudes towards beef and acceptability of enhanced beef. *Meat Science, 65* (2), 721-729.

Rogero, M. M., Borelli, P., Vinolo, M. A. R., Fock, R. A., de Oliveira Pires, I. S., & Tirapegui, J. (2008). Dietary L-glutamine supplementation affects macrophage function, hematopoiesis and nutritional status in early weaned mice. *Clinical nutrition, 27* (3), 386-397.

Roldan-Santiago, P., Martínez-Rodríguez, R., Yáñez-Pizaña, A., Trujillo-Ortega, M., Sánchez-Hernández, M., Pérez-Pedraza, E., & Mota-Rojas, D. (2013). Stressor factors in the transport of weaned piglets: a review. *Veterinarni Medicina, 58* (6).

Rowe, L. J., Maddock, K. R., Lonergan, S. M., & Huff-Lonergan, E. (2004). Oxidative environments decrease tenderization of beef steaks through inactivation of μ-calpain. *Journal of Animal Science, 82* (11), 3254-3266.

Santos, C., Moniz, C., Roseiro, C., Tavares, M., Medeiros, V., Afonso, I.,... da Ponte, D. J. B. (2016). Effects of Early Post-Mortem Rate of pH fall and aging on Tenderness and Water Holding Capacity of Meat from Cull Dairy Holstein-Friesian Cows. [Cull dairy cows; Aging; pH fall rate; Tenderness; Purge loss; Thawing loss]. *2016, 5* (2). doi: 10.5539/jfr.v5n2p1

Sañudo, C., Sierra, I., Olleta, J., Martin, L., Campo, M., Santolaria, P., . . . Nute, G. (1998). Influence of weaning on carcass quality, fatty acid composition and meat quality in intensive lamb production systems. *Animal Science*, 66(1), 175-187.

Savell, J., Branson, R., Cross, H., Stiffler, D., Wise, J., Griffin, D., & Smith, G. (1987). National consumer retail beef study: palatability evaluations of beef loin steaks that differed in marbling. *Journal of Food Science, 52* (3), 517-519.

Scheffler, T., & Gerrard, D. (2007). Mechanisms controlling pork quality development: The biochemistry controlling postmortem energy metabolism. *Meat Science, 77* (1), 7-16.

Schwartzkopf-Genswein, K., Faucitano, L., Dadgar, S., Shand, P., González, L., & Crowe, T. (2012). Road transport of cattle, swine and poultry in North America and its impact on animal welfare, carcass and meat quality: A review. *Meat science, 92* (3), 227-243.

Seideman, S. C., Cross, H. R., Smith, G. C., & Durland, P. R. (1984). Factors associated with fresh meat color: A review. *Journal of Food Quality, 6* (3), 211-237. doi: doi:10.1111/j.1745-4557.1984.tb00826.x

Shackelford, S., Wheeler, T., & Koohmaraie, M. (1995). Relationship between shear force and trained sensory panel tenderness ratings of 10 major muscles from Bos indicus and Bos taurus cattle. *Journal of animal science, 73* (11), 3333-3340.

Sierra, V., & Oliván, M. (2013). Role of mitochondria on muscle cell death and meat tenderization. *Recent patents on endocrine, metabolic & immune drug discovery, 7* (2), 120-129.

Smith, G. C., Tatum, J. D., & Belk, K. E. (2008). International perspective: characterisation of United States Department of Agriculture and Meat Standards Australia systems for assessing beef quality. *Australian Journal of Experimental Agriculture, 48* (11), 1465-1480. doi: http://dx.doi.org/10.1071/EA08198

Soderholm, J. D., Yates, D. A., Gareau, M. G., Yang, P.-C., MacQueen, G., & Perdue, M. H. (2002). Neonatal maternal separation predisposes adult rats to colonic barrier dysfunction in response to mild stress. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 283* (6), G1257-G1263.

Spanier, A., Flores, M., McMillin, K., & Bidner, T. (1997). The effect of post-mortem aging on meat flavor quality in Brangus beef. Correlation of treatments, sensory, instrumental and chemical descriptors. *Food Chemistry, 59* (4), 531-538.

Spreeuwenberg, M., Verdonk, J., Gaskins, H. R., & Verstegen, M. (2001). Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. *The Journal of nutrition, 131* (5), 1520-1527.

Stanton, C., & Light, N. (1990). The effects of conditioning on meat collagen: Part 3—Evidence for proteolytic damage to endomysial collagen after conditioning. *Meat science, 27* (1), 41-54.

Stelzleni, A. M. (2006). Feeding and aging effects on carcass composition, fatty acid profiles and sensory attributes of muscles from cull cow carcasses. PhD Disseratation, University of Florida, Gainseville, Florida.

Strasser, A., O'Connor, L., & Dixit, V. M. (2000). Apoptosis signaling. *Annual review of biochemistry, 69* (1), 217-245.

Sutherland, H., Salmons, S., Ramnarine, I. R., Capoccia, M., Walsh, A. A., & Jarvis, J. C. (2006). Adaptive conditioning of skeletal muscle in a large animal model (Sus domesticus). *Journal of anatomy, 209* (2), 165-177.

Sutherland, M. A., Backus, B. L., & McGlone, J. J. (2014). Effects of transport at weaning on the behavior, physiology and performance of pigs. *Animals, 4* (4), 657-669.

Tang, S., Bao, E., Sultan, K. R., Nowak, B., & Hartung, J. (2013). Transportation stress and expression of heat shock protein affecting pork quality. *Pak. Vet. J, 34*, 112-115.

Teixeira, A., Pereira, E., & Rodrigues, E. (2011). Goat meat quality. Effects of salting, air-drying and ageing processes. *Small Ruminant Research, 98* (1), 55-58.

Teye, G., & Okutu, I. (2009). Effect of ageing under tropical conditions on the eating qualities of Beef. *African Journal of Food, Agriculture, Nutrition and Development, 9* (9).

Thompson, H., Maynard, E., Morales, E., & Scordilis, S. (2003). Exercise-induced HSP27, HSP70 and MAPK responses in human skeletal muscle. *Acta physiologica scandinavica, 178* (1), 61-72.

Utrera, M., & Estévez, M. (2012). Oxidation of myofibrillar proteins and impaired functionality: underlying mechanisms of the carbonylation pathway. *Journal of agricultural and food chemistry, 60* (32), 8002-8011.

Van der Meulen, J., Koopmans, S., Dekker, R., & Hoogendoorn, A. (2010). Increasing weaning age of piglets from 4 to 7 weeks reduces stress, increases post-weaning feed intake but does not improve intestinal functionality. *Animal, 4* (10), 1653-1661.

Veiseth, E., Shackelford, S., Wheeler, T., & Koohmaraie, M. (2001). Effect of postmortem storage on calpain 1 and calpain 2 in ovine skeletal muscle. *Journal of Animal Science, 79* (6), 1502-1508.

Vitale, M., Pérez-Juan, M., Lloret, E., Arnau, J., & Realini, C. (2014). Effect of aging time in vacuum on tenderness, and color and lipid stability of beef from mature cows during display in high oxygen atmosphere package. *Meat science, 96* (1), 270-277.

Wang, B., Wu, G., Zhou, Z., Dai, Z., Sun, Y., Ji, Y.,... Han, F. (2015). L-glutamine and intestinal barrier function. *Amino acids, 47* (10), 2143-2154.

Wang, H., Zhang, C., Wu, G., Sun, Y., Wang, B., He, B.,... Wu, Z. (2015). L-glutamine enhances tight junction protein expression and modulates corticotropin-releasing factor signaling in the jejunum of weanling piglets. *The Journal of nutrition, 145* (1), 25-31.

Wang, J., Chen, L., Li, P., Li, X., Zhou, H., Wang, F., . . . Wu, G. (2008). Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. The Journal of nutrition, 138(6), 1025-1032.

Wang, Q., Zhao, X., Ren, Y., Fan, E., Chang, H., & Wu, H. (2013). Effects of high pressure treatment and temperature on lipid oxidation and fatty acid composition of yak (Poephagus grunniens) body fat. *Meat science, 94* (4), 489-494.

Warner, R. D. (2017). The Eating Quality of Meat—IV Water-Holding Capacity and Juiciness *Lawrie´s Meat Science (Eighth Edition)* (pp. 419-459): Elsevier.

Watanabe, A., Kamada, G., Imanari, M., Shiba, N., Yonai, M., & Muramoto, T. (2015). Effect of aging on volatile compounds in cooked beef. *Meat science, 107*, 12-19.

Watanabe, A., Tsuneishi, E., & Takimoto, Y. (1991). Effects of pithing on pH, creatine phosphate and ATP-related compounds of beef Psoas major and Longissimus dorsi muscles. *Meat science, 29* (3), 221-228.

Wen, J. (2018). Effect of amino acids and vitamin D3 on performance and biological responses in poultry. PhD dissertation, Virginia Tech.

Wiepkema, P., & Koolhaas, J. (1993). Stress and animal welfare. *Animal welfare, 2* (3), 195-218.

Wijtten, P. J., van der Meulen, J., & Verstegen, M. W. (2011). Intestinal barrier function and absorption in pigs after weaning: a review. *British Journal of Nutrition, 105* (7), 967-981.

Wischmeyer, P. E., Kahana, M., Wolfson, R., Ren, H., Musch, M. M., & Chang, E. B. (2001). L-glutamine induces heat shock protein and protects against endotoxin shock in the rat. *Journal of Applied Physiology, 90* (6), 2403-2410.

Wójtowicz, I., Jabłońska, J., Zmojdzian, M., Taghli-Lamallem, O., Renaud, Y., Junion, G.,... Jagla, T. (2015). Drosophila small heat shock protein CryAB ensures structural integrity of developing muscles, and proper muscle and heart performance. *Development, 142* (5), 994-1005.

Wolter, B., & Ellis, M. (2001). The effects of weaning weight and rate of growth immediately after weaning on subsequent pig growth performance and carcass characteristics. *Canadian Journal of Animal Science*, 81(3), 363-369.

Wu, G., Knabe, D. A., Yan, W., & Flynn, N. E. (1995). L-glutamine and glucose metabolism in enterocytes of the neonatal pig. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 268* (2), R334-R342.

Wu, G., Meier, S. A., & Knabe, D. A. (1996). Dietary L-glutamine supplementation prevents jejunal atrophy in weaned pigs. *The Journal of nutrition, 126* (10), 2578-2584.

Wu, G., & Thompson, J. R. (1990). The effect of L-glutamine on protein turnover in chick skeletal muscle in vitro. *Biochemical journal, 265* (2), 593-598.

Wu, J. J., Dutson, T. R., & Carpenter, Z. L. (1981). Effect of postmortem time and temperature on the release of lysosomal enzymes and their possible effect on bovine connective tissue components of muscle. *Journal of Food Science, 46* (4), 1132-1135. doi: 10.1111/j.1365-2621.1981.tb03008.x

Xing, T., Gao, F., Tume, R. K., Zhou, G., & Xu, X. (2019). Stress effects on meat quality: A mechanistic perspective. *Comprehensive Reviews in Food Science and Food Safety, 18* (2), 380-401.

Xing, T., Xu, X., Jiang, N., & Deng, S. (2016). Effect of transportation and pre‑slaughter water shower spray with resting on AMP‑activated protein kinase, glycolysis and meat quality of broilers during summer. *Animal Science Journal, 87* (2), 299-307.

Xiong, Y. L. (2000). Protein oxidation and implications for muscle food quality. *Antioxidants in Muscle Foods: Nutritional Strategies to Improve Quality. New York: Wiley and Sons*, 85-90.

Xu, Y., Jin, M., Wang, L., Zhang, A., Zuo, B., Xu, D.,... Li, F. (2009). Differential proteome analysis of porcine skeletal muscles between Meishan and Large White. *Journal of animal science, 87* (8), 2519-2527.

Yaylayan, V. A., Keyhani, A., & Wnorowski, A. (2000). Formation of sugar-specific reactive intermediates from 13C-labeled l-serines. *Journal of agricultural and food chemistry, 48* (3), 636-641.

Yi, G., Carroll, J., Allee, G., Gaines, A., Kendall, D., Usry, J.,... Izuru, S. (2005). Effect of L-glutamine and spray-dried plasma on growth performance, small intestinal morphology, and immune responses of Escherichia coli K88+-challenged weaned pigs. *Journal of animal science, 83* (3), 634-643.

Yu, J., Tang, S., Bao, E., Zhang, M., Hao, Q., & Yue, Z. (2009). The effect of transportation on the expression of heat shock proteins and meat quality of M. longissimus dorsi in pigs. *Meat Science, 83* (3), 474-478.

Zamora, F., Chaïb, F., & Dransfield, E. (1998). Calpains and calpastatin from cold-shortened bovine M. longissimus lumborum. *Meat science, 49* (1), 127-133.

Zeng, Z., Li, C., & Ertbjerg, P. (2017). Relationship between proteolysis and water-holding of myofibrils. *Meat science, 131*, 48-55.

Zhang, L., Yue, H., Zhang, H., Xu, L., Wu, S., Yan, H.,... Qi, G. (2009). Transport stress in broilers: I. Blood metabolism, glycolytic potential, and meat quality. *Poultry science, 88* (10), 2033-2041.

Zhang, W., Lonergan, S. M., Gardner, M. A., & Huff-Lonergan, E. (2006). Contribution of postmortem changes of integrin, desmin and μ-calpain to variation in water holding capacity of pork. *Meat science, 74* (3), 578-585.

Zhao, C., Tian, F., Yu, Y., Luo, J., Mitra, A., Zhan, F.,... Updike, M. S. (2012). Functional genomic analysis of variation on beef tenderness induced by acute stress in Angus cattle. *Comparative and functional genomics, 2012*.

CHAPTER 2. PROTEOLYTIC CHANGES OF MYOFIBRILLAR AND SMALL HEAT SHOCK PROTEINS IN DIFFERENT BOVINE MUSCLES DURING AGING: THEIR RELEVANCE TO TENDERNESS AND WATER-HOLDING CAPACITY

2.1 Abstract

The objective of this study was to determine the proteolytic changes of myofibrillar and small heat shock proteins (HSPs) in different muscles during aging and to evaluate their relevance to meat quality attributes. From 8 beef carcasses, *longissimus lumborum* (LL), *semimembranosus* (SM), and *psoas major* (PM) muscles were obtained, cut into sections and assigned to various aging periods up to 23d. PM exhibited limited aging potential in quality developments shown by lower extents of shear force, water-holding capacity (WHC), and proteolytic changes, including calpain 1 autolysis, troponin T, and HSP27 compared to LL and SM. Conversely, LL had an increase in tenderization and WHC, which was accompanied with more extended calpain 1 autolysis, proteolysis and HSP27 degradation, compared with other muscles. The results of this study suggest that postmortem proteolytic changes of myofibrillar proteins, small HSPs and apoptotic factors occur in a muscle-specific manner, which is likely attributed to different rate and extent of meat quality developments of each muscle during aging.

Keywords: Beef; aging; Small heat shock proteins; Apoptosis; Meat tenderization

2.2 Introduction

Tenderness is considered one of the most important palatability factors affecting consumers' eating experience (Brewer & Novakofski, 2008; Kim, Warner, & Rosenvold, 2014; Koohmaraie, 1996; Kristensen & Purslow, 2001; Huff-Lonergan, Zhang, & Lonergan, 2010). During postmortem aging, noticeable improvements in eating quality attributes, such as tenderness, juiciness, and/or flavor, occur through the action of endogenous proteolytic systems degrading cytoskeletal myofibrillar proteins in meat (Kim et al., 2018). Although it is well-established that

meat tenderization is primarily influenced by elevated postmortem fragmentation of muscle structure (among other factors such as background toughness and sarcomere length) (Goll, Thompson, Li, Wei, & Cong, 2003; Hopkins & Thompson, 2001; Kemp, Sensky, Bardsley, Buttery, & Parr, 2010), the exact mechanism by which endogenous proteases play roles in tenderness development along with other biochemical processes (e.g. cellular death and survival cascades) has still not been clearly understood (Ouali et al., 2013).

The advances in proteomic techniques have successfully identified some candidate proteins that correlate with variation in meat quality, particularly in meat tenderness. Studies have consistently identified small heat shock proteins (HSPs), chaperone proteins that have diverse anti-stress functions, as one of the viable quality markers related to meat tenderness (Guillemin et al., 2011; Kim et al., 2008; Morzel, Terlouw, Chambon, Micol, & Picard, 2008). Small HSPs are well known anti-apoptotic molecules that are crucial for cells to fight the battle between death and survival (Garrido, Gurbuxani, Ravagnan, & Kroemer, 2001). The roles of small HSPs in protecting cytoskeletal structures, such as actin, titin, and troponin T, have been well-established in postmortem muscles (Kötter et al., 2014; Lomiwes, Farouk, Wiklund, & Young, 2014; Wójtowicz et al., 2015). Ouali et al. (2013) suggested that apoptosis associated biochemical changes may be intrinsically related to postmortem proteolytic systems. In fact, several studies have postulated the relevance of postmortem degradation of small HSPs to the extent of structural/cytoskeletal protein degradation, where an increase in degradation of small HSPs could possibly indicate a loss of its anti-apoptotic (chaperoning) function in postmortem muscles (Balan, Kim, & Blijenburg, 2014; Choe, Stuart, & Kim, 2016; Cramer, Penick, Waddell, Bidwell, & Kim, 2018; Lomiwes, Farouk, Frost, Dobbie, & Young, 2013). As a consequence, more enzymatic proteolysis could occur and result in an increase in aging potential of postmortem muscles (Kim et al., 2018).

Given that metabolism dysregulation plays an important role in apoptosis, it is reasonable to postulate that apoptotic and proteolytic changes of postmortem skeletal muscles may be differently affected by different muscle types (e.g. glycolytic vs. oxidative). Furthermore, it has been well-documented that postmortem aging response occurs in a muscle-specific manner (Bratcher, Johnson, Littell, & Gwartney, 2005). However, most of the published research reporting proteolytic changes of small HSPs (and apoptotic factors) have been primarily studied in *longissimus lumborum* muscles, while little to no information is currently available regarding the proteolytic alterations of small HSPs in different beef muscles during aging. Therefore, the

objective of this study was to 1) determine the proteolytic changes of myofibrillar proteins and alterations of small HSPs and apoptotic factors in different bovine muscles during aging, and to 2) evaluate their relevance to tenderness development and water-holding capacity.

2.3   Materials and methods

2.3.1   Raw materials and processing

Eight Angus × Simmental beef cattle (USDA Select; A maturity) were slaughtered at the Purdue University Meat Laboratory. At 45 minutes postmortem, about 10g of *M. longissimus lumborum* was removed from one of the carcasses, which was used as the early postmortem biochemical reference sample. At 1d postmortem, whole muscles [*longissimus lumborum* (LL), *semimembranosus* (SM), and *psoas major* (PM)] were separated from the left side of each carcass. Approximately 100g of each muscle were snap-frozen and stored at − 80 °C until being analyzed as 1d biochemical reference sample. Then, each muscle was cut into four equal sections in length, vacuum packaged and randomly assigned to one of the four aging periods at 1 °C (2, 9, 16, and 23 days postmortem). At the completion of each aging period, multiple steak cuts were made from each muscle section for measurements of cook loss/shear force (2.54-cm thick), drip loss, and western-blot. The samples assigned for drip loss measurement were used immediately after cutting, and other samples were vacuum-packaged and stored at -80 °C until being used for further analyses.

2.3.2   Warner-Bratzler shear force

Warner-Bratzler shear force (WBSF) was measured using 2.54-cm-thick steaks from each aged muscle, which were also used for cook loss measurement. The steaks were cooked on an electric griddle (Farberware, Walter Idde and Co., Bronx, NY) at a constant surface temperature of 135°C until the internal temperature reached to targeted 41°C, then were flipped to the other side and cooked until reaching the targeted internal temperature of 71°C. The internal temperature was monitored by using a digital temperature logger (OctTemp2000, MadgeTech, Inc., Warner, NH) equipped with thermocouple (T-type, Omega Engineering, Stamford, CT). After chilling at 4°C overnight, six cores from each sample were obtained parallel to the muscle fiber orientation using a hand-held coring device (1.27 cm diameter), then were sheared through using a Warner-

Bratzler shear attachment with TA-XT Plus texture analyzer (Stable Micro System Ltd., UK). Peak shear force (kg) of the six replicates were averaged to represent the maximum force that was required to shear through the sample.

2.3.3 pH and water-holding capacity

The pH of LL, SM, and PM muscles was measured in triplicate using an insertion-type pH meter (HI 99,163, Hanna Instruments Inc., Woonsocket) at 1d, 2d, 9d, 16d, and 23d postmortem after slaughter. Water-holding capacity (WHC) was analyzed by measuring purge, drip, and cook loss of the beef samples. The purge loss of LL, SM, and PM muscle sections was determined by measuring weight differences between an initial weight prior to vacuum-packaging and the final weight after aging conditioning (Kim, Liesse, Kemp, & Balan, 2015). At the completion of each assigned aging time, each muscle section was removed from the vacuum bag, gently blotted off surface liquid, and weighed to calculate the percent purge loss. Drip loss of the muscles was measured at the completion of each aging period following the procedure described by Honikel (1998) with a minor modification. In duplicates, the samples were trimmed off all the visible fat and connective tissue, cut into uniform pieces (3 cm × 3 cm × 3 cm; about 50g), weighed, and suspended in nylon net in sealed containers. After 48 hours of storage in the cooler (2.5°C), the samples were blotted off surface liquid and re-weighed. The percentage drip loss was calculated using the difference between the initial and final weight. Cook loss was determined by measuring weight differences between the weight of uncooked and cooked steaks. Cooked steaks were cooled at room temperature for 30 min and weighed.

2.3.4 Western-blot analysis

Whole muscle protein was extracted using one gram of minced meat sample homogenized with 10 ml of extraction buffer (10 mM phosphate buffer with 2% SDS; pH 7.0). The protein concentration was measured and adjusted to 6.4 mg/ml. The SDS-PAGE samples with a final protein concentration of 4 mg/ml were prepared using 1 ml of protein extract, 500 μL sample loading buffer (0.025% w/v bromophenol blue, 3% w/v SDS, 3 mM EDTA, 30% v/v glycerol, 0.03 M Tris base, pH 8.0) and 100 μL 2-mercaptoethanol, which was incubated in 50°C heat block for 20 min and stored at -80°C.

Western blots were performed in accordance with Carlson et al. (2017) with minor modifications. SDS-PAGE protein samples (40μg for calpain 1, troponin T and desmin, 60μg for cytochrome c, caspase 3, HSP20, HSP27 and αβ-crystallin) were electrophoresed and transferred onto polyvinylidene fluoride (PVDF) membranes (0.2 μm pore size, Millipore, Bedford, MA, USA) using a TE22 Transphor unit (Hoefer Inc, Richmond, CA, USA) for 90 min under 90V at 1°C. The blotted membranes were blocked and incubated with the following primary antibodies, which were diluted with 3% nonfat dry milk: 1:10000 mouse monoclonal anti-desmin IgG (Sigma-Aldrich D1022), 1:10000 anti-troponin T IgG (Sigma-Aldrich T6277), 1:10000 mouse monoclonal anti-calpain 1 IgG (ThermoFisher MA3-940), 1:1000 mouse monoclonal anti-HSP27 IgG (Abcam ab79868); 1:1000 rabbit polyclonal anti-HSP20 IgG (Abcam ab13491); 1:1000 rabbit polyclonal anti-αβ-crystallin IgG (ThermoFisher PA1-16951); 1:1000 rabbit polyclonal anti-cytochrome c IgG (Abcam ab90529); and 1:1000 rabbit polyclonal anti-caspase3 IgG (Abcam ab4051). After primary antibody incubation, the washed membranes (3 × 10 min in PBS-Tween, pH 7.0) were probed with secondary antibodies conjugated with horseradish peroxidase (10,000:1 goat-anti-mouse IgG or 10,000:1 goat-anti-rabbit IgG, ThermoFisher). The targeted protein bands were detected using an enhanced chemiluminescent (ECL) detection reagent (ThermoFisher; Rockford, IL), which was visualized using UVP GelDoc-It imaging system (UVP, Upland, CA, USA). The protein bands were analyzed by densitometry using Visionworks LS Analysis Software (UVP, Upland, CA). Each gel was loaded with a consistent reference sample. The targeted bands intensity was quantified as a relative ratio compared to the reference samples: the 45-min postmortem LL was used for referencing HSP27, HSP20, and cytochrome c; a 23d postmortem LL was used for referencing desmin and troponin T, and a 2d postmortem SM were used for referencing caspase 3. The three bands of calpain 1 at 80, 78, and 76 kDa were quantified and expressed as the ratio of each band relative to the total intensity.

2.3.5   Statistical analysis

The experimental design of this study was a split-plot design, with muscle type effect (LL, SM, and PM) as the whole plot and aging duration effect (1, 2, 9, 16 and 23 days of aging) as the subplot. The data were analyzed using PROC MIXED procedure of SAS 9.4 software, where muscle type and aging time were fixed effects and carcass was a random effect. Least square means

were separated (F test, $P < 0.05$) by least significant differences. Principle Component Analysis (PCA) and Spearman's ranking correlation were analyzed using Metaboanalyst 3.0 (https://www.metaboanalyst.ca/). Correlation matrix were presented as heatmaps, and P-values were adjusted to false discover rate (FDR) through Benjamin-Hochberg procedure. Correlations with FDR < 0.1 were subjected to further biological interpretation.

2.4   Results

2.4.1   WBSF, pH, and WHC

An aging period by muscle type interaction was found in WBSF values (Table 2.1). Overall, a decrease in WBSF was found in all beef muscles during aging ($P < 0.01$; Table 2.1). However, there was a distinct difference in the rate and extent of decreases in WBSF values between muscles: LL and SM showed a continuous decrease in WBSF values throughout the entire aging period, whereas PM showed decrease in WBSF values only from 2d to 9d postmortem and then remained with no further changes until the end of aging (Table 2.1). The result indicated a muscle-specific aging response for the tenderization process.

The pH of beef muscles, which ranged from 5.47 to 5.64 across all treatment groups, was affected by an interaction between aging and muscle type ($P < 0.0001$). In general, the PM muscles showed 0.1 units higher pH value compared to LL and SM muscles. Although significant, the overall numerical change caused by aging treatment was minimal: SM maintained consistent pH value up to 23d, whereas PM muscles had an increase in pH from 2d (5.60) to 9d (5.64) and LL had a decrease from 16d (5.54) to 23d (5.47).

For WHC, an interaction ($P < 0.01$) between aging period and muscle type affected drip loss, purge loss, and cook loss (Table 2.1). With increased aging period, a gradual decrease in drip loss was found in LL and SM, but PM showed no change in drip loss throughout the entire aging period ($P > 0.05$; Table 2.1). For purge loss, while all the beef muscles showed an increase in purge loss during aging, PM exhibited the highest accumulation of purge in 9d and then showed an abrupt decrease (Table 2.1). Cook loss showed minimal changes during aging in all the muscles in general, except for PM that showed the lowest value on 9d. For both purge and cook loss, LL remained overall lower or equivalent values compared to SM and PM counterparts (Table 2.1).

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2.4.2 Myofibrillar protein degradation and calpain 1 autolysis

To investigate muscle-specific features of postmortem proteolysis, the abundance of intact and degradation fragments of troponin T and desmin and calpain 1 autolysis was determined using western blot analysis (Figure 2.1 & Table 2.2). For desmin, both aging period and muscle type affected the extent of desmin fragmentation (Table 2.2), no interaction between aging and muscle type was found ($P > 0.05$, Table 2.2). The extent of intact desmin decreased with increasing aging periods ($P < 0.0001$; Table 2.2). The 42 kDa degradation products were increased as aging duration extended ($P < 0.001$; Figure 2.1 & Table 2.2). In particular, the 42 kDa degradation bands started to appear as early as 1d postmortem (Figure 2.1). In addition, muscle type affected abundance of 42 kDa product of desmin. Regardless of aging treatment, LL showed lowest band intensity followed by SM and PM, (Table 2.2, $P < 0.0001$).

For troponin T fragmentation, a decrease in abundance of intact bands were found as aging duration prolonged ($P < 0.05$, Table 2.2). Troponin T degradation bands at 30 kDa was influenced by muscle $\times$ aging interaction ($P < 0.001$, Table 2.2 & Figure 2.3). In LL and SM muscles, the 30 kDa bands increased over aging ($P < 0.05$, Figure 2.3), but those in PM showed no change over time and remained consistently lower than the other two muscles, regardless of aging duration (Figure 2.1 & Figure 2.3).

Western-blot analyzed autolysis of calpain 1 80 kDa subunit to reveal enzyme activity development. The intact and autolyzed forms of 80 kDa subunit, observed at 80, 78, and 76 kDa respectively, were influenced by both aging and muscle types (Figure 2.2, Table 2.2), but no interaction was found ($P > 0.05$). With prolonged aging period, decline of the 80kDa band and increases of the 78 and 76 kDa bands were observed ($P < 0.0001$, Table 2.2), regardless of muscle type. For the muscle effect, LL exhibited a greater extent of calpain 1 autolysis as indicated by lower intensity of 80 kDa ($P < 0.01$) and higher intensity of 76 kDa bands ($P < 0.001$) compared to SM and PM (Table 2.2). At 23d of aging, LL only showed fully autolyzed form at 76kDa, whereas both SM and PM muscles still exhibited intact 80 kDa and partial autolyzed 78 kDa bands of calpain 1 (Figure 2.2).

2.4.3 Small heat shock proteins, cytochrome c and caspase 3

The abundance of small HSPs including HSP27, HSP20, and αβ-crystallin was determined by using western blot. While HSP20 and αβ-crystallin exhibited a single band throughout the aging duration, only HSP27 showed degradation bands (Figure 2.2). Main effects (muscle and/or aging) were found to affect HSP27 degradation, HSP20, and αβ-crystallin, no interaction effects were observed (Table 2.2). The intact bands (observed at 23 kDa) of HSP27 was not affected by either aging duration or muscle type ($P > 0.05$), but an increase in degradation product of HSP27 (observed at 20 kDa) was shown with extended aging periods ($P < 0.001$, Table 2.2 & Figure 2.2). Furthermore, a muscle effect was also found, where LL and SM had similar HSP27 degradation products, which was higher than PM (Figure 2.2, Table 2.2, $P < 0.001$). Aging decreased HSP20 intact form 9d to 16d, and LL had a higher HSP20 band intensity followed by SM and PM ($P < 0.0001$, Table 2.2). While no muscle effect was found in αβ-crystallin ($P > 0.05$, Table 2.2), aging decreased the protein abundance from 2d to 9d ($P < 0.001$, Table 2.2).

Both muscle type and aging duration affected abundance of cytochrome c, and no interaction effect was found (Table 2.2). PM and SM had higher abundance of cytochrome c compared to LL ($P < 0.001$, Table 2.2). With increasing aging periods, the abundance of cytochrome c decreased up to 23d ($P < 0.01$, Table 2.2).

An interaction between muscle type and aging period was observed in caspase 3. The extent of cleaved caspase 3 bands was increased in abundance at 9d in SM and PM ($P < 0.05$), while LL had higher caspase 3 abundance at 1d but showed a decrease at 2d ($P < 0.05$; Figure 2.4). The cleaved band of caspase 3 at 15 kDa was appeared in 1d, 2d, and 9d postmortem, but was not detected in any of the 16d and 23d aged samples (Figure 2.4).

2.4.4 PCA and correlation based on small HSPs, postmortem proteolysis and meat quality attributes.

Two PCAs were conducted based on different data lay-outs and both score and loading plots are shown in Figure 2.5. In the first PCA, the total dimensions were all the quality and proteolytic attributes from each of the 2d, 9d, 16d, and 23d of aging, whereas three muscles from eight cattle were considered as observations (Figure 2.5A). The second PCA used all the quality and proteolytic attributes as the dimensions, and three muscles with four aging time (Figure 2.5C)

as the observations. In the first PCA, PC1 and PC2 explained 18.1% and 14.9% of the total variation in the dataset, respectively. A cluster representing PM was separated from LL and SM, where observations were separated according to muscle type along PC1 axis. In the second PCA, PC1 alone explained 48.5% of the total variation of the data set, whereas the observations were separated according to different aging periods. The corresponding loading plots showed that desmin degradation products of 42 kDa, troponin T degradation product of 30 kDa, and cleaved caspase 3 were among the features that were mostly responsive to postmortem aging (Figure 2.5 D).

The WBSF, WHC, proteolytic features, small HSPs, and apoptosis related proteins were correlated in each of the muscles with segregated aging by using Spearman's ranking correlation and presented as heatmaps (Figure 2.6A-C). Muscle-specific color patterns were found, where quality and proteolytic traits in LL formed larger clusters than those of SM and PM, meaning that quality/proteolytic traits in LL muscles shared a higher degree of collinearity. Significant correlations were identified between small HSPs, calpain autolysis bands, and structural protein degradation products in all muscles, with detailed interpretations presented in the following section.

2.5   Discussion

The current study aimed to determine the muscle-specific effects on aging response in terms of structural myofibrillar protein degradation, small HSP dynamics, and apoptotic changes in relation to meat quality attributes. LL showed the most extensive tenderization potential, indicated by a 46.3% decrease of WBSF values compared to SM (22.4%) and PM (39.4%). While noticeable tenderization of LL was observed up to 23d aging, PM showed no further change at and beyond 9d aging, which was concurrent with a lack of troponin T 30kDa fragmentation. Similarly, a muscle-specificity in WHC development was found, where drip loss decreased over 1d to 23d postmortem in LL (3.2% to 1.6%) and SM (3.8% to 2.1%), but no change was observed in PM. Moreover, the current result showed more extensive calpain 1 autolysis in LL compared to other muscles. Even after 23d of aging, the calpain 1 in SM and PM were still not fully autolyzed (Figure 2.2).

Calpain 1 enzyme activity escalates when the 80 kDa catalytic subunit converts to autolyzed 76 kDa form through a 78 kDa intermediate band, which was mediated by Ca2+ binding at μM concentration level (Lametsch, Lonergan, & Huff-Lonergan, 2008). Factors hampering the

release of calpain 1 activity in postmortem muscles mainly include allosteric inhibitor (calpastatin), pH, and metabolic factors that likely influence Ca2+ concentration. According to Ouali and Talmant (1990), calpastatin level was negatively associated with glycolytic features between fast vs. slow twitch muscles, supporting the current observation that LL showed the highest calpain 1 activity. Recent studies proposed that muscles with enhanced ATP retention showed prolonged Ca2+ uptake from cytosol via sarcoplasmic reticulum, and thereby inhibited calpain 1 activation and proteolysis (England, Matarneh, Scheffler, Wachet, & Gerrard, 2015; Ramos et al., 2020). As PM is an oxidative, mitochondria-rich muscle type, ATP availability in PM would be higher than in LL (Watanabe, Tsuneishi, & Takimoto, 1991). In fact, the calpain activity and calpain-mediated proteolysis have been reported to vary depend upon muscle types (Delgado, Geesink, Marchello, Goll, & Koohmaraie, 2001; Veiseth-Kent, Pedersen, Rønning, & Rødbotten, 2018). Therefore, the different rate and extent of calpain 1 autolysis in the current study could be ascribed to different oxidative/glycolytic metabolism between LL, SM and PM muscles, at least in part.

While the muscle-specificity in proteolysis is well-documented in the literature (Delgado, Geesink, Marchello, Goll, & Koohmaraie, 2001; Veiseth-Kent, Pedersen, Rønning, & Rødbotten, 2018), it is worthwhile to note that in the present study the different rates and extents of HSP27 degradation were found between the muscles during aging. As HSPs have protective functions as chaperone proteins from proteases' catalytic events, the potential adverse impacts of small HSPs on meat tenderness by reducing degradation of myofibrillar proteins have been suggested (Lomiwes et al., 2014). Several studies reported positive relationships between increases in degradation of small HSPs (HSP27 in particular) and meat tenderness improvements, as degraded HSPs may no longer prevent irreversible damage to myofibrillar proteins (Balan et al., 2014; Choe et al., 2016; Cramer et al., 2018; Lomiwes, Farouk, Frost, Dobbie, & Young, 2013). In the present study, high collinearity between HSP27 intact/degradation and calpain 1 autolysis bands have been found in multiple clusters in all muscles, suggesting an interaction between HSP27 and calpain 1 in mediating proteolytic events. In a recently published study, addition of HSP27 was found to block the degradation of troponin T in calpain 1 and caspase-3 treated myofilaments, but the blockage was not observed in enzymatic degradation of nebulin and titin (Ding et al., 2018). This indicated that HSP27 may modulate proteolytic activities via interacting with either enzymes or substrates, or both. In the present study, LL muscles exhibited higher HSP27 degradation product compared to SM and PM counterparts, regardless of aging periods (Table 2.2). An increase in

degradation products of HSP27 and a decrease in intact HSP20 were also found in all muscles during aging periods (Table 2.2). The observed muscle-specificity in small HSP abundance and degradation could be attributed to muscle-specific metabolic responses. The exact mechanisms explaining muscle specificity in regulating small HSP activities and postmortem degradation remain largely unknow; however, the process could involve different levels of protein phosphorylation modification between muscles. It has been recently reported that the degradation of HSP27 was affected by the level of protein phosphorylation. Li, Li, Du, Shen, and Zhang (2018) found that the troponin T and HSP27 were less degraded in more phosphorylated muscles, concluding that phosphorylation would adversely affect the extent of proteolysis by hindering degradation of HSP27. As HSP27 phosphorylation is primarily mediated by MAPK-p38 signaling pathway (Armstrong, Delacey, & Ganote, 1999; Thompson, Maynard, Morales, & Scordilis, 2003), upregulation of MAPK members has been identified in m. soleus, an oxidative muscle, in comparison to glycolytic longissimus muscle (Li, Xu, Li, Xiong, & Zuo, 2010). This suggested a possibility that oxidative muscle, such as PM, may show higher level of phosphorylation modification of HSP27, which could due to higher ATP availability and protein kinase pathway activation compared to glycolytic muscle. As a result, less degradation of HSP27 occurred in PM. Less extent of HSP27 degradation in PM in comparison of LL could possibly indicate more chaperoning activity retention to protect structural protein from proteolytic events (Balan et al., 2014; Choe et al., 2016; Cramer et al., 2018; Lomiwes et al., 2013), further eliminating the aging potential of PM muscles. This postulation, however, would warrant further investigations for confirmation.

Another noteworthy observation in the present study is the muscle-specificity in proteolytic patterns of desmin vs. troponin T, where although PM had more desmin degradation products at 42 kDa, a lack of troponin T degradation was found. Desmin degradation was recognized as a good indicator of WHC improvement (Huff-Lonergan & Lonergan, 2005), which may possibly explain (at least in part) why the current PM muscles exhibited lower drip loss compared to the other muscle types. Also, the present study showed that drip loss was decreased over aging in LL and SM muscles, but not in PM, corroborating with the muscle specific degradation of troponin T. As discussed above, Li et al. (2018) found troponin T and HSP27 were degraded to a lesser extent in muscles with higher phosphorylation level; however, no difference in desmin degradation was observed in relation to the level of protein phosphorylation in their study. This possibly indicated

that in responding to postmortem proteolysis, different structural/myofibrillar proteins (i.e, troponin T vs. desmin) were processed in a different manner. While troponin T is located at the actin filament, desmin is part of intermediate filaments that connect sarcomeres in-between at Z-disk areas (Ertbjerg & Puolanne, 2017). From this perspective, chaperoning protection of HSP27 could be particularly targeting contractile fibril lattice region rather than inter-sarcomere area. Ouali et al. (2013) noted that the relocation of small HSPs is a crucial event in cytoskeletal protection, which is likely a strictly guided and regulated process rather than universal, random binding. Kötter et al. (2014) also reported a stress-initiated recruitment of HSP27 in protection of skeletal muscle fibers, which was specifically took place in titin of the Z-disk area.

Moreover, the present study found that LL showed an earlier onset of procaspase 3 cleavage compared to SM and PM (Figure 2.4). During apoptosis, caspase 3 is the major protease responsible for degrading key structural and functional cellular proteins (Huang, Huang, Xue, Xu, & Zhou, 2011). After cleavage of 32 kDa pro-caspase 3 zymogen, activated caspase 3 can be observed at 15-17 kDa (Cao et al., 2013; Underwood, Means, & Du, 2008). In the present study, LL had higher 15 kDa caspase 3 abundance compared to PM and SM counterparts at 1d postmortem. PM and SM showed an increase of caspase 3 cleavage at 9d, but no change was found in LL from 2d to 9d. This could be indicative of an earlier release of protease enzyme activity of caspase 3 in LL compared to SM and PM. Our finding was corroborating with Cao et al. (2010), where cellular apoptosis changes were assessed in longissimus, psoas, and semitendinosus of bovine muscles across 7d postmortem periods. The m. longissimus showed higher caspase 3 activity than m. psoas at 1d postmortem, which was concurrent with more extended apoptotic mediated proteolysis and morphological change (Cao et al., 2010). As previously discussed, the phosphorylation of anti-apoptotic molecule HSP27 is well recognized as an indispensable modification in responding to cellular stress to release cytoskeletal protective activities (Ito et al., 2005; Lomiwes et al., 2014; Takayama, Reed, & Homma, 2003). In the present study, less degradation of HSP27 and lower (or delayed) caspase 3 cleavage were concurrently found in PM muscles. Therefore, a cautious notion could be proposed that the muscle-specificity in small HSP modification, activity, and/or degradation may be involved in regulation of the onset of apoptosis. Hence, future research perspectives should be warranted focusing on explaining the specific protein target, the extent, and the time window of phosphorylation modification taken place under

different metabolic environments, and their roles in mediating postmortem apoptosis and proteolysis.

2.6 Conclusion

The results of the present study found muscle specificity in proteolytic changes of myofibrillar structural proteins, small heat shock proteins, and apoptotic factors. LL exhibited an increase in tenderization and higher WHC during postmortem aging compared to other muscles, which was concurrent with more extensive HSP27 degradation and an early onset of caspase 3 activation. PM muscles exhibited lower extent of calpain 1 autolysis and concurrent decreases in HSP27 degradation and troponin T degradation during aging. The muscle-specific proteolytic changes in small HSPs would be likely due to unique metabolic features of each of the muscle types, possibly related to the overall change of protein modification. Furthermore, less degradation of HSP27 was coincided with lower extent of troponin T degradation in PM during aging, while muscle specificity of desmin degradation was overall less evident. This observation could possibly indicate that muscle-specific small HSP activity might be particularly targeting the contractile fibril lattice region rather than the costamere regions. Therefore, interactions between cellular activities, including energy metabolism activities, apoptosis response, and small HSP translocation and/or phosphorylation, in postmortem muscles and corresponding effects on proteolysis, meat tenderization and WHC should be further elucidated.

2.7 Literatures cited

Armstrong, S. C., Delacey, M., & Ganote, C. E. (1999). Phosphorylation state of hsp27 and p38 MAPK during preconditioning and protein phosphatase inhibitor protection of rabbit cardiomyocytes. *Journal of Molecular and Cellular Cardiology, 31* (3), 555-567.

Balan, P., Kim, Y. H. B., & Blijenburg, R. (2014). Small heat shock protein degradation could be an indicator of the extent of myofibrillar protein degradation. *Meat Science, 97* (2), 220-222.

Bratcher, C. L., Johnson, D. D., Littell, R. C., & Gwartney, B. L. (2005). The effects of quality grade, aging, and location within muscle on Warner–Bratzler shear force in beef muscles of locomotion. *Meat Science, 70* (2), 279-284.

Brewer, S., & Novakofski, J. (2008). Consumer sensory evaluations of aging effects on beef quality. *Journal of Food Science, 73* (1), S78-S82.

Cao, J. X., Ou, C. R., Zou, Y. F., Ye, K. P., Zhang, Q. Q., Khan, M. A., Pan, D. D., Zhou, G. (2013). Activation of caspase-3 and its correlation with shear force in bovine skeletal muscles during postmortem conditioning. *Journal of Animal Science, 91* (9), 4547-4552.

Cao, J., Sun, W., Zhou, G., Xu, X., Peng, Z., & Hu, Z. (2010). Morphological and biochemical assessment of apoptosis in different skeletal muscles of bulls during conditioning. *Journal of Animal Science, 88* (10), 3439-3444.

Carlson, K., Prusa, K., Fedler, C., Steadham, E., Outhouse, A., King, D., & Lonergan, S. (2017). Postmortem protein degradation is a key contributor to fresh pork loin tenderness. *Journal of Animal Science, 95* (4), 1574-1586.

Choe, J. H., Stuart, A., & Kim, Y. H. B. (2016). Effect of different aging temperatures prior to freezing on meat quality attributes of frozen/thawed lamb loins. *Meat Science, 116*, 158-164.

Cramer, T., Penick, M. L., Waddell, J. N., Bidwell, C. A., & Kim, Y. H. B. (2018). A new insight into meat toughness of callipyge lamb loins - The relevance of anti-apoptotic systems to decreased proteolysis. *Meat Science, 140*, 66-71.

Delgado, E., Geesink, G., Marchello, J. A., Goll, D., & Koohmaraie, M. (2001). The calpain system in three muscles of normal and callipyge sheep. *Journal of Animal science, 79* (2), 398-412.

Ding, Z., Huang, F., Zhang, C., Zhang, L., Sun, H., & Zhang, H. (2018). Effect of heat shock protein 27 on the in vitro degradation of myofibrils by caspase‐3 and μ‐calpain. *International Journal of Food Science & Technology, 53* (1), 121-128.

England, E. M., Matarneh, S., Scheffler, T., Wachet, C., & Gerrard, D. (2015). Altered AMP deaminase activity may extend postmortem glycolysis. *Meat Science, 102*, 8-14.

Ertbjerg, P., & Puolanne, E. (2017). Muscle structure, sarcomere length and influences on meat quality: A review. *Meat Science*, *132*, 139-152.

Garrido, C., Gurbuxani, S., Ravagnan, L., & Kroemer, G. (2001). Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochemical and Biophysical Research Communications, 286* (3), 433-442.

Goll, D. E., Thompson, V. F., Li, H., Wei, W., & Cong, J. (2003). The Calpain System. *Physiological Reviews, 83* (3), 731-801.

Guillemin, N., Jurie, C., Cassar-Malek, I., Hocquette, J.-F., Renand, G., & Picard, B. (2011). Variations in the abundance of 24 protein biomarkers of beef tenderness according to muscle and animal type. *Animal, 5* (6), 885-894.

Huff-Lonergan, E., & Lonergan, S. M. (2005). Mechanisms of water-holding capacity of meat: The role of postmortem biochemical and structural changes. *Meat Science*, *71* (1), 194-204.

Huff-Lonergan, E., Zhang, W., & Lonergan, S. M. (2010). Biochemistry of postmortem muscle—Lessons on mechanisms of meat tenderization. *Meat Science, 86* (1), 184-195.

Honikel, K. O. (1998). Reference methods for the assessment of physical characteristics of meat. *Meat Science, 49* (4), 447-457.

Hopkins, D. L., & Thompson, J. M. (2001). The relationship between tenderness, proteolysis, muscle contraction and dissociation of actomyosin. *Meat Science, 57* (1), 1-12.

Huang, M., Huang, F., Xue, M., Xu, X., & Zhou, G. (2011). The effect of active caspase-3 on degradation of chicken myofibrillar proteins and structure of myofibrils. *Food Chemistry, 128* (1), 22-27.

Ito, H., Iwamoto, I., Inaguma, Y., Takizawa, T., Nagata, K. i., Asano, T., & Kato, K. (2005). Endoplasmic reticulum stress induces the phosphorylation of small heat shock protein, Hsp27. *Journal of Cellular Biochemistry, 95* (5), 932-941.

Kemp, C. M., Sensky, P. L., Bardsley, R. G., Buttery, P. J., & Parr, T. (2010). Tenderness – An enzymatic view. *Meat Science, 84* (2), 248-256.

Kim, N. K., Cho, S., Lee, S. H., Park, H. R., Lee, C. S., Cho, Y. M., Choy, Y. H., Yoon, D., Im, S.K., & Park, E. W. (2008).Proteins in longissimus muscle of Korean native cattle and their relationship to meat quality. *Meat Science, 80* (4), 1068-1073.

Kim, Y. H. B., Liesse, C., Kemp, R., & Balan, P. (2015). Evaluation of combined effects of ageing period and freezing rate on quality attributes of beef loins. *Meat Science, 110*, 40-45.

Kim, Y. H. B., Ma, D., Setyabrata, D., Farouk, M. M., Lonergan, S. M., Huff-Lonergan, E., & Hunt, M. C. (2018). Understanding postmortem biochemical processes and post-harvest aging factors to develop novel smart-aging strategies. *Meat Science, 144*, 74-90.

Kim, Y. H. B., Warner, R. D., & Rosenvold, K. (2014). Influence of high pre-rigor temperature and fast pH fall on muscle proteins and meat quality: a review. *Animal Production Science, 54* (4), 375-395.

Koohmaraie, M. (1996). Biochemical factors regulating the toughening and tenderization processes of meat. *Meat Science, 43*, 193-201.

Kötter, S., Unger, A., Hamdani, N., Lang, P., Vorgerd, M., Nagel-Steger, L., & Linke, W. A. (2014). Human myocytes are protected from titin aggregation-induced stiffening by small heat shock proteins. *Journal of Cell Biology, 204* (2), 187-202.

Kristensen, L., & Purslow, P. P. (2001). The effect of ageing on the water-holding capacity of pork: role of cytoskeletal proteins. *Meat Science, 58* (1), 17-23.

Lametsch, R., Lonergan, S., & Huff-Lonergan, E. (2008). Disulfide bond within calpain 1 active site inhibits activity and autolysis. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1784* (9), 1215-1221.

Li, Y., Xu, Z., Li, H., Xiong, Y., & Zuo, B. (2010). Differential transcriptional analysis between red and white skeletal muscle of Chinese Meishan pigs. *International Journal of Biological Sciences*, *6* (4), 350.

Li, Z., Li, M., Du, M., Shen, Q. W., & Zhang, D. (2018). Dephosphorylation enhances postmortem degradation of myofibrillar proteins. *Food Chemistry, 245*, 233-239.

Lomiwes, D., Farouk, M. M., Wiklund, E., & Young, O. A. (2014). Small heat shock proteins and their role in meat tenderness: A review. *Meat Science, 96* (1), 26-40.

Lomiwes, D., Farouk, M. M., Frost, D. A., Dobbie, P. M., & Young, O. A. (2013). Small heat shock proteins and toughness in intermediate pHu beef. *Meat Science, 95* (3), 472-479.

Morzel, M., Terlouw, C., Chambon, C., Micol, D., & Picard, B. (2008). Muscle proteome and meat eating qualities of Longissimus thoracis of "Blonde d'Aquitaine" young bulls: A central role of HSP27 isoforms. *Meat Science, 78* (3), 297-304.

Ouali, A., Gagaoua, M., Boudida, Y., Becila, S., Boudjellal, A., Herrera-Mendez, C. H., & Sentandreu, M. A. (2013). Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved. *Meat Science, 95* (4), 854-870.

Ouali, A., & Talmant, A. (1990). Calpains and calpastatin distribution in bovine, porcine and ovine skeletal muscles. *Meat Science, 28* (4), 331-348.

Ramos, P. M., Wright, S. A., Delgado, E. F., van Santen, E., Johnson, D. D., Scheffler, J. M., Elzo, M.A., Carr, C.C., Scheffler, T. L. (2020). Resistance to pH decline and slower calpain 1 autolysis are associated with higher energy availability early postmortem in Bos taurus indicus cattle. *Meat Science, 159*, 107925.

Takayama, S., Reed, J. C., & Homma, S. (2003). Heat-shock proteins as regulators of apoptosis. *Oncogene, 22* (56), 9041.

Thompson, H. S., Maynard, E. B., Morales, E. R., & Scordilis, S. P. (2003). Exercise‐induced HSP27, HSP70 and MAPK responses in human skeletal muscle. *Acta Physiologica Scandinavica*, *178* (1), 61-72.

Underwood, K., Means, W., & Du, M. (2008). Caspase 3 is not likely involved in the postmortem tenderization of beef muscle. *Journal of Animal Science, 86* (4), 960-966.

Veiseth-Kent, E., Pedersen, M. E., Rønning, S. B., & Rødbotten, R. (2018). Can postmortem proteolysis explain tenderness differences in various bovine muscles? *Meat Science, 137*, 114-122.

Watanabe, A., Tsuneishi, E., & Takimoto, Y. (1991). Effects of pithing on pH, creatine phosphate and ATP-related compounds of beef Psoas major and Longissimus dorsi muscles. *Meat Science, 29* (3), 221-228.

Wójtowicz, I., Jabłońska, J., Zmojdzian, M., Taghli-Lamallem, O., Renaud, Y., Junion, G., Daczewska, M., Huelsmann, S., Jagla, K., & Jagla, T. (2015). Drosophila small heat shock protein CryAB ensures structural integrity of developing muscles, and proper muscle and heart performance. *Development, 142* (5), 994-1005.

Table 2.1 Warner-Bratzler shear force, pH and water-holding capacity in three bovine muscles aged for different periods

| Parameters | Aging (A) | Interaction effect Muscle (M)[1] | | | SE[2] | P-value | |
		LL	SM	PM			
WBSF (kg)	2d	4.73^{ax}	4.42^{ax}	3.63^{bx}	0.23	M	<.0001
	9d	3.20^{by}	4.46^{ax}	2.30^{cy}		A	<.0001
	16d	2.86^{byz}	3.58^{ay}	2.53^{by}		M×A	0.005
	23d	2.54^{bz}	3.43^{ay}	2.20^{cy}			
pH*	1d	5.54^{bx}	5.55^{b}	5.59^{ay}	0.013	M	<.0001
	2d	5.50^{cy}	5.54^{b}	5.60^{ay}		A	0.003
	9d	5.56^{bx}	5.53^{b}	5.64^{ax}		M×A	<.0001
	16d	5.54^{bx}	5.55^{b}	5.65^{ax}			
	23d	5.47^{cy}	5.54^{b}	5.64^{ax}			
Cook Loss (%)	2d	22.77^{b}	30.39^{ax}	28.99^{ax}	1.08	M	<.0001
	9d	21.98^{b}	30.74^{ax}	22.24^{by}		A	0.02
	16d	23.01^{b}	27.25^{ay}	26.15^{ax}		M×A	0.007
	23d	22.22^{b}	28.06^{axy}	24.59^{abx}			
Purge Loss (%)	2d	1.61^{z}	2.22^{y}	2.89^{z}	0.54	M	0.0006
	9d	2.40^{byz}	3.21^{by}	6.14^{ax}		A	<.0001
	16d	3.29^{bxy}	4.97^{ax}	3.82^{abyz}		M×A	0.002
	23d	4.31^{x}	5.48^{x}	4.53^{y}			
Drip Loss (%)	2d	3.15^{ax}	3.79^{ax}	1.28^{b}	0.29	M	<.0001
	9d	3.29^{ax}	3.48^{ax}	1.91^{b}		A	<.0001
	16d	1.70^{y}	2.39^{y}	1.96		M×A	0.0005
	23d	1.60^{by}	2.10^{ay}	1.49^{b}			

* pH of 9d, 16d and 23d muscles were partially retrieved from Ma et al. (2017)
[1]Muscles: LL, *longissimus lumborum*; SM, *semimembranosus*; PM, *psoas major*
[2]Standard errors of means
a-c Means with different letters in each row and different muscles are significantly different ($P < 0.05$)
x-z Means with different letters in each column and different aging are significantly different ($P < 0.05$)

Table 2.2 Quantified relative abundance of myofibrillar structural proteins, calpain 1 autolysis, small heat shock proteins, and apoptotic proteins in three bovine muscles from different aging periods

| | Main Effects | | | | | | | | | | | | |
| | Aging (A) | | | | | SE[4] | Muscle[3] (M) | | | SE | P - value | | |
Parameter [1,2]	1d	2d	9d	16d	23d		LL	SM	PM		M	A	M×A
Structural protein degradation													
Desmin intact	1.54[a]	1.48[a]	1.21[b]	1.01[c]	1.11[bc]	0.091	1.21	1.25	1.36	0.082	0.084	<.0001	0.41
Desmin degradation 42kDa	0.40[d]	0.68[c]	0.98[b]	1.20[ab]	1.28[a]	0.014	0.67[c]	0.91[b]	1.15[a]	0.12	<.0001	0.0003	0.74
Desmin degradation 38kDa	0.061[d]	0.061[d]	0.32[c]	0.50[b]	0.71[a]	0.084	0.34	0.36	0.28	0.077	0.31	<.0001	0.47
Troponin T intact	1.40[a]	1.17[b]	0.94[c]	1.02[bc]	1.19[b]	0.085	1.19	1.21	1.04	0.085	0.64	0.02	0.86
Troponin T degradation 30kDa	0.21[c]	0.24[c]	0.47[b]	0.65[a]	0.51[b]	0.069	0.59[a]	0.51[a]	0.15[b]	0.063	<.0001	<.0001	0.0008
Calpain 1 80kDa	0.27[a]	0.20[b]	0.19[bc]	0.15[c]	0.17[c]	0.016	0.17[b]	0.20[a]	0.22[a]	0.014	0.001	<.0001	0.77
Calpain 1 78kDa	0.39[a]	0.36[a]	0.34[b]	0.29[c]	0.31[c]	0.023	0.30[b]	0.35[a]	0.37[a]	0.021	0.0002	<.0001	0.20
Calpain 1 76kDa	0.33[c]	0.44[b]	0.48[b]	0.56[a]	0.52[a]	0.035	0.54[a]	0.45[b]	0.41[b]	0.031	<.0001	<.0001	0.60
Small Heat Shock Proteins													
HSP27 intact	1.19	1.19	1.04	1.06	1.03	0.012	1.11	1.04	1.16	0.11	0.18	0.11	0.96
HSP27 degradation 20kDa	0.32[c]	0.48[bc]	0.53[b]	0.67[a]	0.62[a]	0.079	0.63[a]	0.56[a]	0.39[b]	0.070	0.0006	0.0003	0.76
HSP20	0.79[a]	0.87[a]	0.79[a]	0.63[b]	0.61[b]	0.065	0.89[a]	0.78[b]	0.55[c]	0.060	<.0001	<.0001	0.71
αβ-crystallin	0.59[ab]	0.69[a]	0.49[bc]	0.42[c]	0.44[c]	0.053	0.59	0.47	0.52	0.045	0.087	0.0002	0.70
Apoptotic Factors													
Cytochrome c	1.04[a]	1.15[a]	0.92[b]	0.98[b]	0.72[c]	0.105	0.80[b]	0.97[a]	1.11[a]	0.095	0.0006	0.002	0.77
Caspase 3	0.98[b]	0.89[b]	1.24[a]	-	-	0.098	0.99	1.08	1.05	0.098	0.62	0.001	0.03

[1] Protein abundance except calpain 1 was expressed as relative ratio of band intensity compared to the corresponding bands of the reference samples

[2] Three bands of 80, 78, and 76 kDa of calpain 1 were quantified and expressed as the ratio of each band that relative to the total intensity

[3] Muscle: LL, *longissimus lumborum*; SM, *semimembranosus*; PM, *psoas major*

[4] Standard errors of means

[a-d] Different letters within a row in each main effect indicate significant differences ($P < 0.05$)
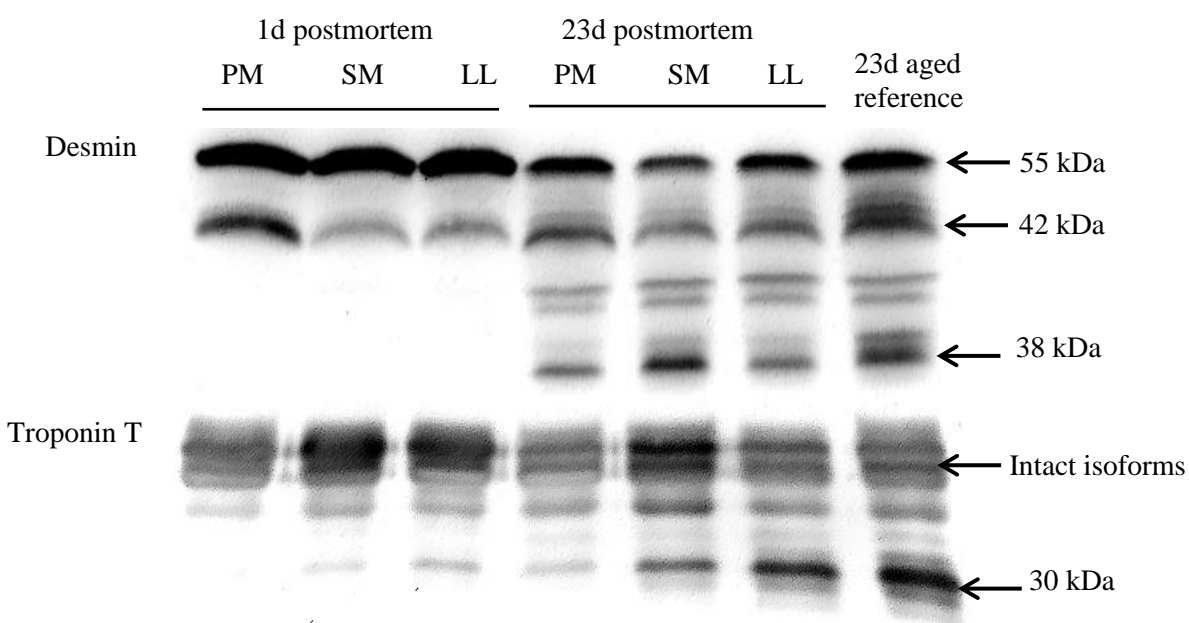
Figure 2.1 A representative set of western blot images showing postmortem change of desmin and troponin T in three bovine muscles. LL, longissimus lumborum; SM, semimembranosus; PM, psoas major. 23d aged reference: an LL sample aged for 23d postmortem
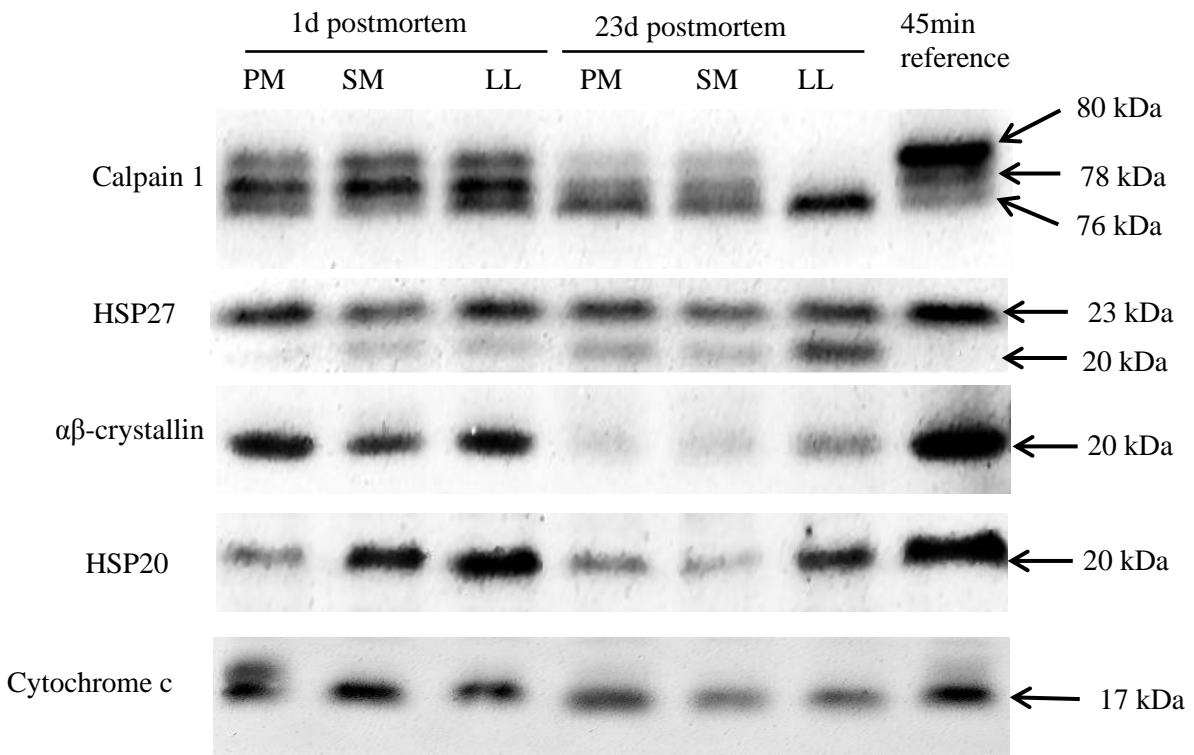
Figure 2.2 A representative set of western blot images showing postmortem change of calpain 1, small heat shock proteins, and cytochrome c in three bovine muscles. LL, longissimus lumborum; SM, semimembranosus; PM, psoas major. 45 min reference: an LL sample collected at 45 min postmortem
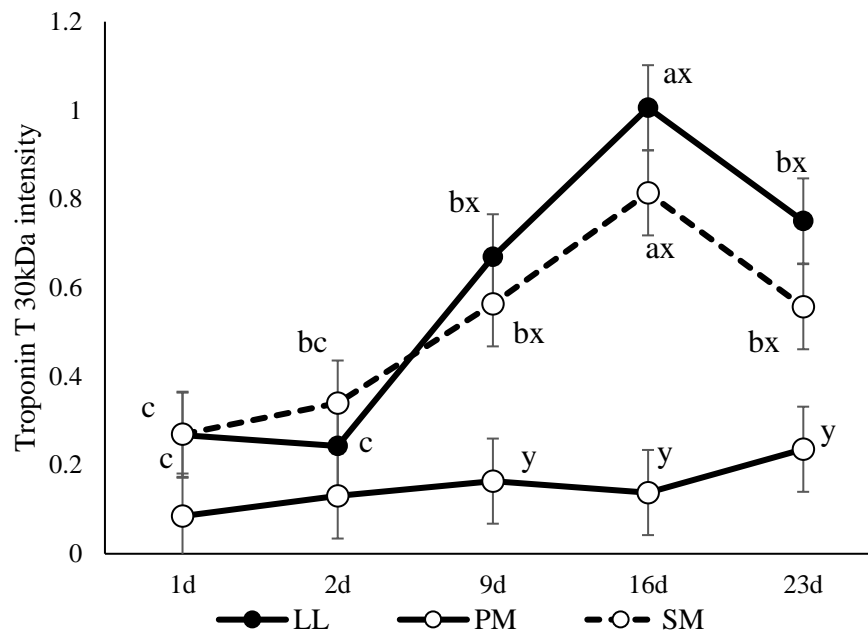
Figure 2.3 Interaction effect of muscle type and aging time on troponin T degradation products at 30 kDa, Densitometric analysis of the band in bovine LL, SM, and PM muscles during postmortem aging. [a-c] Different letters indicating that values within the same muscle are significantly different [x-z] Different letters indicating that values within the same aging are significantly different. LL, *longissimus lumborum*; SM, *semimembranosus*; PM, *psoas major*
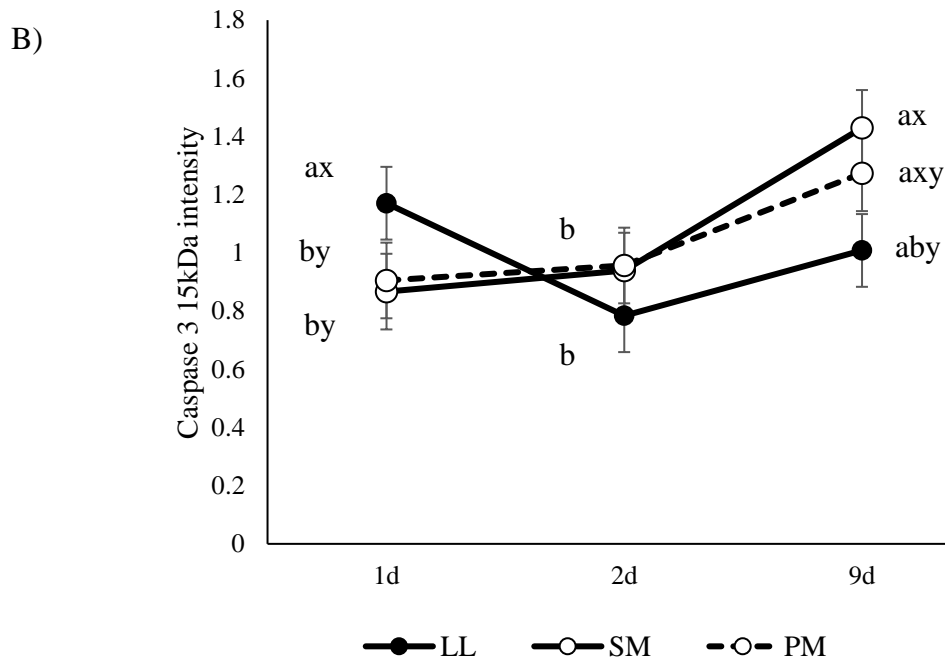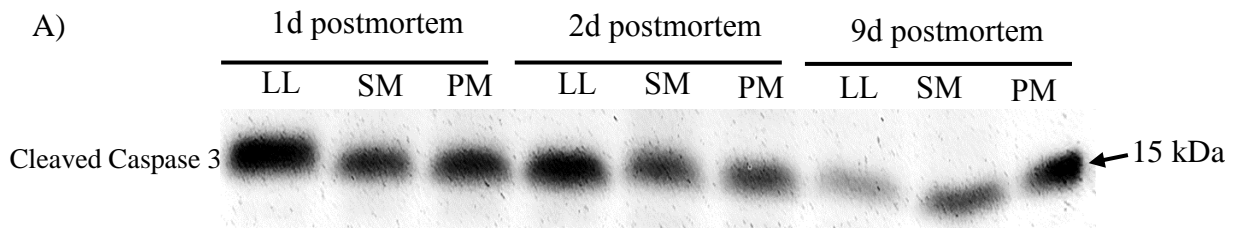
Figure 2.4 Interaction effect of muscle type and aging time on cleaved caspase 3. A. Representative western blot image of cleaved caspase 3 bands in bovine LL, SM, and PM muscles during postmortem aging; B. Densitometric analysis of cleaved caspase 3 in bovine LL, SM, and PM muscles during postmortem aging; a-c Different letters indicating that values within the same muscle are significantly different (P < 0.05). x-z Different letters indicating that values within the same aging are significantly different (P < 0.05). LL, longissimus lumborum; SM, semimembranosus; PM, psoas major
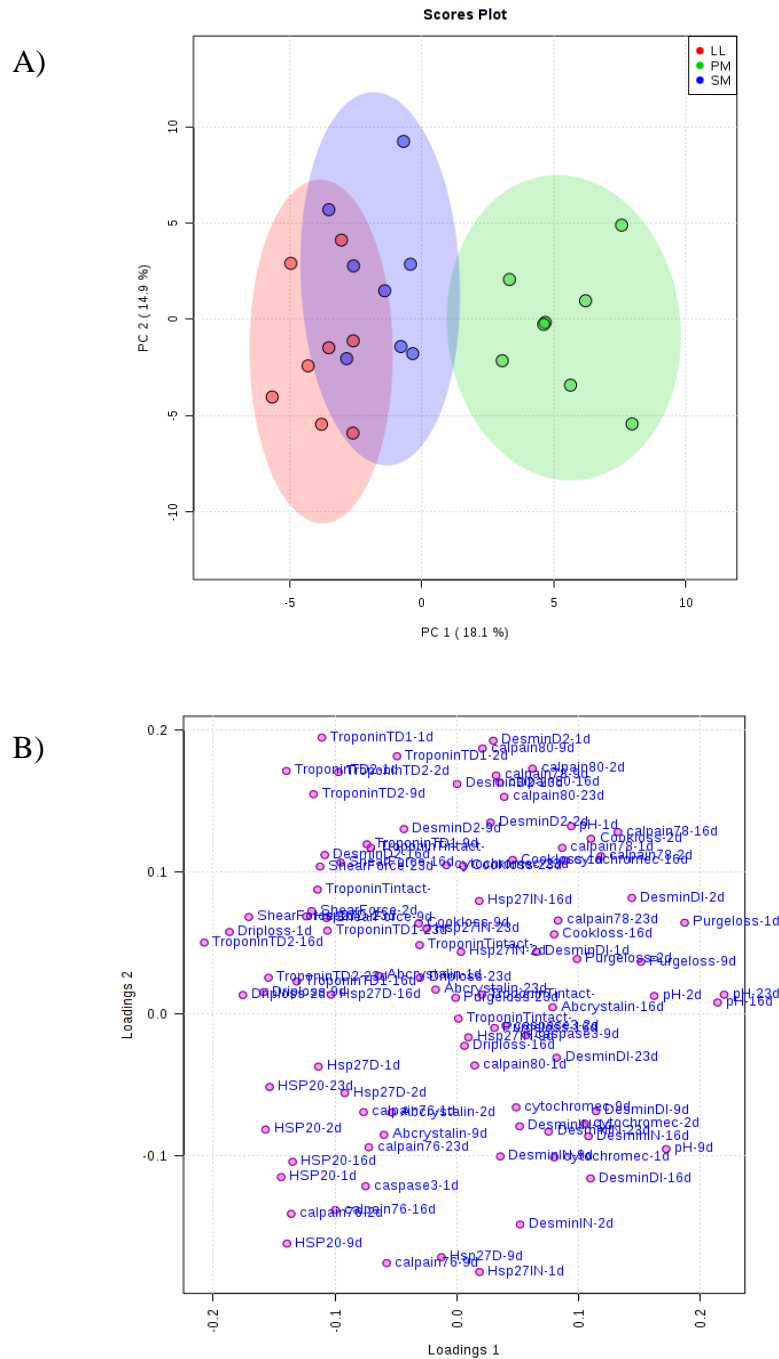
Figure 2.5 PCA analysis and loading plots based on meat quality and proteolytic attributes in three bovine muscles. (A-B) PCA based on quality and protein features with aging effect; 3 muscles from 8 animals as observations; (C-D) PCA based on quality and protein features without aging effect; 3 muscles from 8 animals with 2, 9, 16, and 23d postmortem aging as observations.

Figure 2.5 continued

C)

Scores Plot

D)
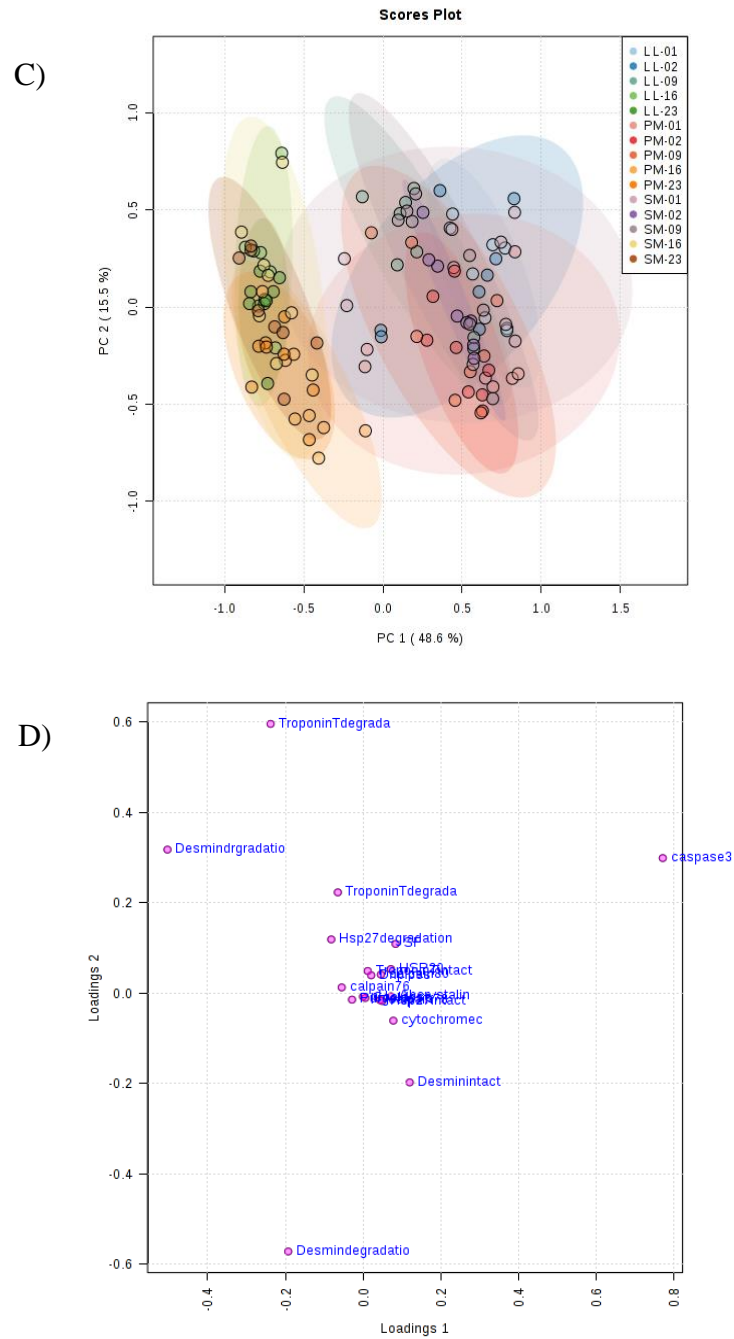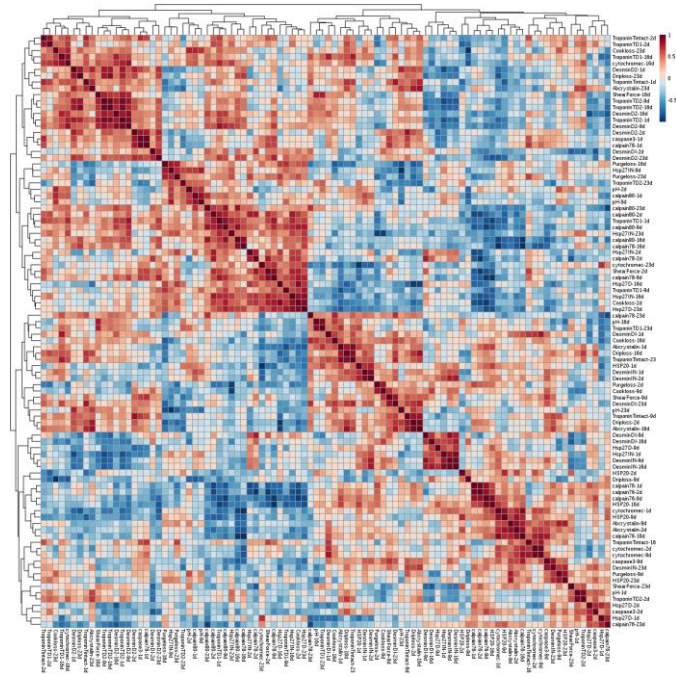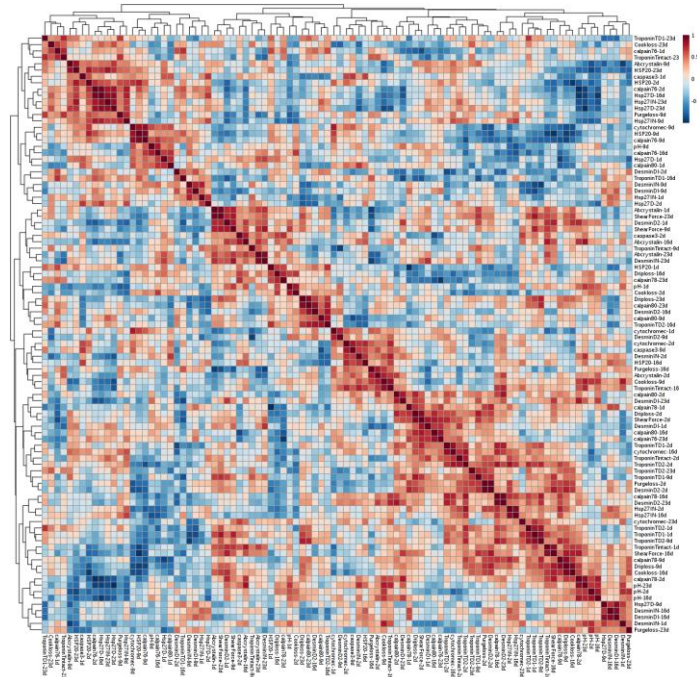
Figure 2.6 Figure 2.7 Correlation matrix heatmaps of meat quality attributes and proteolytic features in three bovine muscles; (A) LL, *longissimus lumborum*; (B) SM, *semimembranosus*; (C) PM, *psoas major*

Figure 2.6 continued

C)
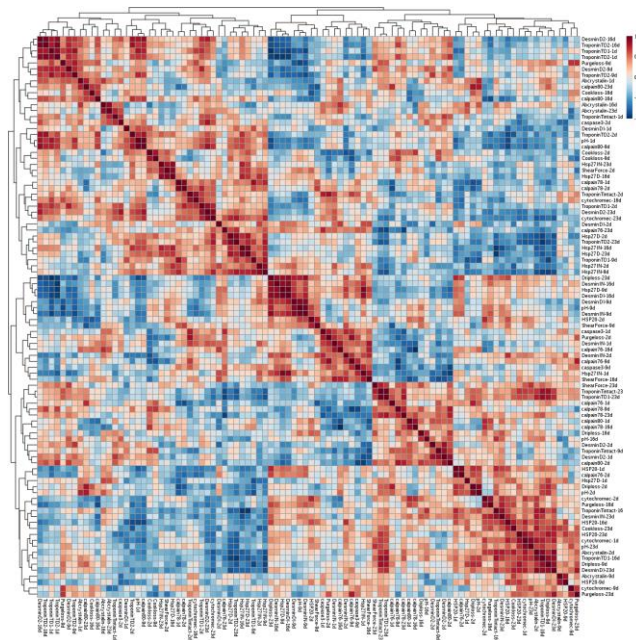
CHAPTER 3.   PROTEOMIC AND METABOLOMIC PROFILING REVEALS THE INVOLVEMENT OF APOPTOSIS IN MEAT QUALITY CHARACTERISTICS OF OVINE *M. LONGISSIMUS* FROM DIFFERENT CALLIPYGE GENOTYPES

3.1   Abstract

Proteome and metabolome changes in muscles from callipyge mutation (+/*C*) and non-callipyge phenotype (+/+, *C*/+, and *C*/*C*) lambs were profiled to provide insight into the biochemical changes affecting meat quality attributes. *M. longissimus thoracis* from lambs with all four possible callipyge genotype (n = 4, *C*/+, *C*/*C*, +/*C*, and +/+) were collected after 3d aging and analyzed using mass-spectrometry based platforms. Among identified proteomes, cytochrome c (pro-apoptotic protein) was detected with significantly lower abundances in +/*C*. Anti-apoptotic HSP70, BAG3, and PARK7 were over-abundant in +/*C*, which could result in delayed apoptosis and possibly attributed to tougher meat in callipyge lambs. Eight glycolysis enzymes were overabundant in +/*C* lambs, whereas 3 enzymes involved in TCA cycle were overabundant in non-callipyge ones (*C*/*C* and/or *C*/+). Twenty-five metabolites were affected by genotypes ($P < 0.05$), including metabolic co-factors, polyphenols, and AA/short peptides. Our omics results provided insightful information for revealing the differences in biochemical attributes caused by callipyge mutation.

Keywords: Proteomics; Metabolomics; Apoptosis; Callipyge lambs

3.2   Introduction

The callipyge sheep is an unique animal model for the study of muscle development and biology, as the callipyge genotype is characterized with postnatal muscle hypertrophy in specific loin and hindquarter areas (Bidwell et al., 2014; Koohmaraie, Doumit, Shackelford, Lonergan, & Wheeler, 1995). The unique phenotype, where the reduced body fat accretion and elevated fast-

twitch glycolytic type IIB muscle fibers occur, is only observed when paternally inherited callipyge mutation allele combines with a normal maternal allele, which is noted as $+/C$ (Jackson, Green, & Miller, 1997; Jackson, Miller, & Green, 1997; Li et al., 2014). Conversely, the other three genotype combinations (C/C, $C/+$, and $+/+$), exhibit normal phenotype (Beattie et al., 1998; Keele, Leymaster, Freking, & Nielsen, 1998).

The callipyge genotype ($+/C$) shows an overexpression of calpastatin, which is an allosteric inhibitor of calpain 1, producing tougher meat compared to normal genotypes (C/C, $C/+$, and $+/+$) (Koohmaraie et al., 1995). The role of calpain/calpastatin system in meat tenderization process has been extensively studied, as enzymatic degradation of myofibrillar structures via calpain 1 is the major contributor of postmortem tenderization of meat (Geesink & Koohmaraie, 1999; Maddock, Huff-Lonergan, Rowe, & Lonergan, 2005). Moreover, recent studies proposed that the ante-mortal cell fate determining process, namely apoptotic and anti-apoptotic response of the muscle cells, could be the preceding step that modulate calpain 1 activation (Maddock et al., 2005; Ouali et al., 2013). In our recent published studies, we found that hypertrophied callipyge muscles showed delayed onset of apoptosis with less degradation of anti-apoptotic small heat shock proteins compared to muscles from normal genotypes (Cramer, Penick, Waddell, Bidwell, & Kim, 2018). Furthermore, *m. longissimus* of callipyge lamb retained greater color stability compared to meat from other non-callipyge genotypes (Penick et al., 2017).

Recently, omics techniques have been increasingly applied to analyze biochemical changes in postmortem muscles and their relevance to meat quality attributes. Various pre-slaughter and postmortem processing conditions have been found to influence meat quality developments in various animal species, including color, water-holding capacity, oxidative stability, tenderness and flavor. To obtain further insights how these quality traits are developed and regulated, mass-spectrometry (MS) based proteomics and metabolomics platforms have been widely used to delineate the underlying mechanisms via profiling the biological/physiochemical reaction networks in various fresh muscle foods (Kim, Kemp, & Samuelsson, 2016; Ma et al., 2017; Subbaraj, Kim, Fraser, & Farouk, 2016).

The callipyge lamb model could provide a novel platform to parse the associated key metabolism features and protein networks that are closely modulating postmortem proteolysis as well as other meat quality attributes. However, there is little to no published information using omics approaches to understand the unique biochemical and quality characteristics of callipyge

lamb meat. Therefore, the objective of this study was to elucidate the effects of the callipyge mutation on differentially expressed protein/metabolites profiles and their possible involvement in affecting meat quality attributes. Four genotypes of lambs (*+/C*, *C/C*, *C/+*, and *+/+*) were used in the present study, and both proteome and metabolome were analyzed utilizing HPLC-MS-based platform

3.3   Materials and methods

3.3.1   Raw materials and sample preparation

The Purdue Animal Care and Use Committee (PACUC) approved the use of animals in this study (Protocol # 1112000493). Sixteen dark face crossed lambs (wethers and ewes, averaged 48.1 kg, 7 months old) were harvested (n = 4 for *C/+*, *C/C*, *+/C*, and *+/+*). After 24 h post-mortem, the right-side loin (*m. longissimus thoracis*) was separated and aged for 3 days in 4 °C. After the completion of aging period, samples were immersed in liquid nitrogen immediately and subsequently stored at -80 °C until sample preparation of proteomics and metabolomics analysis. Four replicates of each genotype were used for proteomics analysis, and three replicates of each genotype were used for metabolomics analysis. All the muscle samples were snap frozen powered, then crushed with mortar and pestle.

3.3.2   Analysis of proteomics

3.3.2.1 Protein extraction and digestion

The loin sample (10 mg) was added to 50 µL of 100 mM ammonium bicarbonate, sonicated for 30 minutes and centrifuged for 5 minutes to collect the supernatant. Proteins were precipitated using cold acetone (-20 °C). Samples were reduced and alkylated followed by the addition of sequence grade Lyc-C/Trypsin (Promega) on a scale of protein/trypsin 50:1 (w/w) for enzymatic digestion, which was carried out in the Barocycler NEP2320 (PBI) at 50 °C under 20,000 psi for 60 minutes. The digested samples were then cleaned over C18 MicroSpin columns (Nest Group), dried and resuspended in a diluent composed of 97% water and 3% acetonitrile (v/v) with 0.1% formic acid (w/v).

3.3.2.2 HPLC-MS/MS analysis for proteomics

Separation of peptides was separated by a nanoLC system (1100 Series LC, Agilent Technologies, Santa Clara, CA). The peptides were loaded on the Agilent 300SB-C18 enrichment column for concentration. After 5 minutes, the peptides were separated with the C18 reversed phase ZORBAX 300SB-C18 analytical column (0.75 μm × 150 mm, 3.5 μm) from Agilent. For the first 5 minutes, the column was equilibrated with 95% water/0.1% formic acid (mobile phase A) followed by the linear gradient of 5% acetonitrile/0.1% formic acid (mobile phase B) to 35% in 95 minutes, 40% B in 97 minutes, and 95% B in 100 minutes. The column remained at 95% B for 2 minutes before being brought back to 95% B and held for 20 minutes. A blank injection was run between samples to avoid carryover and keep the system clean. The LTQ-Orbitrap mass spectrometer was used to identify peptides. The data-dependent positive acquisition mode was operated in which each full MS scan (30.000 resolving power) was followed by eight MS/MS scans, where the eight most abundant molecular ions were selected and fragmented by collision-induced dissociation (CID) using normalized collision energy of 35%.

3.3.3 Analysis of metabolomics

3.3.3.1 Metabolites extraction

Protein removal and sample extraction were performed according to Bligh-Dyer with a minor change. Chloroform (200 μL) with an equal volume of methanol was added to 100 mg of tissue. Samples were extracted in tissue homogenizer, and 200 μL of water was added to the extraction, which was then shaken for 2 hours. After that, samples were centrifuged at 16,000 ×g and 4 °C for 8 minutes. The upper methanol and water phase contained the metabolites. The lower chloroform phase contained the lipids. Each phase was removed and separately transferred to its own vial. Vials were evaporated to dryness in a SpeedVac Concentrator. The metabolite fraction was reconstituted in 50 μL of a diluent composed of 95% water and 5% acetonitrile, containing 0.1% formic acid. The lipid fraction was reconstituted in a diluent composed of 50% water and 50% acetonitrile.

3.3.3.2 HPLC-MS analysis of metabolomics.

For metabolomics profiling, separations were performed on an Agilent 1100 system (Palo Alto, CA) using a Waters T3 column (3 µm, 150 × 2.1 mm i.d). A binary mobile phase consisting of solvent systems A and B were used in gradient elution, where A was 0.1% formic acid (v/v) in double distilled water and B was 0.1% formic acid (v/v) in acetonitrile. The mobile phase flow rate was set at 0.3 mL/min. Initial conditions were 100:0 (A:B) and were held for 1 min, followed by a linear gradient to 5: 95 at 21 min and were held until 26 min. Column re-equilibration returned to 100:0 at 30 min and was held until 35 min. The lipids were assayed using a Waters Xterra MS C18 column (5 µm, 2.1 × 150 mm). A binary mobile phase consisting of solvent systems A and B were used, where A was 0.1% formic acid (v/v) and 10 mM ammonium acetate in water and B was 0.1% formic acid (v/v) and 10 mM ammonium acetate in acetonitrile. Initial conditions were 65:35 (A:B) and were held for 1 min, followed by a linear gradient to 20:80 at 10 min, 0:100 at 20 min and was held until 32 min. Column re-equilibration returned to 65:35 at 33 min and was held until 40 min.

Following chromatographic separation, the compounds were identified by electrospray ionization (ESI) into an Agilent MSD-TOF spectrometer. ESI capillary voltage was 3.5 kV, nitrogen gas temperature was set to 350 °C, drying gas flow rate was 9.0 L/min, nebulizer gas pressure was 35 psig, fragmentor voltage was 135 V, skimmer was 60 V, and OCT RF was 250 V. Mass data (from m/z 70-1100) were collected using Agilent MassHunter software (v. B.03).

3.3.4   Data processing and statistical analysis

For proteomics, the.RAW files from LC-MS/MS analysis were processed using the MaxQuant computational proteomics platform version 1.5.3.17 (Cox & Mann, 2008). The peak list generated was screened against the *Bos taurus* and *Ovis aries* sequence from UniProt. Data were analyzed by MaxQuant with the following settings: initial precursor and fragment mass tolerance set to 0.07 and 0.02 Da respectively, minimum peptides length of seven amino-acid. The fasta databases were randomized and the protein FDR was set to 1 %. Enzymatic trypsin digestion allowed for two missed cleavages and three modifications per peptide; the fixed modifications were iodoethanol (C), variable modifications were set to Acetyl (Protein N-term) and Oxidation

(M). Further multivariate statistical analyses were performed by online software of MetaboAnalyst 3.5 (http://www.metaboanalyst.ca/). The features (proteins) which have more than 50% missing values were removed. The remaining missing values of those proteins were estimated by a small value (half of the minimum positive value in the original data). Prior to statistical analyses, all variables were normalized by the constant sum, transformed by generalized log transformation, and scaled to range variance. The principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and hierarchical cluster analysis (HCA) were performed. The deferentially presented proteins were initially screened using ANOVA ($P < 0.05$) or pairwise fold change comparison (FC > 1.5), then cross validated using PLS-DA, with VIP >1 (importance of projection scores) as criteria. High to low fold change was calculated between two genotypes with highest and lowest protein abundance, respectively.

For metabolomics, the Agilent.d files from HPLC-MS analysis were processed using Mass Profile Professional 13.1.1 (Agilent, Santa Clara, CA, USA). The metabolite abundance data were normalized (log scale) using an external scalar (individual loading weight of each sample). Metabolites that have more than 50% missing values were excluded from the analysis. For each feature, the equal variance assumption was checked using Barlett's test and normality of residual term was checked using Shapiro-Wilk's test. Metabolites that satisfied both assumptions were analyzed using ANOVA, those did not satisfy the assumptions of ANOVA were analyzed using Kruskal-Wallis test. Differentially presented metabolites were determined using FDR < 0.05.

3.4 Results

3.4.1 Proteins identification and comparison

A total of 178 proteins were identified and checked for data integrity to be used for PCA, HCA, and PLS-DA analyses. Distinct proteome profiles of different callipyge genotypes were visualized in PCA score plot (Figure 3.1), which showed that 46.9% and 13% of the total variance were explained by the first two principle components, respectively. The callipyge genotype (*+/C*) formed a cluster that was separated from other non-callipyge genotypes (*C/C*, *C/+*, and +/+), indicating its unique proteome profile. HCA heatmap was performed to visualize the intensity of each protein, samples from different genotype demonstrated different color distributions (Figure 3.2). The *C/+* and +/+ samples showed similar proteome profiles and were aggregated into a cluster

firstly. When the Euclidean distance was increased, *C/C* was then aggregated into the former cluster, and separated from +/*C*. The results of HCA were consistent with PCA, which all indicated the distinctive proteome profile of +/*C* comparing to other normal genotypes (*C/C*, *C/+*, and +/+).

ANOVA (*P* < 0.05) and pairwise fold-change (FC > 1.5) were initially performed to find proteins with possible abundance difference across the four genotypes. A total of 63 proteins were determined, 16 of which were identified by both methods, and 13 and 34 proteins were determined by only ANOVA or pairwise method, respectively. Due to the inconsistency in validating differentially presented proteins by the two method, the 63 proteins were cross validated using Variable Importance in Projection (VIP) score in PLS-DA model using criteria of VIP > 1. Proteins with at least one of the first two VIP values larger than 1 were considered as differentially presented across 4 genotypes. A total of 38 proteins were identified as the final ones, which can be classified into six categories: metabolic enzymes, proteins related to stress response and apoptosis, muscle structure and function related proteins, binding and transporting proteins, and signal transduction/regulation.

Eight enzymes in glycolysis (pyruvate kinase, glucose-6-phosphate isomerase, phosphoglucomutase-1, phosphoglycerate mutase, enolase, fructose-bisphosphatase-2, fructose-bisphosphate aldolase, and L-lactate dehydrogenase) were identified with more than 1.5-fold higher in +/*C* muscles than the others (Table 3.1); whereas proteins and enzymes involved in the arobic respiration were less abundant in +/*C*, including malate dehydrogenase 2, aldehyde dehydrogenase, and cytochrome c. Among those, phosphoglucomutase-1 showed significantly overabundant expression in the +/*C* muscle sample than all other normal genotype muscles. In addition, stress response and apoptosis related proteins were affected by +/*C* genotype, where HSPA1A (HSP70), PARK7 (Protein DJ), and BAG3 (BCL2 associated athanogene 3) presented the highest abundance in +/*C* genotypes, and HSPB1 (HSP27) exhibited the lowest content in +/*C* samples compared to other normal genotypes (Table 3.1). Three structural proteins titin, nebulin, and myozenin 1 presented lower abundance in +/*C* genotype, all of which were associated with z-disc/myofibrillar microstructure (Table 3.1). Also, eight proteins that retained general binding and/or involved in cell cycle regulation, including elongation factor 2, galectin, 14-3-3 protein isoforms, PDZ and LIM domain protein 7 and valosin-containing protein (Table 3.1), all of which uniquely presented highest or lowest abundance in +/*C* genotype compared to other normal genotypes. Overall, the identification of differentially presented protein was corroborate with PCA

and HCA analysis, as most of the proteins exhibited distinctively high or low abundance in the callipyge (*+/C*) genotype.

To evaluate the major protein network pathways that affected by callipyge genotype, the STRING 9.1 was used to characterize the interactions among the 38 differentially abundant proteins in the ovine muscle proteome (*Ovis aries* database, confidence level 0.5, Figure 3.3.). Visually, the network comprised of 3 major clusters with 38 nodes (proteins) and 75 edges (interactions). The major clusters represented glucose metabolism, muscle function, and stress response, respectively, with the central nodes comprised of glycolytic enzymes enolase, pyruvate kinase, fructose-bisphosphate aldolase and others. Moreover, KEGG enrichment showed similar findings (Figure 3.4). Nine pathways ($P < 0.05$) were revealed, where glycolysis/gluconeogenesis was the most significant enrichment pathway, and 12 proteins were enriched in this pathway. Other important pathways were involved in pyruvate metabolism, pentose phosphate pathway, TCA cycle, and biosynthesis of amino acids.

3.4.2 Metabolomics

The HPLC-MS analysis initially detected 425 nonpolar and 665 polar features in lamb loin muscle samples with four genotypes. Of the 1088 m/z-rt features from the initial data set, 854 were satisfied the two assumptions of ANOVA. For the remaining 234 features, the Kruskal-Wallis test was used for comparison across the genotype groups. Twenty-five metabolites were identified with significant difference (FDR < 0.05) in abundance across different genotypes. These metabolites consisted of amino acids and dipeptides, dietary-derived phenolic compounds, lipid and lipid oxidation product, energy metabolism related compounds, and oligopeptides (Table 3.2). Leu-Pro, L-anserine, Met-Phe, homoarginine, Tyr-Ala, resveratrol 4'- (2-galloylglucoside), octadienoic acid, inosine 5'-monophosphate, Pro Gln Asn, and Glu Arg Pro were found with the highest content in *+/C* muscles. Whereas Pro-Val, Val-Val, abscisic aldehyde, PC (16:1 (9E)/0:0), dephospho-CoA, pivaloylcarnitine, Tyr Pro Lys, and Lys Lys Pro Arg were found to be the lowest content in *+/C* muscles.

3.5 Discussion

The results of the present study elucidated the effects of callipyge mutation on differentially expressed protein/metabolites profiles, which could possibly reveal the combined effects of (anti-) apoptotic cascades, metabolism activities, and proteolytic systems in meat quality development. According to STRING interaction analysis, a group of proteins with pro- or anti-apoptotic functions was annotated as one of the major clusters (Figure 3.3, green nodes), including cytochrome c (CYCS), HSP70 (HSPA1A), HSP27 (HSPB1), BAG3 (BCL2 associated athanogene 3), protein DJ-1 (PARK7), and elongation factor (EEF2). These proteins strongly link the apoptotic response and mitochondria activity with the meat aging process, which was previously reported in literatures (Becila et al., 2010; Ouali et al., 2013).

In the present study, cytochrome c was identified with higher abundance in non-callipyge lambs than that in callipyge ones. This observation was consistent with our previous study, where lower cytochrome c contents in loin muscles from callipyge lambs were found throughout postmortem aging compared to the muscles from non-callipyge lambs (Cramer et al., 2018). Previously, it has been shown that the release of cytochrome c from mitochondria to cytoplasm is the initiating step of apoptotic cascade (Skulachev, 1998). Elevated cytosolic cytochrome c escalates the enzymatic activities of caspases - the major protease system responsible for degrading cell structural and functional compartments (Jiang & Wang, 2004; Zhang, Zheng, Nussinov, & Ma, 2017). Caspases can also cleave calpastatin, resulting in an increase in calpain activity (Pörn-Ares, Samali, & Orrenius, 1998; Wang et al., 1998). Therefore, it is reasonable to postulate that less cytochrome c in callipyge lamb meat could result in lower activity of caspases or delayed onset of apoptosis, which could in turn partially contribute to higher calpastatin activity compared to non-callipyge counterparts. As a result, the higher activity of calpastatin in callipyge lamb leads to tougher meat due to inhibited calpain 1 activation (Cramer et al., 2018).

Moreover, BAG3 and HSP70 were found with significantly higher abundance in $+/C$, whereas HSP27 was lower in $+/C$ compared to other normal genotypes. HSP70 is a well-known chaperone protein to modulate apoptosis by interfering with cytochrome c release, and BAG3 potentially suppress cell apoptosis via its inhibitory activity to BCL2, a fundamental pro-apoptotic molecule (Hishiya, Kitazawa, & Takayama, 2010). One example of BAG3 and HSP70 interaction modulating muscle structure and function is that the deletion of BAG3 gene leads to mislocalization and destabilization of the HSP70 substrate CapZβ1, and eventually causes

myofibrillar degeneration (Hishiya et al., 2010). Therefore, overabundance of BAG3 and HSP70 in callipyge lambs is protective to live muscle structural integrity, and could potentially retard postmortem apoptosis due to their anti-apoptotic activities. This, in turn, may lead to decreased proteolysis during postmortem aging, resulting in tougher meat compared with non-callipyge genotypes. In addition, HSP27 is another chaperone protein that was identified as one of the quality markers that related to meat tenderness characteristics (Guillemin et al., 2011; Kim et al., 2008; Morzel, Terlouw, Chambon, Micol, & Picard, 2008). The overall abundance of HSP27 in the present study was shown decreased in *+/C*. Due to the limitation of a gel-free, mass-spec based proteomics system, the protein abundance of the current study cannot be further identified as whether intact or degradative forms of HSP27. However, Cramer et al. (2018) found that *longissimus* muscle from callipyge lamb showed a significantly lower band intensity of HSP27 degradation products compared to the muscle from non-callipyge lamb based on their targeted western-blot analysis. Therefore, it would be reasonable to conclude the observed lower abundance of protein would be likely related to HSP27 degradation product, implying that the muscle from callipyge might maintain its chaperoning activity possibly hindering myofibrillar protein degradation. In fact, the lower abundance of myofibrillar structural proteins, such as titin, nebulin, and myozenin 1, was found in loins from *+/C* genotype compared to others (P < 0.05), indicating the lower degradation of these cytoskeletal myofibrillar proteins occurred in the callipyge lamb loins. This observation is in good agreement with the findings from Cramer et al. (2018), where they also reported the lower degradation of desmin and troponin-T in the loins from callipyge compared to loins from non-callipyge lambs.

Several studies found that HSP27 was among the substrates of postmortem proteolysis (Lametsch, Roepstorff, & Bendixen, 2002; Morzel et al., 2004), and postulated its relevance to the extent of postmortem protein degradation (Balan, Kim, & Blijenburg, 2014; Choe, Stuart, & Kim, 2016; Cramer et al., 2018; Lomiwes et al., 2014). Ma and Kim (2019) recently reported that beef *longissimus* muscles showed extended myofibrillar degradation coupled with increases in HSP27 degradation during aging, indicating that HSP27 degradation is a robust indicator of postmortem proteolysis (Balan et al., 2014).

The current study also found that PARK7 was overabundant in *+/C* lambs, consistent with previous reports (Yu et al., 2018). PARK7 was identified as an important modulator of muscle hypertrophy in callipyge sheep (Yu et al., 2018). It also serves as a redox-sensitive molecular

chaperone, a sensor of oxidative stress, and a positive regulator of cytoprotective pathways among other functions (Vavougios et al., 2018). Gene research indicated that in callipyge lambs, the up-regulation of PARK7 could enhance activities of phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) pathway, consequentially increase protein synthesis and muscle mass (Yu et al., 2018). Proteomics investigation also indicated PARK7 as a marker of compromised meat tenderness (Gagaoua, Terlouw, Boudjellal, & Picard, 2015), corroborating its essential role in anti-apoptotic and anti-oxidant activities (Picard & Gagaoua, 2017). Therefore, overabundant PARK7 in *+/C* in present study was in agreement with previous studies, which suggested an association between PARK7 and down-regulated proteolysis, probably due to its biochemical activities promoting protein synthesis and defending against apoptotic response (Kemp, King, Koohmaraie, Shackelford, & Wheeler, 2009; Yu et al., 2018).

Besides the apoptotic vs. anti-apoptotic systems, STRING interactions showed that glucose metabolism enzymes formed the central nodes with most interactions being annotated (Figure 3.3, red nodes). Eight glycolytic enzymes were overabundant in loins from *+/C* lambs compared to that from non-callipyge counterparts. Glycolysis/gluconeogenesis were as well the most significantly enriched pathway from KEGG result (Figure 3.4). Moreover, MYL3 (Myosin light chain 3, typical MYL isoform representing Type IIA fast-oxidative fiber) was significantly down-regulated in *+/C* with 7.285-fold, compared to *+/+*, confirming the oxidative to glycolytic fiber type shifting.

The current metabolomics analysis also identified lower abundance of dephospho-CoA and pivaloylcarnitine in *+/C*, where both compounds were important participants in aerobic respiration (TCA and electron transport chain) activities. These results suggested a significant distinction in postmortem metabolic processes between muscles from callipyge and non-callipyge lamb carcasses, which subsequently would result in different quality attributes in terms of oxidative stability, particularly color stability in lamb meat. Our previous research found that the loins from callipyge lambs showed initially lower redness, but more color-stable during display, when compared to loins from non-callipyge lambs (Penick et al., 2017). As slow twitch, oxidative muscle fibers have more abundant myoglobin and mitochondria content (Choi & Kim, 2009), ante-mortal aerobic metabolism could be facilitated by oxygen residuals from oxymyoglobin (Pösö & Puolanne, 2005), generating massive reactive oxygen species through the incomplete oxidation of oxygen to water (Holzerová & Prokisch, 2015). This in turn could increase oxidative stress of

slow-red muscles (Ke et al., 2017), accelerating the oxidation of myoglobin and lipid during the subsequent display (Ke et al., 2017; Ramanathan & Mancini, 2018). Thus, less oxidative metabolism in callipyge could partially explain more stable color during aging/display compared to other normal genotypes. Moreover, the current metabolomics analysis identified antioxidant metabolites, including dietary polyphenol resveratrol and histidyl dipeptide L-anserine, with higher abundance in *+/C* muscle samples, corroborating with the notion that callipyge lamb meat had higher oxidative stability.

3.6   Conclusion

The results of the current study found that several novel factors including energy metabolism enzymes and co-enzymes, apoptosis related factors, and anti-oxidant metabolite compounds were significantly affected by the callipyge genotypes. Omics results give us a better understanding of the meat quality trait differences arisen from callipyge mutation, such as tenderness and meat color stability. Superior oxidative stability of callipyge muscles could be likely due to the shift from oxidative metabolism toward glycolytic metabolism, coupled with alteration in apoptotic and anti-stress responses. Our results also revealed that compromised proteolytic aging potential in postmortem muscles from callipyge lamb carcasses would be partly attributed to more elevated anti-apoptotic features compared to muscles from non-callipyge counterparts. Further studies are needed to confirm the roles of identified proteins and metabolites in explaining the distinct meat quality development of callipyge mutation and applying to muscles from other specices (e.g. beef muscles) with limited aging potential.

3.7   Literatures cited

Balan, P., Kim, Y. H. B., & Blijenburg, R. (2014). Small heat shock protein degradation could be an indicator of the extent of myofibrillar protein degradation. *Meat Science, 97* (2), 220-222. doi: https://doi.org/10.1016/j.meatsci.2014.01.019

Beattie, C. W., Keele, J. W., Leymaster, K. A., Kappes, S. M., Sonstegard, T. S., Smith, T. P. L.,... Nielsen, M. K. (1998). Evaluation of the ovine callipyge locus: I. Relative chromosomal position and gene action. *Journal of Animal Science, 76* (8), 2062-2071. doi: 10.2527/1998.7682062x

Becila, S., Herrera-Mendez, C. H., Coulis, G., Labas, R., Astruc, T., Picard, B.,... Ouali, A. (2010). Postmortem muscle cells die through apoptosis. *European Food Research and Technology, 231* (3), 485-493. doi: 10.1007/s00217-010-1296-5

Bidwell, C. A., Waddell, J. N., Taxis, T. M., Yu, H., Tellam, R. L., Neary, M. K., & Cockett, N. E. (2014). New insights into polar overdominance in callipyge sheep. *Animal Genetics, 45* (s1), 51-61. doi: doi:10.1111/age.12132

Choe, J.-H., Stuart, A., & Kim, Y. H. B. (2016). Effect of different aging temperatures prior to freezing on meat quality attributes of frozen/thawed lamb loins. *Meat science, 116*, 158-164.

Choi, Y. M., & Kim, B. C. (2009). Muscle fiber characteristics, myofibrillar protein isoforms, and meat quality. *Livestock Science, 122* (2), 105-118. doi: https://doi.org/10.1016/j.livsci.2008.08.015

Cox, J., & Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized ppb-range mass accuracies and proteome-wide protein quantification. *Nature biotechnology, 26* (12), 1367-1372.

Cramer, T., Penick, M. L., Waddell, J. N., Bidwell, C. A., & Kim, Y. H. B. (2018). A new insight into meat toughness of callipyge lamb loins - The relevance of anti-apoptotic systems to decreased proteolysis. *Meat Science, 140*, 66-71. doi: https://doi.org/10.1016/j.meatsci.2018.03.002

Gagaoua, M., Terlouw, E. C., Boudjellal, A., & Picard, B. (2015). Coherent correlation networks among protein biomarkers of beef tenderness: What they reveal. *Journal of Proteomics, 128*, 365-374.

Geesink, G. H., & Koohmaraie, M. (1999). Postmortem proteolysis and calpain/calpastatin activity in callipyge and normal lamb biceps femoris during extended postmortem storage. *Journal of Animal Science, 77* (6), 1490-1501.

Guillemin, N., Jurie, C., Cassar-Malek, I., Hocquette, J.-F., Renand, G., & Picard, B. (2011). Variations in the abundance of 24 protein biomarkers of beef tenderness according to muscle and animal type. *Animal, 5* (6), 885-894.

Hishiya, A., Kitazawa, T., & Takayama, S. (2010). BAG3 and Hsc70 Interact With Actin Capping Protein CapZ to Maintain Myofibrillar Integrity Under Mechanical Stress. *Circulation Research, 107* (10), 1220-1231. doi: 10.1161/CIRCRESAHA.110.225649

Holzerová, E., & Prokisch, H. (2015). Mitochondria: Much ado about nothing? How dangerous is reactive oxygen species production? *The International Journal of Biochemistry & Cell Biology, 63*, 16-20. doi: https://doi.org/10.1016/j.biocel.2015.01.021

Jackson, S., Green, R., & Miller, M. (1997). Phenotypic characterization of Rambouillet sheep expressing the callipyge gene: I. Inheritance of the condition and production characteristics. *Journal of Animal Science, 75* (1), 14-18.

Jackson, S., Miller, M., & Green, R. (1997). Phenotypic characterization of Rambouillet sheep expressing the callipyge gene: II. Carcass characteristics and retail yield. *Journal of Animal Science, 75* (1), 125-132.

Jiang, X., & Wang, X. (2004). Cytochrome C-Mediated Apoptosis. *Annual Review of Biochemistry, 73* (1), 87-106. doi: 10.1146/annurev.biochem.73.011303.073706

Ke, Y., Mitacek, R. M., Abraham, A., Mafi, G. G., VanOverbeke, D. L., DeSilva, U., & Ramanathan, R. (2017). Effects of Muscle-Specific Oxidative Stress on Cytochrome c Release and Oxidation–Reduction Potential Properties. *Journal of agricultural and food chemistry, 65* (35), 7749-7755.

Keele, J. W., Leymaster, K. A., Freking, B. A., & Nielsen, M. K. (1998). Evaluation of the ovine callipyge locus: II. Genotypic effects on growth, slaughter, and carcass traits. *Journal of Animal Science, 76* (10), 2549-2559. doi: 10.2527/1998.76102549x

Kemp, C. M., King, D. A., Koohmaraie, M., Shackelford, S. D., & Wheeler, T. L. (2009). The caspase proteolytic system in callipyge and normal lambs in longissimus, semimembranosus, and infraspinatus muscles during postmortem storage1. *Journal of Animal Science, 87* (9), 2943-2951. doi: 10.2527/jas.2009-1790

Kim, N. K., Cho, S., Lee, S. H., Park, H. R., Lee, C. S., Cho, Y. M.,... Park, E. W. (2008). Proteins in longissimus muscle of Korean native cattle and their relationship to meat quality. *Meat Science, 80* (4), 1068-1073.

Kim, Y. H. B., Kemp, R., & Samuelsson, L. M. (2016). Effects of dry-aging on meat quality attributes and metabolite profiles of beef loins. *Meat Science, 111*, 168-176. doi: https://doi.org/10.1016/j.meatsci.2015.09.008

Koohmaraie, M., Doumit, M. E., Shackelford, S. D., Lonergan, S. M., & Wheeler, T. L. (1995). A muscle hypertrophy condition in lamb (callipyge): characterization of effects on muscle growth and meat quality traits1. *Journal of Animal Science, 73* (12), 3596-3607. doi: 10.2527/1995.73123596x

Lametsch, R., Roepstorff, P., & Bendixen, E. (2002). Identification of protein degradation during post-mortem storage of pig meat. *Journal of Agricultural and Food Chemistry, 50* (20), 5508-5512.

Li, J., Greenwood, P. L., Cockett, N. E., Hadfield, T. S., Vuocolo, T., Byrne, K.,... Schirra, H. J. (2014). Impacts of the Callipyge Mutation on Ovine Plasma Metabolites and Muscle Fibre Type. *PLOS ONE, 9* (6), e99726. doi: 10.1371/journal.pone.0099726

Lomiwes, D., Hurst, S., Dobbie, P., Frost, D., Hurst, R., Young, O., & Farouk, M. (2014). The protection of bovine skeletal myofibrils from proteolytic damage post mortem by small heat shock proteins. *Meat Science, 97* (4), 548-557.

Ma, D., & Kim, Y. (2019). Effects of Postmortem Aging on Small Heat Shock Protein Degradation of 3 Bovine Muscles. *Meat and Muscle Biology, 1* (3), 142-142.

Ma, D., Kim, Y. H. B., Cooper, B., Oh, J.-H., Chun, H., Choe, J.-H.,... Min, B. (2017). Metabolomics Profiling to Determine the Effect of Postmortem Aging on Color and Lipid Oxidative Stabilities of Different Bovine Muscles. *Journal of Agricultural and Food Chemistry, 65* (31), 6708-6716. doi: 10.1021/acs.jafc.7b02175

Maddock, K., Huff-Lonergan, E., Rowe, L., & Lonergan, S. M. (2005). Effect of pH and ionic strength on μ-and calpain 2 inhibition by calpastatin. *Journal of Animal Science, 83* (6), 1370-1376.

Morzel, M., Chambon, C., Hamelin, M., Santé-Lhoutellier, V., Sayd, T., & Monin, G. (2004). Proteome changes during pork meat ageing following use of two different pre-slaughter handling procedures. *Meat science, 67* (4), 689-696.

Morzel, M., Terlouw, C., Chambon, C., Micol, D., & Picard, B. (2008). Muscle proteome and meat eating qualities of Longissimus thoracis of "Blonde d'Aquitaine" young bulls: a central role of HSP27 isoforms. *Meat Science, 78* (3), 297-304.

Ouali, A., Gagaoua, M., Boudida, Y., Becila, S., Boudjellal, A., Herrera-Mendez, C. H., & Sentandreu, M. A. (2013). Biomarkers of meat tenderness: present knowledge and perspectives in regards to our current understanding of the mechanisms involved. *Meat science, 95* (4), 854-870.

Penick, M., Kim, H.-W., Setyabrata, D., Waddell, J. N., Bidwell, C. A., & Kim, Y. H. B. (2017). Callipyge genotypic effects on meat quality attributes and oxidation stability of ovine M. longissimus. *Small Ruminant Research, 146*, 5-12. doi: https://doi.org/10.1016/j.smallrumres.2016.11.011

Picard, B., & Gagaoua, M. (2017). Proteomic investigations of beef tenderness *Proteomics in Food Science* (pp. 177-197): Elsevier.

Pörn-Ares, M. I., Samali, A., & Orrenius, S. (1998). Cleavage of the calpain inhibitor, calpastatin, during apoptosis. [Original Paper]. *Cell Death And Differentiation, 5*, 1028. doi: 10.1038/sj.cdd.4400424

Pösö, A. R., & Puolanne, E. (2005). Carbohydrate metabolism in meat animals. *Meat Science, 70* (3), 423-434. doi: https://doi.org/10.1016/j.meatsci.2004.12.017

Ramanathan, R., & Mancini, R. A. (2018). Role of mitochondria in beef color: A review. *Meat and Muscle Biology, 2* (1), 309-320.

Skulachev, V. P. (1998). Cytochrome c in the apoptotic and antioxidant cascades. *FEBS Letters, 423* (3), 275-280. doi: 10.1016/S0014-5793 (98)00061-1

Subbaraj, A. K., Kim, Y. H. B., Fraser, K., & Farouk, M. M. (2016). A hydrophilic interaction liquid chromatography–mass spectrometry (HILIC–MS) based metabolomics study on colour stability of ovine meat. *Meat Science, 117*, 163-172. doi: https://doi.org/10.1016/j.meatsci.2016.02.028

Vavougios, G. D., Zarogiannis, S. G., Krogfelt, K. A., Gourgoulianis, K., Mitsikostas, D. D., & Hadjigeorgiou, G. (2018). Novel candidate genes of the PARK7 interactome as mediators of apoptosis and acetylation in multiple sclerosis: An in silico analysis. *Multiple Sclerosis and Related Disorders, 19*, 8-14. doi: https://doi.org/10.1016/j.msard.2017.10.013

Wang, K. K. W., Posmantur, R., Nadimpalli, R., Nath, R., Mohan, P., Nixon, R. A.,... Allen, H. (1998). Caspase-Mediated Fragmentation of Calpain Inhibitor Protein Calpastatin during Apoptosis. *Archives of Biochemistry and Biophysics, 356* (2), 187-196. doi: https://doi.org/10.1006/abbi.1998.0748

Yu, H., Waddell, J. N., Kuang, S., Tellam, R. L., Cockett, N. E., & Bidwell, C. A. (2018). Identification of genes directly responding to DLK1 signaling in Callipyge sheep. *BMC Genomics, 19* (1), 283. doi: 10.1186/s12864-018-4682-1

Zhang, M., Zheng, J., Nussinov, R., & Ma, B. (2017). Release of Cytochrome C from Bax Pores at the Mitochondrial Membrane. *Scientific Reports, 7* (1), 2635. doi: 10.1038/s41598-017-02825-7

Table 3.1. Differentially abundant proteins across four genotypes with an emphasize on distinct callipyge characteristics.

Accession	Gene names	Protein names[1]	Highest in[2]	Lowest in	High to low FC	VIP1	VIP2
Metabolism enzymes							
W5PCV0	ADSSL1	Adenylosuccinate synthetase	+/C	+/+	2.97	1.105	0.846
W5PLV2	ALDH2	Aldehyde dehydrogenase 2	C/C	+/C	2.18	1.004	1.563
W5PC00	AKR1B1	Aldo-keto reductase	+/C	C/+	3.31	1.996	1.520
W5PTU7	CA2	Carbonic anhydrase	C/C	+/C	2.07	0.877	1.512
W5PIG7	ENO1	Enolase	+/C	+/+	2.45	1.282	0.976
W5PFT7	FBP2	Fructose-bisphosphatase 2	+/C	+/+	2.13	1.335	1.023
Q3ZBY4	ALDOC	Fructose-bisphosphate aldolase	+/C	+/+	2.29	1.484	1.109
W5P1X9	ALDOA	Fructose-bisphosphate aldolase	+/C	+/+	1.65	1.067	0.953
W5P323	GPI	Glucose-6-phosphate isomerase	+/C	+/+	2.42	1.727	1.306
W5P5C5	GYG1	Glycogenin 1	+/C	C/+	2.46	1.115	0.876
W5PIN6	LDHA	L-lactate dehydrogenase	+/C	+/+	2.65	1.782	1.358
B3IVN4	PKM	M1-type Pyruvate kinase	+/C	+/+	3.25	2.075	1.583
W5PW05	MDH2	Malate dehydrogenase 2	C/C	+/C	1.90	0.584	1.143
Q08DP0	PGM1	Phosphoglucomutase-1	+/C	C/+	2.72	1.918	1.434
W5PVY5	PGAM2	Phosphoglycerate mutase 2	+/C	+/+	2.81	1.805	1.367
A5D984	PKM2	Pyruvate kinase	+/C	+/+	2.10	1.233	0.948
B2ZA84	GLRX/ LOC10110	Thioltransferase	+/C	+/+	5.78	2.158	1.666
W5QDD0	8092	Glutathione S-transferase 1	+/C	C/+	2.19	1.654	1.244
Stress response and apoptosis							
W5NYH4	BAG3	BCL2 associated athanogene 3	+/C	C/+	3.04	1.572	1.180
P62896	CYCS	Cytochrome c	C/C	+/C	1.69	1.034	0.962
Q3T149	HSPB1	Hsp 27	C/+	+/C	3.49	2.217	1.682
W5PG95	HSPA1A	Hsp70	+/C	C/+	1.42	1.078	1.247
W5PK66	PARK7	Protein DJ	+/C	+/+	3.93	2.693	2.014

Table 3.1 continued

Accession	Gene names	Protein names[1]	Highest in[2]	Lowest in	High to low FC	VIP1	VIP2
Cell cycle and cell fate regulation							
P62262	YWHAE	14-3-3 protein epsilon	*+/C*	*C/+*	4.01	2.833	2.152
P68252	YWHAG	14-3-3 protein gamma	*+/C*	*C/+*	2.36	1.640	1.241
Q3SYU2	EEF2	Elongation factor 2	*+/C*	*C/+*	2.73	1.937	1.454
W5PWZ2	GAL-1	Galectin	*+/C*	*+/+*	2.13	1.089	0.814
W5QEL8	NDRG2	N-myc downstream regulated gene 2	*+/C*	*C/+*	4.19	2.072	1.553
Q3SX40	PDLIM7	PDZ and LIM domain protein 7	*C/+*	*+/C*	2.53	1.186	0.887
Q3ZBT1	VCP	Valosin-containing protein	*+/C*	*+/+*	2.96	1.345	1.035
P29701	AHSG	Alpha-2-HS-glycoprotein	*C/C*	*+/+*	3.89	1.070	1.329
Muslce structure and function							
W5PDD8	MYOZ1	Myozenin 1	*+/+*	*+/C*	3.73	1.822	1.373
W5PFV9	NEB	Nebulin	*+/+*	*+/C*	2.81	1.555	1.162
W5Q754	TTN	Titin	*C/+*	*+/C*	3.15	1.758	1.313
Binding and transporting							
Q1KYZ6	HBBB	Beta-B globin chain	*C/C*	*+/C*	1.69	0.462	1.057
W5PF65	TF	Transferrin	*C/C*	*+/C*	2.24	0.535	1.330
P12303	TTR	Transthyretin (prealbumin)	*C/C*	*+/C*	4.28	1.435	1.834
W5NZ10	NUTF2	Nuclear transport factor 2	*+/C*	*C/+*	2.22	1.663	1.262

[1]Peptides were identified comparing against Uniprot *Bos Taurus* and *Ovis aries* database (www.uniprot.org).

[2]Maternal/Paternal (C: mutant allele, +: wildtype allele); +/C (callipyge hypertrophied phenotype); C/C, C/+ and +/+ (non-hypertrophied phenotype).

Table 3.2 Metabolites that were significantly different across four callipyge genotypes.

Mass_ Input	Mass_ Database	Putative metabolites[1]	Formula	ΔPPM	P- Value	Highest in[2]	Lowest in
Amino acids and dipeptides							
214.1294	214.1317	Prolyl-Valine	C10H18N2O3	10	0.034	*C*/+	+/*C*
216.1471	216.1474	Valyl-Valine	C10H20N2O3	1	0.006	+/+	+/*C*
228.1472	228.1474	Leucyl-Proline	C11H20N2O3	0	0.035	+/*C*	+/+
240.1155	240.1222	L-Anserine	C10H16N4O3	8	0.038	+/*C*	*C*/+
296.1198	296.1195	Methionyl-Phenylalanine	C14H20N2O3S	1	0.035	+/*C*	*C*/*C*
188.1244	188.1273	homoarginine	C7H16N4O2	10	0.027	+/*C*	*C*/+
252.1082	252.111	Tyr-Ala	C12H16N2O4	10	0.016	+/*C*	+/+
Dietary derived phenolics and other secondary metabolites							
168.0788	168.0786	2,6-Dimethoxy-4-methylphenol	C9H12O3	0	0.011	+/+	*C*/*C*
208.1448	208.1463	4-Heptyloxyphenol	C13H20O2	7	0.018	+/+	*C*/+
234.1601	234.162	Macrophyllic acid A	C15H22O2	8	0.005	+/+	*C*/+
248.1427	248.1412	Abscisic aldehyde	C15H20O3	5	0.040	+/+	+/*C*
311.2477	311.246	N-tetradecanoyl-L-homoserine lactone	C18H33NO3	5	0.037	+/+	*C*/+
542.1371	542.1424	Resveratrol 4'- (2-galloylglucoside)	C27H26O12	9	0.000	+/*C*	+/+

Table 3.2 continued

Mass_ Input	Mass_ Database	Putative metabolites[1]	Formula	ΔPPM	P-Value	Highest in[2]	Lowest in
Lipid and lipid oxidation product							
140.0835	140.0837	Octadienoic acid	C8H12O2	1	0.041	*+/C*	*+/+*
279.2558	279.2562	Linoleamide	C18H33NO	1	0.017	*+/+*	*C/+*
493.3171	493.3168	PC (16:1 (9E)/0:0)	C24H48NO7P	0	0.031	*C/C*	*+/C*
771.6069	771.6142	PC (P-18:0/18:1 (9Z))	C44H86NO7P	9	0.045	*+/+*	*C/+*
Major energy metabolism related compounds							
315.2756	315.2773	Dehydrophytosphingosine	C18H37NO3	5	0.004	*+/+*	*C/+*
348.0435	348.0471	Inosine 5'-monophosphate	C10H13N4O8P	10	0.009	*+/C*	*C/+*
687.1487	687.1489	Dephospho-CoA	C21H35N7O13P2S	0	0.038	*+/+*	*+/C*
245.1638	245.1627	Pivaloylcarnitine	C12H23NO4	4	0.018	*+/+*	*+/C*
Oligopeptides							
357.1683	357.1648	Pro Gln Asn	C14H23N5O6	9	0.002	*+/C*	*+/+*
400.2083	400.207	Glu Arg Pro	C16H28N6O6	3	0.030	*+/C*	*C/+*
406.2255	406.2216	Tyr Pro Lys	C20H30N4O5	9	0.039	*C/+*	*+/C*
527.3574	527.3544	Lys Lys Pro Arg	C23H45N9O5	5	0.002	*+/+*	*+/C*

[1]Compounds were tentatively identified using METLIN (metlin.scripps.edu) and HMDB (www.hmdb.ca) metabolite database with a mass error ≤ 10 ppm

[2]Maternal/Paternal (*C*: mutant allele, +: wildtype allele); *+/C* (callipyge hypertrophied phenotype); *C/C*, *C/+* and +/+ (non-hypertrophied phenotype).
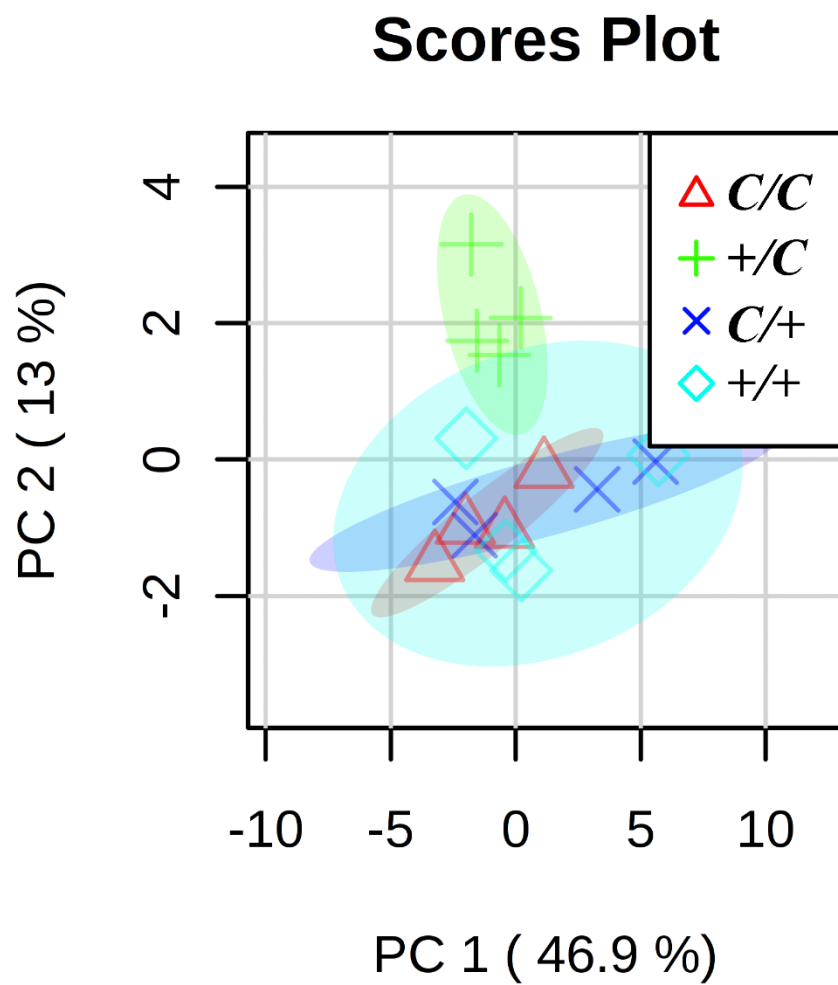
Figure 3.1 The PCA score plot of 178 mutual proteins from different four callipyge genotypes (Maternal/Paternal; *C*: mutant allele, +: wild type allele; *+/C*: callipyge hypertrophied phenotype; *C/C*, *C/+* and *+/+*: non-hypertrophied phenotype).
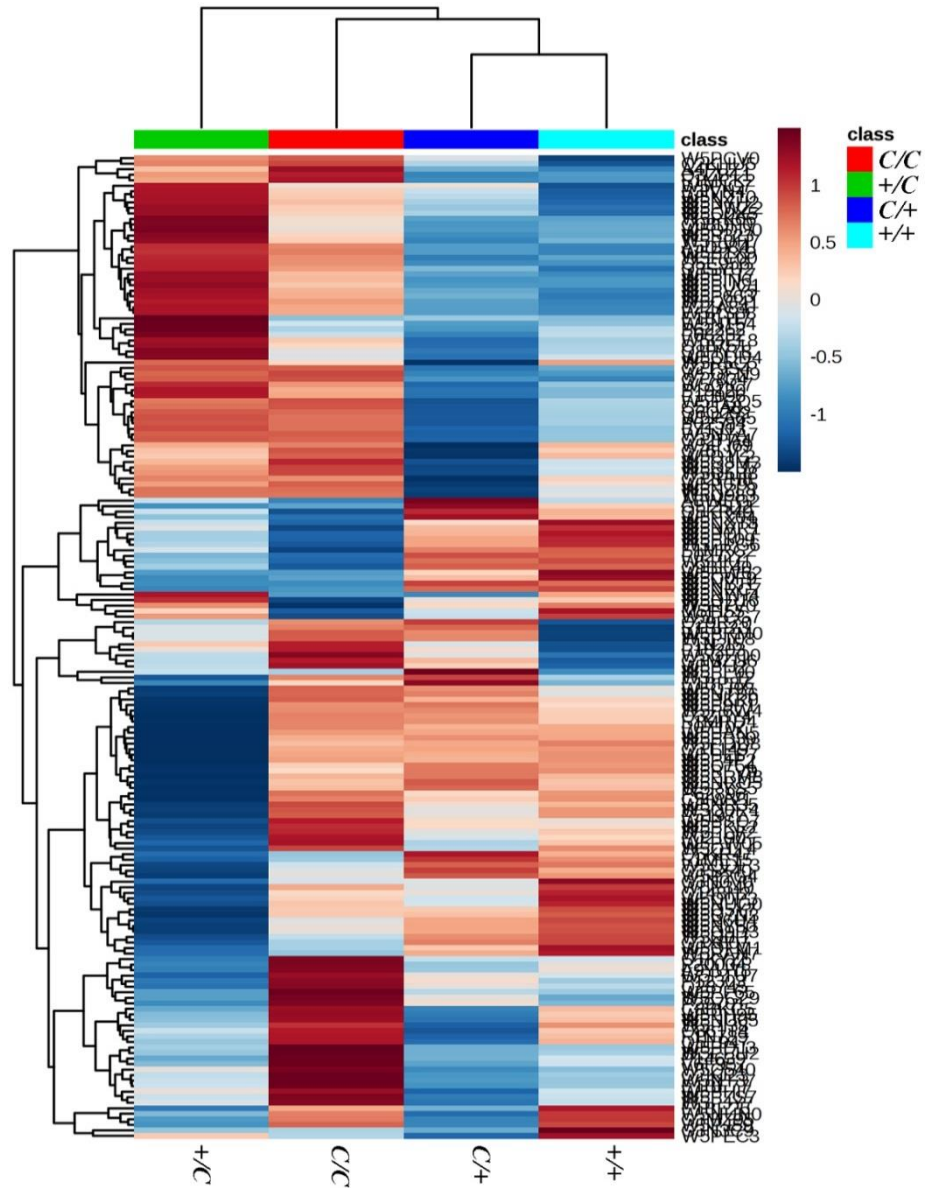
Figure 3.2 Hierarchical clustering analysis (HCA) of 178 mutual proteins from lambs with four callipyge genotypes. The heatmap presents mean relative abundance of the proteins (logarithmic scale in base 2) with different colors, where deeper red represents higher intensity and deeper blue represents lower intensity. (Maternal/Paternal; C: mutant allele, +: wild type allele; +/C: callipyge hypertrophied phenotype; C/C, C/+ and +/+: non-hypertrophied phenotype).
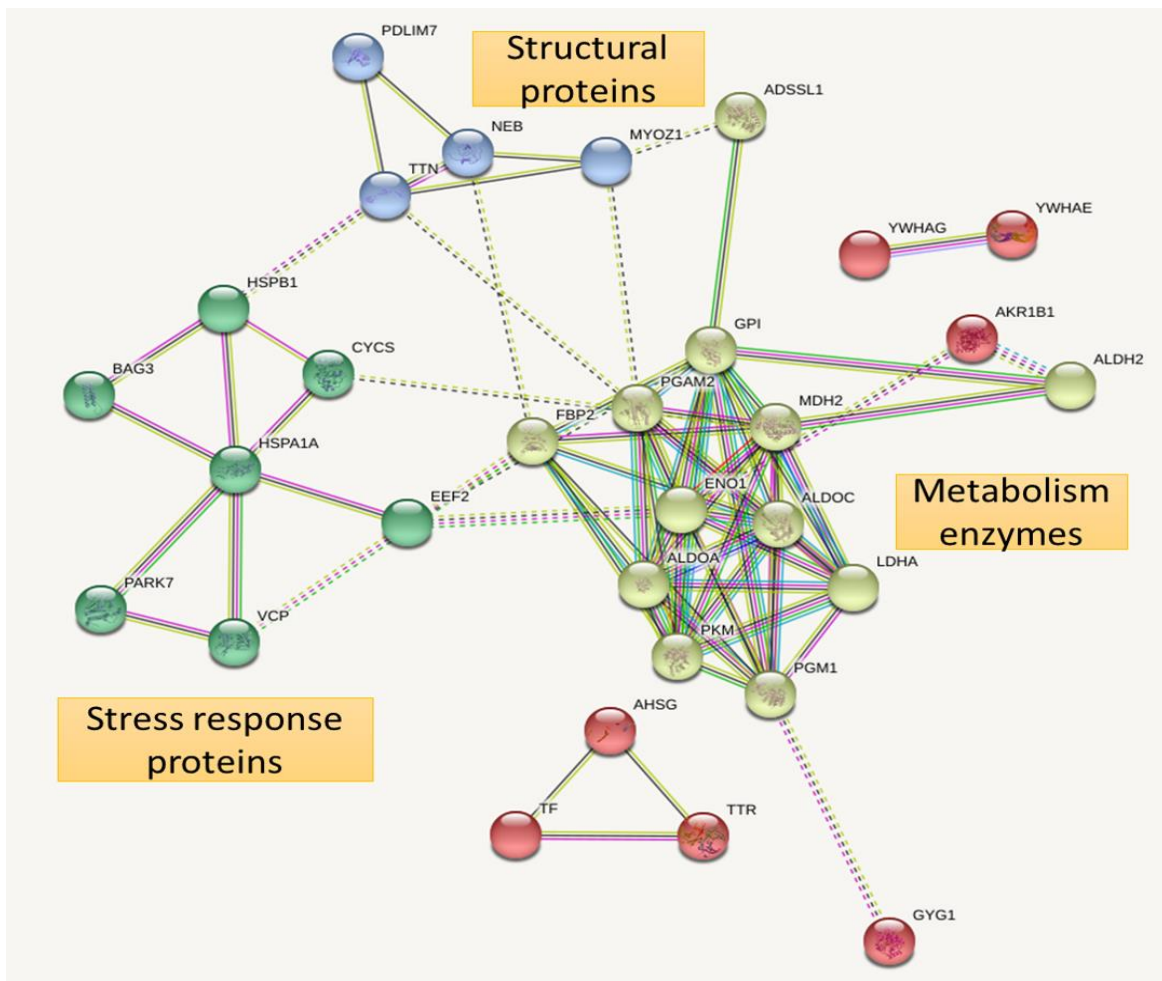
Figure 3.3 Protein interaction network for the callipyge lamb muscle proteome using STRING analysis. Thirty-eight differentially presented proteins were analyzed to form three main clusters with miscellaneous nodes. The network nodes represent proteins, and the lines represent protein-protein interactions.
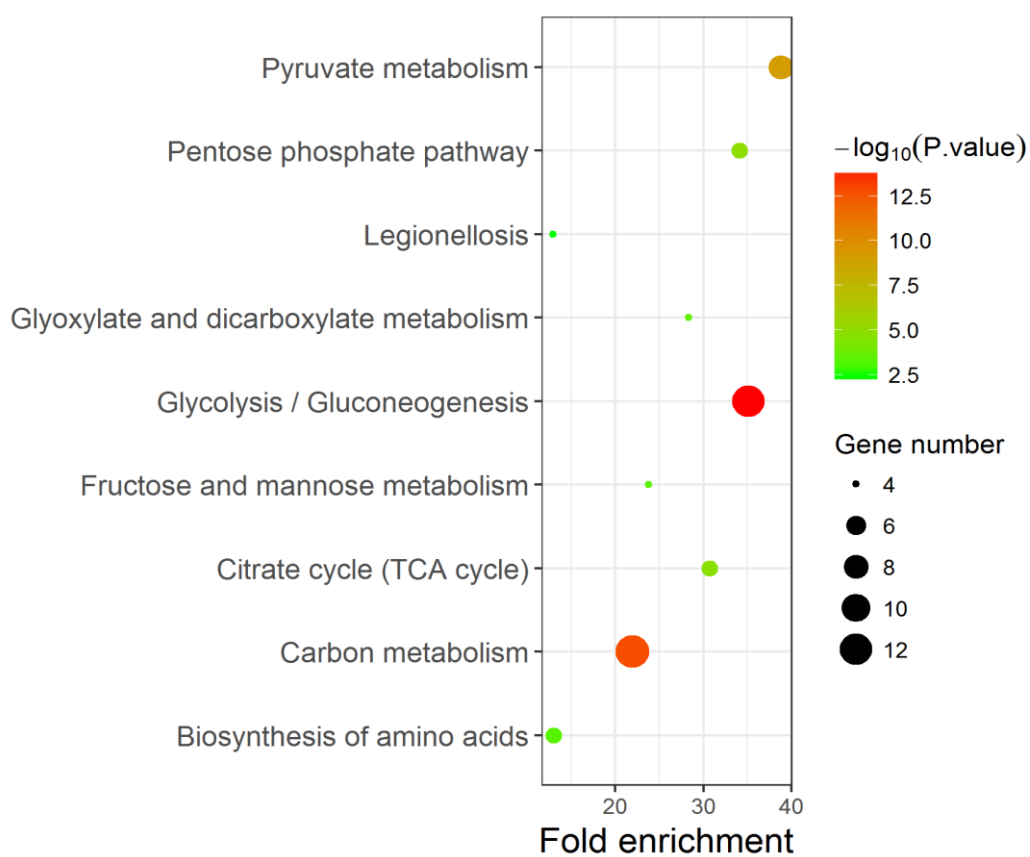
Figure 3.4 KEGG pathway enrichment of the thirty-eight differentially presented proteins among callipyge genotypes

CHAPTER 4. 0.20% L-GLUTAMINE IN REPLACEMENT OF DIETARY ANTIBIOTICS DURING POST WEANING AND TRANSPORT RECOVERY: IMPACTS ON CARCASS AND MEAT QUALITY ATTRIBUTES

4.1 Abstract

Dietary L-glutamine could be potentially used as an antibiotics alternative to alleviate post transport and wean stress. The study objective was to evaluate the impacts of 0.2% dietary L-glutamine supplementation during post weaning and transport recovery on carcass and meat quality characteristics of pigs. A total of 480 pigs were weaned and transported in two production seasons, SUMMER (weaned in July, 2016 and harvested in January, 2017) vs. SPRING (weaned in April, 2017 and harvested in September 2017) , and fed 3 different diets (Non: no antibiotic, Anti: 441 ppm chlortetracycline and 38.6 ppm tiamulin, Gln: 0.20% L-glutamine with no antibiotics) for 14 days after transport. The animals then fed a common basal diet until reaching market weight. Pairs of *longissimus dorsi* (LD) and *psoas major* (PM) muscles were harvested from 1-day and 7-day chilled carcass sides (n=10 pigs/dietary treatment/season trial). Overall, no negative impacts of Gln were found in carcasses characteristics including LEA, BF, muscling score or visceral organ weights. Dietary Gln did not affect chemical and physical attributes of porcine muscles including pH, protein and lipid content, SF, and WHC (P > 0.05). Gln showed marginally decreased CIE L* and hue, suggesting enhanced color stability. SPRING trial showed faster pH decline, paler surface color, higher intra-muscular fat deposition, improved tenderness and water-holding capacity as indicated by lower shear force values, thaw-purge loss, and cooking loss (P < 0.05). The result suggested that Gln supplementation as a nutraceutical mitigation strategy could have equivalent impacts on fat and lean accretion, SF, and WHC and slight improvements in meat color attributes of fresh pork products compared to those obtained from antibiotics treated pigs.

Key words: Early-life stress; Antibiotics replacement; Oxidation stability

4.2 Introduction

Weaning is a susceptible and challenging life stage characterized by abrupt environmental and physiological alternations of piglets (Campbell, Crenshaw, & Polo, 2013; Lewis & Berry, 2006). Because multi-site production system is widely applied due to the benefit of reducing pathogen transfection and improving overall farming efficiency (Harris, 2008), weaning is commonly concurrent with transporting, the most stressful and injurious management event in livestock operation chain (Lewis & Berry, 2006). It is well documented that poor transporting conditions of pigs are related to excessive muscle contraction, causing acidosis and oxidation damage of muscle tissues. Rough handling during transport and lairage also results in carcass and meat quality defects, such as skin lesions, bruises, tenderness issue, PSE (pale, soft, exudative) meat and compromised protein functionality (Faucitano & Goumon, 2018). Unfortunately, such in-transient loss can be magnified as the environmental ambient temperature increases. Higher temperature (35 °C compared to 20 °C) accompanied with longer transport duration results in nearly 50% more weight loss in weaning piglets (Berry & Lewis, 2001). Sutherland, McDonald, and McGlone (2009) found that the rate of dead-on-arrival (DOA) pigs increased with ambient temperatures that above 20 °C, with the highest DOA percentage being recorded at temperatures 25 °C and above. Besides, seasonality is well acknowledged as one of the primary contributors to the variations of pork quality characteristics, which is partially attributed to seasonal change of environmental temperatures during transport and lairage (Arkfeld et al., 2017). Even heat stress as short as 2 hours imposes measurable effects on muscle proteome profile, impairs meat quality and protein functionality (Cruzen, Baumgard, Gabler, Pearce, & Lonergan, 2017).

Sutherland, Backus, and McGlone (2014) argued that weaning pigs are likely to experience similar stressors as older pigs during transport, on top of weaning-associated stressors. Therefore, dietary antibiotics are utilized in newly weaned and transported pigs to help coping with stress recovery (Lewis & Berry, 2006).Although non-therapeutic use of dietary antibiotics was a common practice to promote production efficiency, public concerns and legislative drives are calling for nutraceutical alternatives to reduce antibiotic use (Landers, Cohen, Wittum, & Larson, 2012). One possibility is to provide substances that can support structural integrity and function of gastro-intestinal (GI) tracks - the core site of nutrient absorption and the primary barrier against environmental microbes (Hanczakowska, Niwinska, Grela, Weglarzy, & Okon, 2014). L-

glutamine has been recognized as an efficient energy source of intestinal epithelium cells (Wu, Knabe, Yan, & Flynn, 1995). In neonatal pigs, L-glutamine (among glutamate and aspartate) is preferentially utilized as energy source by enterocytes over glucose and free fatty acids (Burrin & Stoll, 2002). Dietary supplementation of L-glutamine in nursery pigs improved intestinal epithelium integrity (Ewaschuk, Murdoch, Johnson, Madsen, & Field, 2011; Lescano et al., 2013), health status (Jiang et al., 2009), and growth performance (Zou, Zheng, Fang, & Jiang, 2006). It also servs as an immune-modulator (Newsholme, 2001) and improved tissue antioxidant capacity (Lv et al., 2018; Zhang, Wang, Gao, Jiang, & Zhang, 2002). Particularly, recent studies have been determined that dietary L-glutamine at cost-effective level of 0.2% in newly weaned and transported pigs have improved or equivalent effect on animal growth, well-being, and productivity compared to dietary antibiotics (Duttlinger, Kpodo, Lay Jr, Richert, & Johnson, 2019; Johnson & Lay Jr, 2017).

While previous studies have been mostly focused on physiological and nutritional consequences of utilizing dietary L-glutamine as growth promoter or stress mitigation strategy, little to no information is available on how early-life stress events and corresponding intervention/mitigation practice could affect final carcass and meat quality characteristics. It is possible that early-life nutrition may leave long term effects on muscle protein turn-over, energy metabolism, or redox balance systems, potentially compromising the final meat product quality attributes (Andersen, Oksbjerg, Young, & Therkildsen, 2005). Hence, utilizing L-glutamine as antibiotics alternative should be further evaluated from a meat production perspective. Therefore, the study objective was to determine the impacts of 0.2% dietary L-glutamine supplementation during post weaning and transport recovery on carcass and meat quality characteristics of pigs

4.3   Materials and Methods

4.3.1   Live animal processing and diet

All live animal procedures and dietary treatments have been described previously (Duttlinger et al., 2019). Animal husbandry and the following procedures were approved by the Purdue University Animal Care and Use Committee (protocol #1603001385). Briefly, a total of

480 pigs [5.62 ± 0.06 kg initial BW, Duroc × (Landrace × Yorkshire)] were weaned at 18.4 ± 0.2 d of age and transported in a truck trailer for 12 h in central Indiana. The experiment was conducted in two different seasonal cycles as trials, namely weaned in July, 2016 and harvested in January, 2017 (SUMMER trial, N=240 pigs), and weaned in April, 2017 and harvested in September, 2017 (SPRING trial, N=240 pigs), respectively. On the day of weaning and transport, pigs were removed from sows and loaded onto a gooseneck livestock trailer at a density of 0.07 m^2/pig. During the 12h and 819 km transport, the ambient temperature (TA) and relative humidity (RH) of the trailer were recorded, where on average SUMMER showed TA 29.4 ± 0.2 °C and RH 64.3 ± 0.8%, and SPRING showed TA 11.0 ± 0.2°C and RH 63.1 ± 0.9%, respectively. In each trial (SUMMER vs. SPRING), all the pigs were transported as one group and feed and water were withheld. Pigs were individually weighed prior to the weaning and transport procedure, BW blocked, and randomly allocated to 60 pens, each pen contained 8 pigs. In each trial, 30 pens were assigned randomly to 1 of 3 dietary treatments for 14 days after weaning and transport: regular diets with no antibiotics (Non), dietary antibiotics supplemented (Anti: Non with 441 ppm chlortetracycline and 38.6 ppm tiamulin), or dietary L-glutamine supplemented (Gln: Non with 0.2% dietary L-glutamine and no antibiotics). Following the dietary treatments, pigs were fed with regular antibiotic-free diets until being harvested at 159d post transport. All the diets were formulated to meet or exceed nutrient requirement (NRC, 2012) during each of the growth phase. In brief, 4 diets were formulated accordingly in 34 days of nursery phase, and 6 were in 125 days of grow-finish phase (Duttlinger et al., 2019).

4.3.2   Carcass characteristics evaluation and sample collection

At the end of the grow-finish phase, one pig were randomly taken from each pen (BW 120.28 ± 1.23kg, a total of 10 pigs/treatment/trial) and were transported to Purdue University Meat Laboratory for slaughter. Carcass temperature and pH decline was measured in *m. longissimus* muscles. At 24 hours postmortem, the left sides of the carcasses were ribbed between the 10th and 11th thoracic vertebrae, where loin eye area (LEA), backfat depth (BF) and loin depth were measured. Subjective carcass characteristics including color, intramuscular fat content (marbling), and muscling score were evaluated by an experienced evaluator using National Pork Producers

Council (NPPC) standards (color: 1 very pale to 6 very dark; marbling score: 1 devoid to 10 excessive; muscle score: 1 thin 1.5 to 2.5 average 3 thick). Head, heart, liver, and spleen were weighed during slaughter and expressed as percent of HWC. The right sides of the carcasses were stored in a cooler for 7 days at 2.5 °C. Each sides of the carcasses were fabricated at the completion of 1d or 7d carcass aging periods, respectively. Upon completion of each aging time, *longissimus dorsi* (LD) and *psoas major* (PM) were separated from each carcass side. Multiple steak cuts were made from each muscle to measure physical characteristics of pork qualities including water-holding capacity (WHC), instrumental tenderness, proximate composition, display color, and lipid oxidation.

4.3.3　pH and water-holding capacity

The carcass pH decline was measured in duplicate by inserting the probe of an insertion-type hand-held pH meter (HI 99,163, Hanna Instruments Inc., Woonsocket) between the 7th to 11th ribs at 45min, 2hr, 6hr, and 12hr postmortem. The pH of muscle sections (LD and PM) was measured at 1d and 7d postmortem, respectively.

Water-holding capacity (WHC) was assessed by measuring drip loss, display loss, freeze-thaw purge loss, and cooking loss of the pork samples. Drip loss was measured using modified Honikel drip loss method (Honikel, 1998): cubic shaped samples (about $3 \times 3 \times 3$ cm^3, 40g) free from fat and connective were suspended using nylon net inside an airtight container for 48 h. Drip loss were expressed as the percent differences between weights measured prior to and after suspension. Display weight loss was calculated from the percentage difference between initial weight at 1 d display and final weight at 5 d display (Kim et al., 2016). LD and PM steaks were frozen under 80°C for 5 days and thawed under 2°C overnight. Thaw-purge loss was determined by measuring percent differences between initial and final weights. The thawed samples were then used to determine cooking loss and Warner-Bratzler shear force (WBSF). Cooking loss was calculated as the percentage difference between the sample weights prior to and after cooking using 2.45-cm-thick chops. The samples were cooked on an electric griddle (Farberware, Walter Idde and Co., Bronx, NY) at a constant surface temperature of 135°C until the internal temperature reached to targeted 41°C, then were flipped to the other side and cooked until reaching the targeted

internal temperature of 71°C. The cooking temperature was monitored by using a digital temperature logger (OctTemp2000, MadgeTech, Inc., Warner, NH) equipped with thermocouple (T-type, Omega Engineering, Stamford, CT).

4.3.4   Warner-Bratzler shear force

WBSF was measured using the cooked chops after being chilled at 4°C for overnight. From each chop, six cores were obtained parallel to the muscle fiber orientation using a hand-held coring device (1.27 cm diameter), then were sheared through using a Warner-Bratzler shear attachment with TA-XT Plus texture analyzer (Stable Micro System Ltd., UK). Peak shear force (kg) of six replicates were averaged to represent the maximum force that was required to shear through the sample.

4.3.5   Proximate analysis

Proximate composition including moisture, protein, fat, and ash were evaluated in 1d and 7d aged samples. Moisture content was measured in triplicates, which was obtained via drying the samples in a force-aired oven (Precision Scientific Co., Chicago, IL) at 95 °C for 24 h (method 934.01; AOAC, 2006). Fat content was measured by the Soxhlet diethyl ether extraction method without prior acid hydrolysis using dried samples (method 934.01; AOAC, 2000). Protein content was determined in dry matter samples by the combustion method (model FP2000; Leco Corp., St. Joseph, MI) using EDTA as an internal standard; percent protein was calculated by multiplying total percent nitrogen by coefficient of 6.25 (AOAC, 2006; method 992.15). The mineral content was measured by ashing the samples in a muffle furnace at 550°C for 8 h (Method 934.01; AOAC, 2006).

4.3.6   Instrumental color measurement

At the completion of 1d and 7d postmortem carcass chilling, instrumental color CIE L*, CIE a* and CIE b* were evaluated in a 7-day retail display. Chops (2.45cm thickness) from LD and PM were packaged in Styrofoam trays with oxygen-permeable polyvinyl chloride film (23,000

cm^3 O$_2$ m^{-2} 24 h^{-1} at 23 °C) and displayed under fluorescent white light (1450 lx; color temperature = 3500 K) at 2°C. Samples were relocated daily on display table to minimize any confounding effect that associated with different display locations. Surface color of LD and PM samples was determined on a daily basis using a Hunter MiniScan EZ colorimeter (Hunter, Reston, VA) in triplicates (3 random locations per muscle). The colorimeter was calibrated using black (CIE L*=97.06, CIE a*=0.41, CIE b*=1.72) and white (CIE L*=97.06, CIE a*=0.41, CIE b*=1.72) calibration tiles according to the manufactory manual. The illuminant setting was A10 source and 25mm diameter aperture 10° standard observer. Chroma and hue angle values were calculated using the following expression (AMSA, 2012): chroma = $(a^2+b^2)^{1/2}$; hue angle = $\tan^{-1}$ (b/a).

4.3.7   2-Thiobarbituric acid reactive substances (TBARS)

Lipid oxidation was determined in triplicate by 2-thiobarbituric acid reactive substance (TBARS) assay as described by Buege and Aust (1978). TBARS was determined in LD and PM samples prior to and after the retail display treatment. Sample (5g) were homogenized with 3 volumes of deionized-distilled water (DDW) and 50 µL of 10% butylated hydroxyl anisole (BHA) - ethanol solution. Homogenate was mixed with 2 volume of 20 mM 2-thiobarbituric acid (TBA) solution in 15% trichloroacetic acid (TBA/TCA solution), heated in 80 °C water bath for 15 min and cooled on ice for 10 min. The cooled mixture was centrifuged at 2000×g for 10 min and filtered (Whatman No. 4 paper). Absorbance of supernatant was measured at 538 nm using the microplate spectrophotometer (Epoch, Biotek Instruments Inc., USA), which was compared to a blank prepared with 2 mL of DDW and 4 mL of the TBA/TCA solution. TBARS value was expressed as mg MDA/kg meat, which was calculated by multiplying the absorbance at 538 nm by a factor of 5.54.

4.3.8   Statistical analysis

The experimental design of this study was randomized complete block design with split-plot factors: diet treatment was the main treatment factor, seasonal trials was served as a block factor, muscle type effect (LD vs. PM) was whole plot and aging time effect (1d vs. 7d of aging) as subplot.

Display time (1 vs. 7 days for TBARS and 1 through 7 days for color) was considered as sub-sub plot. Animals were considered as a random effect and was used as experimental unit. The statistical model included main factors and all the possible interactions. Data were analyzed using the PROC MIXED of SAS 9.4. Least square means were separated (F test, $P < 0.05$) by using least significant differences via PDIFF option.

4.4   Results

4.4.1   Initial pH decline and muscle ultimate pH

No dietary treatment effect or related interactions were found in pH or temperature decline processes (P > 0.1, Figure 4.1). A seasonal trial by postmortem time interaction was found in both pH and temperature decline (P < 0.05, Figure 4.1): Compared to SPRING, SUMMER pigs showed lower pH values at 45min and 2h but higher value at 6h and 12h postmortem (P < 0.05, Figure 4.1A), indicating a slower decline rate during pre-rigor time window. The most apparent difference was observed at 12h, where SUMMER pH was shown as 5.91 compared to 5.76 in SPRING (P < 0.05, Figure 4.1A). The two seasonal trials shared similar value of initial temperature at 45 min postmortem. As postmortem time prolonged, SUMMER exhibited a higher rate of temperature decrease (P < 0.05, Figure 4.1B).

Dietary treatment did not affect final muscle pH in LD and PM at 1d and 7d postmortem aging (P > 0.1). A 3-way interaction of seasonal trial, muscle, and postmortem aging was found (P < 0.001, Figure 4.2). In July-Jan, PM at 1d postmortem showed the highest pH value among all the treatment groups, whereas a drastic decrease of pH from 5.92 to 5.7 was found at 7d.

4.4.2   Carcass characteristics and organ weights

Pork carcasses from the Anti treated group showed a tendency to have a lower backfat depth (BF) at 10th rib compared to pork carcasses from the Non group (P = 0.06, Table 4.1), but no difference was observed in BF at last rib (P > 0.1, Table 4.1). Marbling score tend to be lower in Anti as well (P = 0.08, Table 4.1). SPRING pigs showed increases in LEA and marbling (P < 0.05, Table 4.1) and decreased muscling score compared to SUMMER (P < 0.05, Table 4.1).

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SPRING pigs also exhibited paler color compared to SUMMER according to NPPC visual color standard (P < 0.05, Table 4.1).

Dietary treatment did not affect organ weights, regardless of seasonal trials (P > 0.1, Table 4.1). Except for spleen, which showed higher weights in SUMMER pigs compared to SPRING counterparts (P < 0.001, Table 4.1), no difference due to seasonal trials was observed in other organ characteristics.

4.4.3 Proximate composition

No dietary treatment effects were observed in any of the proximate composition (P > 0.1, Table 4.2), including moisture content, dry-matter based crude protein, crude fat, and ash. A trial by aging interaction in moisture indicates that LD and PM muscles in SPRING lose more moisture content during 7d postmortem aging compared to SUMMER (P < 0.0001, Table 4.2). On a dry-matter basis, crude protein, ether extractable lipid and ash content were primarily affected by seasonal trials (P < 0.01, Table 4.2). Specifically, SPRING pigs exhibited increased ether extractable lipid and decreased protein and ash content (Table 4.2).

4.4.4 WBSF and WHC

Dietary treatment and related interactions had no impact on WBSF. Overall, regardless of muscle type or aging treatment, SPRING had lower WBSF values than SUMMER (P<0.01, Figure 4.3). A 3-way interaction between trial, muscle type and aging period was found ($P = 0.043$, Figure 4.3), where decreased WBSF over 7d aging was only found in LD, but not PM. For WHC, dietary treatment and related interactions did not affect any of the parameters including drip loss, thaw-purge loss, display loss, and cooking loss (P > 0.05). A muscle effect was shown that PM to lower in drip, thaw-purge, display, and cooking loss compared to LD counterpart, regardless of aging or trial effects (P<0.05, Table 4.3). An interaction (P < 0.01) between aging period and seasonal trial affected drip, display, and thaw-purge loss. Although overall numerical changes were small, drip loss and display loss were increased by 1/5 and 1/6 in SPRING, respectively (drip loss: 3.31%vs.

4.10%, display loss: 5.32% vs. 6.36%, P < 0.05). Thaw-purge loss and cooking loss were increased in SUMMER (P < 0.01; Table 4.3).

4.4.5   Color and oxidative stability

Main treatment effects including diet, muscle, season and aging and multiple interactions were found to affect CIE L* (lightness), a* (redness), b* (yellowness), hue angle (discoloration), and chroma (color saturation). Overall, although statistically significant, the difference of each parameters between diet and/or aging treatment were numerically small (mostly below 1-unit difference) and therefore should not be considered practically meaningful (Figure 4.4). In general, Gln supplementation showed marginal improvements in color characteristics CIE L*, chroma and hue angle. Regardless of seasonal trial, Gln showed lower hue angle compared to Anti counterparts (P < 0.0001, Figure 4.4). Interaction effects between dietary treatment and seasonal trial were found in CIE L* and chroma: In SPRING, both Gln and Non exhibited lower L* compared to Anti, whereas in July-Jan, Gln showed lowest L* (P < 0.0001, Figure 4.4). Increased chroma was found in Gln supplemented pigs only in SPRING trial, but not in July-Jan. SPRING pigs showed increase in L*, a*, b*, and chroma (P < 0.0001, Figure 4.4), while hue angle was decreased in SPRING for both muscles (P < 0.0001, Figure 4.4).

TBARS increased from display day 1 to day 7 (0.11 vs. 0.13 mg MDA/g muscle, respectively, P < 0.001), no display-related interactions were observed. SPRING samples showed overall higher TBARS than SUMMER trials (P<0.0001, Figure 4.5). Despite of 2-way and 3-way interactions between diet, muscle, and seasonal trials, lower TBARS values were found in Anti treated muscles, while higher TBARS values were more likely observed Gln (P < 0.01, Figure 4.5). Especially in PM, Gln showed highest TBARS compared to Anti or Non counterparts, regardless of seasonal trials. In both trials, PM muscles from Non showed lower TBARS compared to other dietary treatments in the same muscle, whereas in LD, lower TBARS were observed in Anti.

4.5   Discussion

As weaning is associated with an abrupt feed and liquid intake reduction and loss of passive immunity due to a sudden cessation in milk supply from the sows, devastating consequences of

poor weaning management are commonly seen in intestinal barrier and absorption functions (Wijtten, van der Meulen, & Verstegen, 2011), whereas long-term damages remain as a possibility (Boudry, Péron, Le Huërou-Luron, Lallès, & Sève, 2004; O'Mahony et al., 2009). L-glutamine is well recognized as a functional amino acid which serves important roles in maintaining small intestinal health and function and therefore can be applied in weaning pigs to ameliorate weaning associated intestinal dysfunctions (Ji, Wang, Yang, Hu, & Yin, 2019). In the present study, dietary Anti or Gln supplementation had no effect on body weight, loin eye area, muscling score, visual color or visceral organ weights. This observation corroborated with the growth performance evaluations during nursery and grow-finish stages (previously reported in Duttlinger et al., 2019): Gln and Anti supplementation during 14-day post-transport nursery phase improved growth performance as indicated by higher ADFI, ADG, F:G ratio and BW compared to Non; however, the positive effects of both pharmaceutical (Anti) and nutraceutical (Gln) intervention diminished when the dietary treatments were ceased upon the animals reached growing phase. From a carcass composition perspective, temporary supplementation of dietary Gln in post-transport weaning pigs marginally increased BF and decreased marbling score (Table 4.1, $P < 0.1$) in market ready pigs. Consistently, Hanczakowska et al. (2014) found that L-glutamine supplementation in weaning pigs lead to a numerical increase in average BF thickness and a significant change in loin eye BF. A few studies evidenced that dietary L-glutamine supplementation improves piglets' feed efficiency (Kitt et al., 2002) or growth performance (Abreu et al., 2010). Hence, mechanistic studies are still needed to elucidate the impacts of L-glutamine intake in nursery stage on fat and lean accretion and final carcass yield and composition characteristics.

Similar to previous studies (Hu et al., 2017; Hanczakowska et al., 2014), our study showed no difference between dietary treatments in pork muscle pH, WBSF and WHC attributes. While the numerical changes were minimal, Gln showed lower (or lowest) CIE L* and hue in both seasonal trials ($P < 0.05$), indicating a marginal effect of Gln in improving color characteristics. In broilers, studies have been found that dietary L-glutamine supplementation could potentially alleviate heat-stress induced meat quality defects, compensate pH decrease, improve meat texture and reduce water-loss (Dai, Wang, Wen, Wang, & Jin, 2009; Dai et al., 2012; Dai et al., 2018). While little evidence was found in pigs in terms of long-term effects of L-glutamine on final meat quality. The discrepancies are likely due to the timing of dietary treatment and species difference.

In the present study, dietary supplementation only covered 14 days of nursery phase. In pigs, stressors such as acute heat stress, poor transporting conditions and rough handling during preslaughter are among the most risky factors to cause meat quality defects, but the meat production outcomes are likely interactions between external stressors, genetic background and individual variance (Solomon, Van Laack, & Eastridge, 1998). The results of the present study indicate that early life weaning and transport events, if being reasonably managed, may not be a major contributor to variations in pork quality defects in market -ready animals

The current study showed that dietary antibiotics intervention seemed to offer improved oxidative stability as indicated by lower TBARS level, whereas no difference was seen in Gln supplementation group. In fact, during the 14-day post-transport recovery phase, Anti and Gln showed plasma tumor necrosis factor alpha (TNF-α) to decrease compared to Non, indicating lower extent of immune activation or inflammatory response. Further, TNF-α plays a critical role in inducing oxidative stress in tissues (Shiraki et al., 2012; Zhou et al., 2003). It is possible that different intervention strategies in alleviating early-life weaning and transport stress may impact the oxidation susceptibility of the skeletal muscles in the long run (Lv et al., 2018). While in the present study, Gln did not change muscle lipid oxidation stability in long term, previous studies do find dietary L-glutamine to ameliorate oxidative damages that induced by various stressors and enhance antioxidant capacity in different species and tissues (Dai et al., 2018; Dong, Zou, Hu, Qiao, & Sun, 2009; Olaniyi, Sabinari, & Olatunji, 2020; Petry et al., 2019; Xiao et al., 2016). Moreover, feeding weaning pigs with dietary L-glutamate and L-aspartate, pivotal substrates in L-glutamine synthesis, can effectively alleviate weaning associated growth suppression and hydrogen peroxide induced oxidative stress (Duan et al., 2016). Overall, the numerical changes of meat quality, color and oxidation indexes are minimal, therefore we postulate that weaning and transport as early-life stress events may not necessarily lead to noticeable alternation in postmortem muscle biochemistry activities and physical characteristics, if animals were given reasonable recovery.

Seasonality in carcass composition and meat quality variation have been well recognized (Arkfeld et al., 2017). Overall, improved production and meat quality outcomes were observed in SPRING over July-Jan. Noting that in the present study, the production season of SPRING was late-spring to early-autumn compared to SUMMER as late-summer to late-winter, decreased

protein and increased lipid content in SPRING pigs could be partially attributed to seasonal ambient temperature change. Environmental temperature was found to influence lipid metabolism in pigs, where hyperthermia challenges are consistently found to be associate with promoting lipid storage (Qu & Ajuwon, 2018). Environmental temperature above thermoneutral zone decreases lean accretion and inhibit β-oxidation of fatty acid in skeletal muscles to reduce metabolic heat production, leaving more energy (or non-esterized free fatty acids, NEFA) available for fat deposition, resulting in increased fat content in the carcasses (Le Bellego, Van Milgen, and Noblet, 2002; Wu et al., 2016).

According to the growth performance evaluation (Duttlinger et al., 2019), the seasonal discrepancies of animal growth were only observed when the animals reached market stage, but not in nursery or growing phase. Therefore, the difference in animal growth between the two trials were likely due to cumulative effects of management events that occurred during the early-life stage of the animals. In SUMMER, markedly increased pharmaceutical antibiotic treatments were applied when the pigs entered finishing period due to respiratory infection outbreak (Duttlinger et al., 2019). It should be noted that activated immune system is energetically expensive; glucose and other energy substance could be repartitioned to support activated immune function (Kvidera et al., 2017). It is plausible to infer that poorer health status combined with elevated immune response could reduce energy resource that support growth, adversely impact animal performance and final yield and productivity in SUMMER.

In July-Jan, PM at 1d postmortem showed the highest pH value among all the treatment groups, while a drastic decrease of pH from 5.92 to 5.7 was found at 7d. This may indicate that at 24h postmortem, glycolysis of SUMMERPM was not fully terminated. It is recognized that existence of ATP is needed for postmortem muscle to proceed glycolysis (England, Matarneh, Scheffler, Wachet, & Gerrard, 2015). As such, the delayed termination of pH decrease in SUMMERPM could be due to multiple reasons: First, SUMMER pigs were lower in body weight (Table 4.1), accelerating the carcass chilling process. Lower carcass temperature may to some extent slow-down ATP consumption rate. Second, PM as an oxidative red muscle, retained higher glycogen content and possibly longer ATP retention during muscle to meat conversion. Taken together, higher ATP availability may result in delayed cease of postmortem glycolysis in July-Jan, especially in PM muscles.

Consistent with lower pH values in SPRING, increased CIE L* and lower NPPC visual color score indicated a lighter/paler color in SPRING particularly in LD, which was in line with an increased water loss shown as higher drip loss and display loss (P < 0.05) on 1d postmortem aging. Dalla Costa et al. (2007) compared summer vs. winter pig production in Brazil and found warmer production season to show higher light reflectance, representing more PSE-like meat. On the other hand, frozen-thaw loss and cooking loss were lower in SPRING (P < 0.05). It is noteworthy that frozen-thaw and cooking process were analyzed on the same steak samples, therefore the total water loss during the processing (purge + cooking) was increased by 14% (26.8% vs. 31.1%) in SPRING compared to SUMMER pigs. In addition, WBSF was increased SUMMER compared to SPRING. From a proteolytic view to explain WHC (Huff-Lonergan & Lonergan, 2005), increased freeze/thaw and cooking water loss could be corroborated with a lower extent of proteolysis as indicated by higher WBSF, because myofibrillar structural protein fragmentation would provide more intramuscular space to hold water (Farouk, Mustafa, Wu, & Krsinic, 2012; Kristensen & Purslow, 2001). Seasonal discrepancies in WBSF could also be partially attributed to seasonality effect on calpain/calpastatin system. Calpastatin level were found to decrease as pigs experienced chronic exposure of high ambient temperature from 22 to 35 ° C (Shi et al., 2017), potentially increase enzymatic activity of calpain system, accelerating protein turn over and facilitating postmortem proteolysis.

SUMMER trials showed 17.5% lower TBARS than SPRING, which is possibly due to decreased intramuscular fat in July-Jan. Also, considering that SPRING animals were raised under a higher ambient temperature, temperature challenge above thermoneutral zone leads to the production of reactive oxygen species and disruption of antioxidant: prooxidant balance (Yang, Tan, Fu, Feng, & Zhang, 2010). Yang et al. (2014) found MDA content to increase in *m. longissimus* muscles of heat-stressed porcine muscles, indicating possible oxidative stress.

4.6   Conclusion

The results of present study show that 0.2% L-glutamine temporarily administered to nursery pigs after weaning and transport had no negative impact on pork carcass and meat quality characteristics including fat and lean composition, WBSF, and WHC; however, it marginally

improved color stability as indicated by lower CIE L* and hue angle. In SUMMER, concurrence of compromised growth/productivity and meat quality attributes was observed. Further investigations could be directed to understand the underlying mechanism how altered metabolism and growth would result in differentiated physical and quality characteristics in porcine muscles.

4.7   Literatures cited

Andersen, H. J., Oksbjerg, N., Young, J. F., & Therkildsen, M. (2005). Feeding and meat quality–a future approach. Meat science, 70 (3), 543-554.

Arkfeld, E., Mohrhauser, D., King, D., Wheeler, T., Dilger, A. C., Shackelford, S., & Boler, D. D. (2017). Characterization of variability in pork carcass composition and primal quality. *Journal of Animal Science, 95* (2), 697-708.

Berry, R., & Lewis, N. (2001). The effect of duration and temperature of simulated transport on the performance of early-weaned piglets. *Canadian Journal of Animal Science, 81* (2), 199-204.

Boudry, G., Péron, V., Le Huërou-Luron, I., Lallès, J. P., & Sève, B. (2004). Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. *The Journal of nutrition, 134* (9), 2256-2262.

Buege, J. A., & Aust, S. D. (1978). [30] Microsomal lipid peroxidation. In *Methods in enzymology* (Vol. 52, pp. 302-310): Elsevier.

Burrin, D. G., & Stoll, B. (2002). Key nutrients and growth factors for the neonatal gastrointestinal tract. *Clinics in perinatology, 29* (1), 65-96.

Campbell, J. M., Crenshaw, J. D., & Polo, J. (2013). The biological stress of early weaned piglets. *Journal of animal science and biotechnology, 4* (1), 19.

Cruzen, S., Baumgard, L., Gabler, N., Pearce, S., & Lonergan, S. (2017). Temporal proteomic response to acute heat stress in the porcine muscle sarcoplasm. *Journal of animal science, 95* (9), 3961-3971.

Dai, S., Bai, X., Zhang, D., Hu, H., Wu, X., Wen, A.,... Zhao, L. (2018). Dietary glutamine improves meat quality, skeletal muscle antioxidant capacity and glutamine metabolism in broilers under acute heat stress. *Journal of Applied Animal Research, 46* (1), 1412-1417.

Dai, S., Wang, L., Wen, A., Wang, L., & Jin, G. (2009). Dietary glutamine supplementation improves growth performance, meat quality and colour stability of broilers under heat stress. *British poultry science, 50* (3), 333-340.

Dalla Costa, O., Faucitano, L., Coldebella, A., Ludke, J., Peloso, J., Dalla Roza, D., & da Costa, M. P. (2007). Effects of the season of the year, truck type and location on truck on skin bruises and meat quality in pigs. *Livestock science, 107* (1), 29-36.

Dong, J., Zou, X., Hu, J., Qiao, Y., & Sun, K. (2009). Effects of dietary supplementation of glutamine on meat quality and antioxidant indexes of broilers. *Chinese Journal of Animal Nutrition, 21* (2), 245-250.

Duan, J., Yin, J., Ren, W., Liu, T., Cui, Z., Huang, X.,... Wu, X. (2016). Dietary supplementation with L-glutamate and L-aspartate alleviates oxidative stress in weaned piglets challenged with hydrogen peroxide. *Amino Acids, 48* (1), 53-64.

Duttlinger, A. W., Kpodo, K. R., Lay Jr, D. C., Richert, B. T., & Johnson, J. S. (2019). Replacing dietary antibiotics with 0.20% l-glutamine in swine nursery diets: impact on health and productivity of pigs following weaning and transport. *Journal of animal science, 97* (5), 2035-2052.

England, E. M., Matarneh, S., Scheffler, T., Wachet, C., & Gerrard, D. (2015). Altered AMP deaminase activity may extend postmortem glycolysis. *Meat science, 102*, 8-14.

Ewaschuk, J.B., Murdoch, G. K., Johnson, I. R., Madsen, K. L., & Field, C. J. (2011). Glutamine supplementation improves intestinal barrier function in a weaned piglet model of Escherichia coli infection. *British Journal of Nutrition, 106* (6), 870-877.

Farouk, M., Mustafa, N. M., Wu, G., & Krsinic, G. (2012). The "sponge effect" hypothesis: An alternative explanation of the improvement in the waterholding capacity of meat with ageing. *Meat Science, 90* (3), 670-677.

Faucitano, L., & Goumon, S. (2018). Transport of pigs to slaughter and associated handling *Advances in pig welfare* (pp. 261-293): Elsevier.

Hanczakowska, E., Niwinska, B., Grela, E., Weglarzy, K., & Okon, K. (2014). Effect of dietary glutamine, glucose and/or sodium butyrate on piglet growth, intestinal environment, subsequent fattener performance, and meat quality. *Czech Journal of Animal Science, 59* (59), 460-470.

Harris, D.L. (2008). *Multi-site pig production*: John Wiley & Sons.

Honikel, K.O. (1998). Reference methods for the assessment of physical characteristics of meat. Meat science, 49 (4), 447-457.

Hu, C., Jiang, Q., Zhang, T., Yin, Y., Li, F., Deng, J.,... Kong, X. (2017). Dietary supplementation with arginine and glutamic acid modifies growth performance, carcass traits, and meat quality in growing-finishing pigs. *Journal of Animal Science, 95* (6), 2680-2689.

Huff-Lonergan, E., & Lonergan, S. M. (2005). Mechanisms of water-holding capacity of meat: The role of postmortem biochemical and structural changes. *Meat science, 71* (1), 194-204.

Jiang, Z., Sun, L., Lin, Y., Ma, X., Zheng, C., Zhou, G.,... Zou, S. (2009). Effects of dietary glycyl-glutamine on growth performance, small intestinal integrity, and immune responses of weaning piglets challenged with lipopolysaccharide. *Journal of Animal Science, 87* (12), 4050-4056.

Ji, F., Wang, L., Yang, H., Hu, A., & Yin, Y. (2019). The roles and functions of glutamine on intestinal health and performance of weaning pigs. *animal, 13* (11), 2727-2735.

Johnson, J., & Lay Jr, D. (2017). Evaluating the behavior, growth performance, immune parameters, and intestinal morphology of weaned piglets after simulated transport and heat stress when antibiotics are eliminated from the diet or replaced with L-glutamine. *Journal of animal science, 95* (1), 91-102.

Kristensen, L., & Purslow, P.P. (2001). The effect of ageing on the water-holding capacity of pork: role of cytoskeletal proteins. *Meat Science, 58* (1), 17-23.

Kvidera, S., Horst, E., Mayorga, E., Sanz-Fernandez, M., Abuajamieh, M., & Baumgard, L. (2017). Estimating glucose requirements of an activated immune system in growing pigs. *Journal of Animal Science, 95* (11), 5020-5029.

Lallès, J.-P., Boudry, G., Favier, C., Le Floc'h, N., Luron, I., Montagne, L.,... Sève, B. (2004). Gut function and dysfunction in young pigs: physiology. *Animal Research, 53* (4), 301-316.

Landers, T. F., Cohen, B., Wittum, T. E., & Larson, E. L. (2012). A review of antibiotic use in food animals: perspective, policy, and potential. *Public health reports, 127* (1), 4-22.

Le Bellego, L., Van Milgen, J., & Noblet, J. (2002). Effect of high ambient temperature on protein and lipid deposition and energy utilization in growing pigs. *Animal Science, 75* (1), 85-96.

Lescano, D., Albino, L., Hannas, M., Salguero, S., Kutschenko, M., Nogueira, F., & Rostagno, H. (2013). Effect of glutamic acid plus glutamine on the intestinal morphology of piglets. *Journal of Animal Science, 91*.

Lewis, N. J., & Berry, R. J. (2006). Effects of season on the behaviour of early-weaned piglets during and immediately following transport. *Applied Animal Behaviour Science, 100* (3-4), 182-192.

Lv, D., Xiong, X., Yang, H., Wang, M., He, Y., Liu, Y., & Yin, Y. (2018). Effect of dietary soy oil, glucose, and glutamine on growth performance, amino acid profile, blood profile, immunity, and antioxidant capacity in weaned piglets. *Science China Life Sciences, 61* (10), 1233-1242.

Ma, D., Chao, Y., Duttlinger, A., Richert, B., Johnson, J., & Kim, Y. H. B. (2018). *Effect of transporting weaned pigs under different season on meat quality and postmortem proteolysis of two porcine muscles.* Paper presented at the 64th ICoMST, Melbourne, Australia.

Newsholme, P. (2001). Why is L-glutamine metabolism important to cells of the immune system in health, postinjury, surgery or infection? *The Journal of nutrition, 131* (9), 2515S-2522S.

O'Mahony, S. M., Marchesi, J. R., Scully, P., Codling, C., Ceolho, A.-M., Quigley, E. M.,... Dinan, T. G. (2009). Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biological psychiatry, 65* (3), 263-267.

Olaniyi, K. S., Sabinari, I. W., & Olatunji, L. A. (2020). Oral L-glutamine restores adenosine and glutathione content in the skeletal muscle and adipose tissue of insulin-resistant pregnant rats. *Nutrition*, 110789.

Petry, É. R., de Freitas Dresch, D., Carvalho, C., Medeiros, P. C., Rosa, T. G., de Oliveira, C. M.,... Guma, F. C. R. (2019). Oral glutamine supplementation attenuates inflammation and oxidative stress-mediated skeletal muscle protein content degradation in immobilized rats: Role of 70 kDa heat shock protein. *Free Radical Biology and Medicine, 145*, 87-102.

Qu, H., & Ajuwon, K. M. (2018). Metabolomics of heat stress response in pig adipose tissue reveals alteration of phospholipid and fatty acid composition during heat stress. *Journal of Animal Science, 96* (8), 3184-3195.

Shi, Z., Ma, X., Zheng, C., Hu, Y., Yang, X., Gao, K.,... Jiang, Z. (2017). Effects of high ambient temperature on meat quality, serum hormone concentrations, and gene expression in the longissimus dorsi muscle of finishing pigs. *Animal Production Science, 57* (6), 1031-1039.

Shiraki, A., Oyama, J.-i., Komoda, H., Asaka, M., Komatsu, A., Sakuma, M.,... Hirase, T. (2012). The glucagon-like peptide 1 analog liraglutide reduces TNF-α-induced oxidative stress and inflammation in endothelial cells. *Atherosclerosis, 221* (2), 375-382.

Solomon, M., Van Laack, R., & Eastridge, J. (1998). Biophysical basis of pale, soft, exudative (PSE) pork and poultry muscle: A review. *Journal of Muscle Foods, 9* (1), 1-11.

Sutherland, M., McDonald, A., & McGlone, J. (2009). Effects of variations in the environment, length of journey and type of trailer on the mortality and morbidity of pigs being transported to slaughter. *Veterinary Record, 165* (1), 13-18.

Wijtten, P. J., van der Meulen, J., & Verstegen, M. W. (2011). Intestinal barrier function and absorption in pigs after weaning: a review. *British Journal of Nutrition, 105* (7), 967-981.

Wu, G., Knabe, D. A., Yan, W., & Flynn, N. E. (1995). Glutamine and glucose metabolism in enterocytes of the neonatal pig. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 268* (2), R334-R342.

Wu, X., Li, Z.-y., Jia, A.-f., Su, H.-g., Hu, C.-h., Zhang, M.-h., & Feng, J.-h. (2016). Effects of high ambient temperature on lipid metabolism in finishing pigs. *Journal of integrative agriculture, 15* (2), 391-396.

Xiao, L., Cao, W., Liu, G., Fang, T., Wu, X., Jia, G.,... Wu, C. (2016). Arginine, N-carbamylglutamate, and glutamine exert protective effects against oxidative stress in rat intestine. *Animal Nutrition, 2* (3), 242-248.

Yang, L., Tan, G.-Y., Fu, Y.-Q., Feng, J.-H., & Zhang, M.-H. (2010). Effects of acute heat stress and subsequent stress removal on function of hepatic mitochondrial respiration, ROS production and lipid peroxidation in broiler chickens. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 151* (2), 204-208.

Yang, P., Hao, Y., Feng, J., Lin, H., Feng, Y., Wu, X.,... Gu, X. (2014). The expression of carnosine and its effect on the antioxidant capacity of longissimus dorsi muscle in finishing pigs exposed to constant heat stress. *Asian-Australasian journal of animal sciences, 27* (12), 1763.

Zhang, J.-m., Wang, L.-d., Gao, Z.-c., Jiang, Y.-x., & Zhang, Q. (2002). Effect of Dietary Glutamine Supplementation on Antioxidant Capacity of Tissue in the Early-weaned Piylets. *Acta Veterinaria et Zootechnica Sinica, 33* (2), 105-109.

Zhou, Z., Wang, L., Song, Z., Lambert, J. C., McClain, C. J., & Kang, Y. J. (2003). A critical involvement of oxidative stress in acute alcohol-induced hepatic TNF-α production. *The American journal of pathology, 163* (3), 1137-1146.

Zou, X., Zheng, G., Fang, X., & Jiang, J. (2006). Effects of glutamine on growth performance of weanling piglets. *Czech Journal of Animal Science, 51* (10), 444.

Table 4.1 Effects of dietary antibiotics and L-glutamine supplementation on carcass characteristics and organ weights

Parameters	Trial[1]		Diet[2]			S.E.M[3]	P-value		
	SPRING	SUMMER	Anti	Gln	Non		T	D	T×D
Carcass characteristics									
Live weight, kg	129.63	121.77	124.94	124.50	127.66	3.06	0.03	0.73	0.84
Hot carcass weight, kg	100.01	94.31	97.52	94.58	99.38	2.09	0.02	0.27	1.00
Dressing percentage, %	77.16	77.74	78.07	76.39	77.88	0.68	0.47	0.17	0.56
Carcass moisture loss, %	6.06	5.67	5.67	6.21	5.71	0.28	0.21	0.29	0.65
Color score[4]	2.08	3.33	2.63	2.75	2.75	0.11	<.0001	0.67	0.30
Marbling score[4]	1.37	1.07	1.35	1.13	1.18	0.08	0.0009	0.08	0.23
Muscling score[4]	2.00	2.75	2.43	2.30	2.40	0.06	<.0001	0.27	0.27
BF 10th rib, cm[5]	2.40	1.82	1.89	2.15	2.29	0.12	<.0001	0.06	0.66
BF last rib, cm[5]	2.92	2.71	2.72	2.82	2.90	0.15	0.17	0.66	0.96
Loin depth 10th rib, cm	7.11	6.88	7.11	6.88	7.00	0.13	0.09	0.39	0.63
Loin eye area, cm[2]	59.54	51.95	55.99	54.47	56.77	1.44	<.0001	0.51	0.85
Organ weight (Scaled to HCW, %)									
Head	6.11	6.30	6.22	6.21	6.18	0.10	0.06	0.95	0.17
Heart	0.39	0.39	0.40	0.39	0.37	0.01	0.99	0.43	0.33
Spleen	0.18	0.22	0.19	0.20	0.21	0.01	0.0005	0.69	0.46
Liver	1.76	1.76	1.77	1.77	1.74	0.04	0.96	0.80	0.17

[1]**SPRING**: Pigs experienced weaning and transport stress in April 2017; **SUMMER**: Pigs experienced weaning and transport stress in July 2016
[2]**Anti**: Pigs fed dietary antibiotics [chlortetracycline (441 ppm) + tiamulin (38.6 ppm)] for 14 d post-weaning and transport; **Gln**: Pigs fed dietary L-glutamine (0.2%) for 14 d post-weaning and transport; **Non**: Pigs fed non- antibiotics diet for 14 d post-weaning and transport
[3]Pooled standard error of means
[4]Subjective pork carcass traits were evaluated according to National Pork Producers Council standards (NPPC, 2000); Color score: 1 very pale to 6 very dark; Marbling score: 1 devoid to 10 excessive; Muscling score: 1 thin, 1.5 to 2.5 average, 3 thick
[5]BF=backfat

Table 4.2 Effects of dietary antibiotics and L-glutamine supplementation on proximate composition of postmortem porcine muscles

Factors		Moisture %	Dry-matter based		
			Protein %	Lipid %	Ash %
Trial (T)[1]					
SPRING		73.63	84.26	11.16	3.99
SUMMER		73.41	85.41	9.76	4.56
Diet (D)[2]					
Anti		73.40	84.30	10.46	4.26
Gln		73.69	84.85	10.26	4.32
Non		73.47	85.36	10.66	4.24
Muscle (M)[3]					
LD		73.08	84.54	10.76	4.21
PM		73.96	85.13	10.16	4.34
Aging (A)					
1 d		73.62	84.88	10.76	4.32
7 d		73.42	84.79	10.16	4.23
Aging × Trial					
SPRING	1d	73.49	84.17	11.86	3.87
	7d	73.77	84.36	10.45	4.11
SUMMER	1d	73.76	85.59	9.66	4.76
	7d	73.07	85.23	9.86	4.35
S.E.M[4]		0.07	0.22	0.27	0.03
P Value					
D		0.12	0.15	0.82	0.11
M		<.0001	0.19	0.26	0.0003
T		0.08	0.01	0.01	<.0001
A		0.10	0.85	0.25	0.19
T×A		<.0001	0.53	0.13	<.0001
A×M		0.01	0.08	0.35	0.28
T×M		0.02	0.48	0.98	0.54
T×A×M		0.77	0.73	0.68	0.02

[1]**SPRING**: Pigs experienced weaning and transport stress in April 2017; **SUMMER**: Pigs experienced weaning and transport stress in July 2016

[2]**Anti**: Pigs fed dietary antibiotics [chlortetracycline (441 ppm) + tiamulin (38.6 ppm)] for 14 d post-weaning and transport; **Gln**: Pigs fed dietary L-glutamine (0.2%) for 14 d post-weaning and transport; **Non**: Pigs fed non-antibiotics diet for 14 d post-weaning and transport

[3]**LD**: *Longissimus dorsi*; **PM**: *Psoas Major*

[4]Pooled standard error of means

Table 4.3 Effects of dietary antibiotics and L-glutamine supplementation on pH and water-holding capacity of porcine muscles

Factors		pH	Drip loss %	Display loss %	Thaw-purge loss %	Cook loss %
Trial (T)[1]						
SPRING		5.69	4.10	6.36	5.35	21.41
SUMMER		5.72	3.31	5.32	7.52	23.58
Diet (D)[2]						
Anti		5.71	3.67	5.80	6.43	22.50
Gln		5.71	3.53	5.67	6.13	22.41
Non		5.70	3.92	6.06	6.74	22.56
Muscle (M)[3]						
LD		5.63	5.81	7.50	8.41	23.88
PM		5.78	1.60	4.18	4.43	21.09
Aging (A)						
1d		5.72	3.68	5.43	7.19	22.54
7d		5.69	3.73	6.25	5.66	22.43
Aging × Trial						
SPRING	1d	5.65	4.50	5.43	5.38	21.38
	7d	5.73	3.70	7.30	5.32	21.43
SUMMER	1d	5.78	2.86	5.44	9.00	23.70
	7d	5.66	3.75	5.20	6.01	23.45
S.E.M[4]						
P Value						
D		0.34	0.20	0.48	0.50	0.97
M		<.0001	<.0001	<.0001	<.0001	<.0001
T		0.0004	<.0001	0.0002	<.0001	<.0001
A		0.027	0.80	0.0028	0.0004	0.81
T×A		<.0001	<.0001	0.0001	0.0006	0.73
A×M		<.0001	0.092	0.12	0.73	0.74
T×M		0.0005	0.34	0.34	0.13	0.12
T×A×M		<.0001	0.0013	0.29	0.48	0.97

[1]**SPRING**: Pigs experienced weaning and transport stress in April 2017; **SUMMER**: Pigs experienced weaning and transport stress in July 2016

[2]**Anti**: Pigs fed dietary antibiotics [chlortetracycline (441 ppm) + tiamulin (38.6 ppm)] for 14 d post-weaning and transport; **Gln**: Pigs fed dietary L-glutamine (0.2%) for 14 d post-weaning and transport; **Non**: Pigs fed non- antibiotics diet for 14 d post-weaning and transport

[3]**LD**: *Longissimus dorsi*; **PM**: *Psoas Major*

[4]Pooled standard error of means

[5]Total processing loss was obtained by adding thaw-purge loss and cook loss. The data was not subjected to statistical analysis

A)

B)
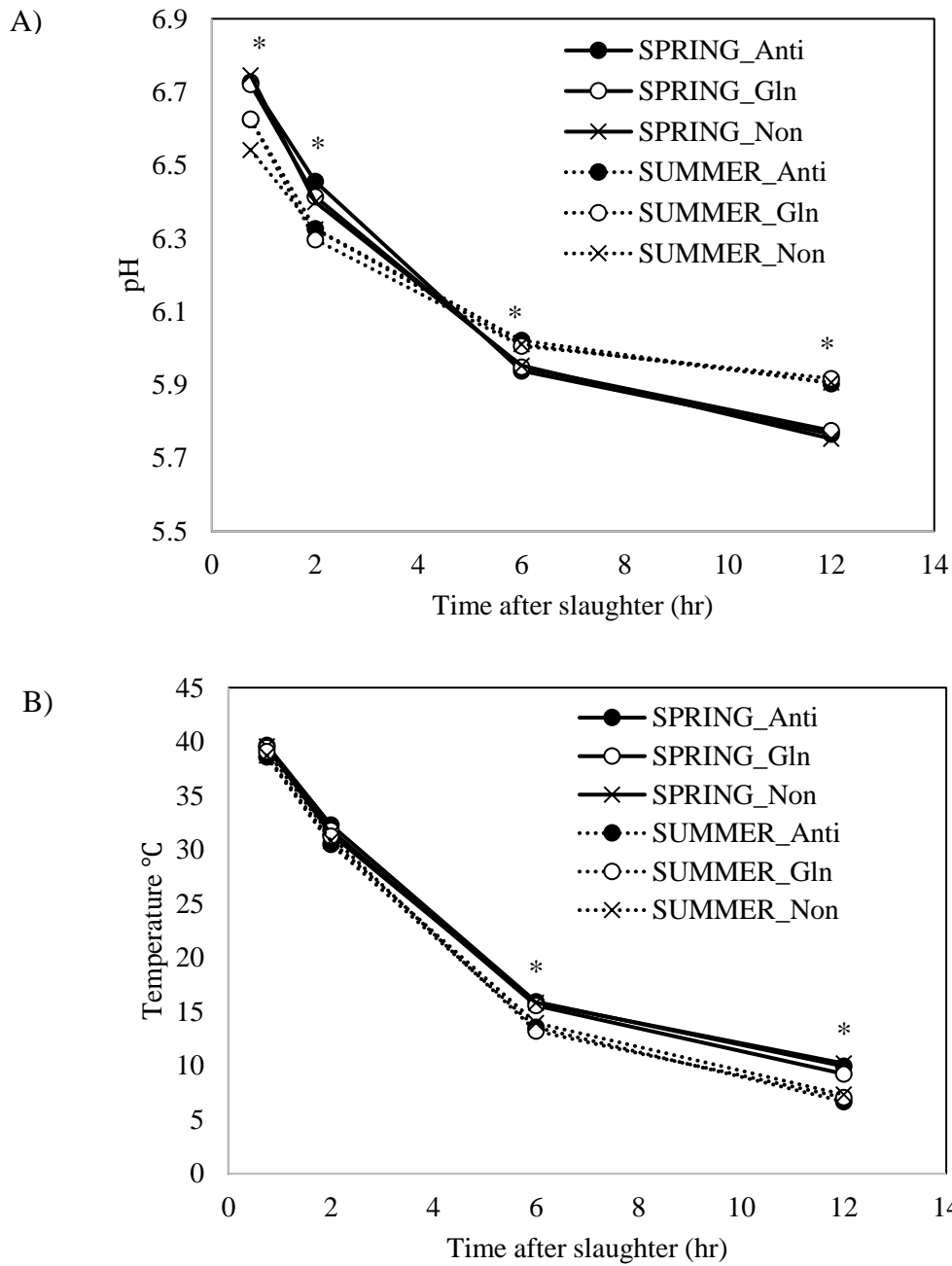
Figure 4.1 Initial pH and temperature decline of *M. Longissimus* during 12 hr postmortem of pigs experienced weaning and transport stress and different dietary treatment. An asterisk (*) on each timepoint indicates overall seasonal trial difference
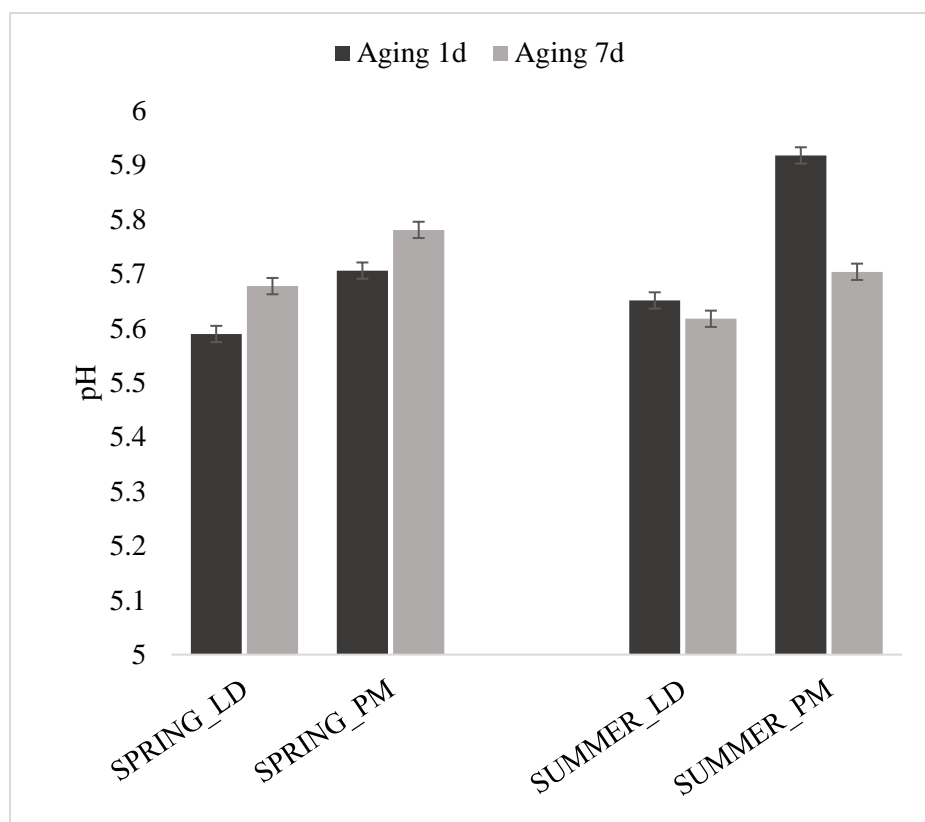
Figure 4.2 Final pH values of LD (longissimus dorsi) and PM (psoas major) muscles at 1d vs. 7d postmortem aging in pigs experienced weaning and transport stress and different dietary treatment. Results were displayed as means ± standard error.
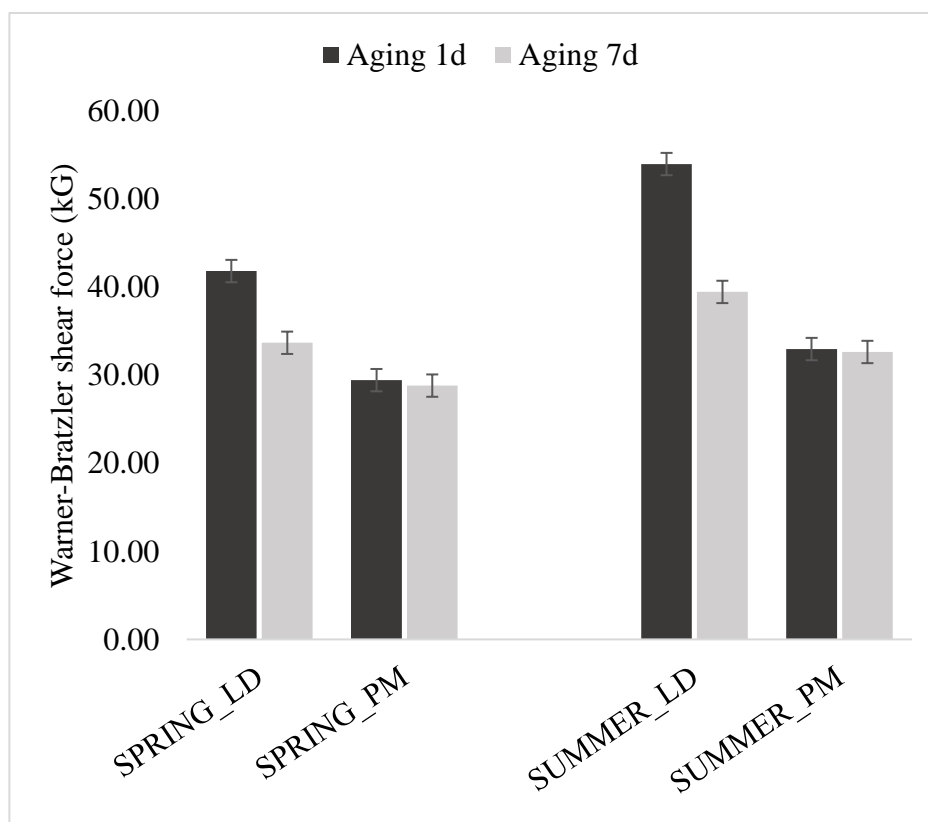
Figure 4.3 Warner-Bratzler shear force of LD (longissimus dorsi) and PM (psoas major) muscles at 1d vs. 7d postmortem aging in pigs experienced weaning and transport stress and different dietary treatment. Results were displayed as means ± standard error.
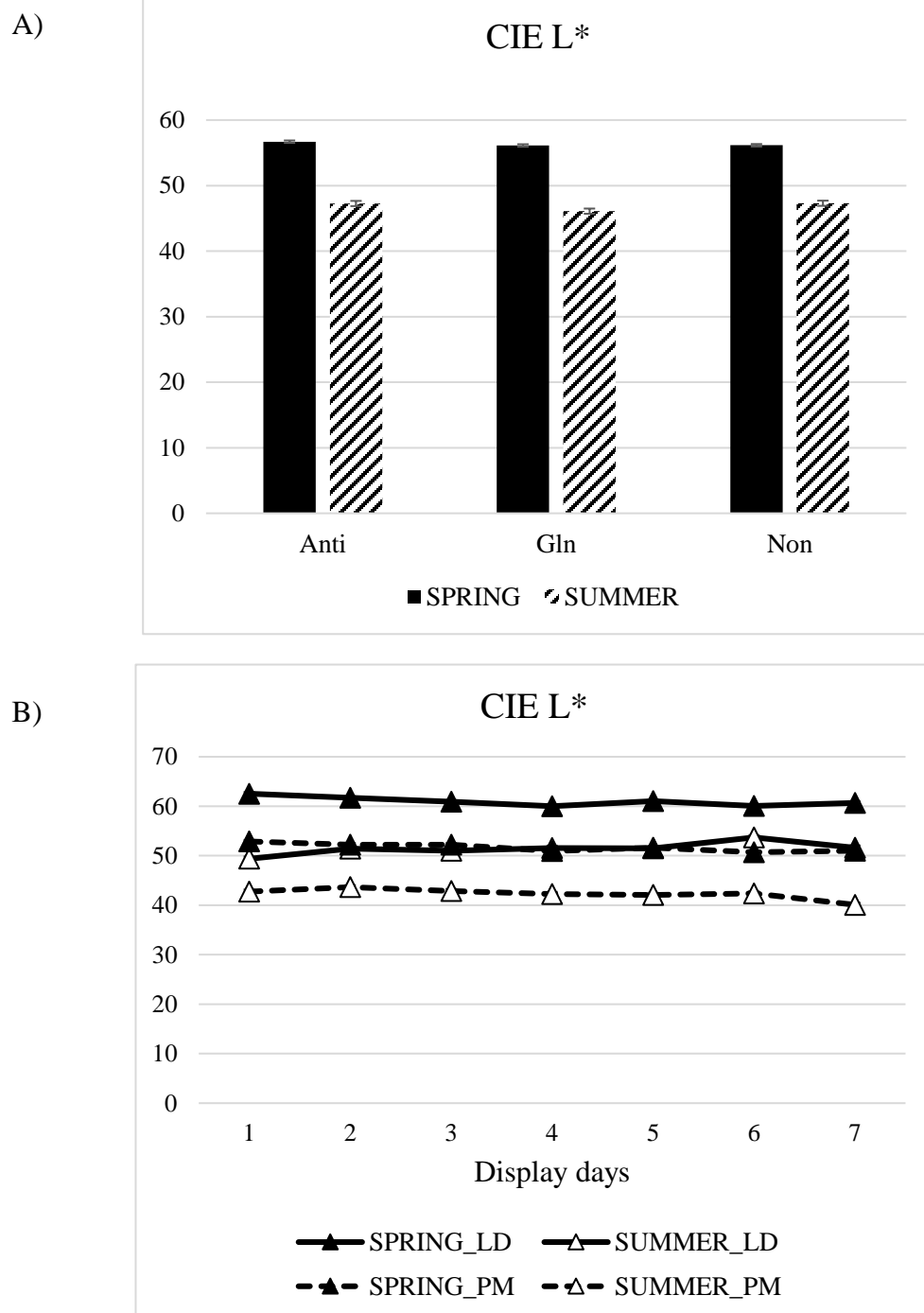
Figure 4.4 The effects of trial by diet and trial by muscle by display interactions on instrumental color characteristics of fresh pork from pigs experienced weaning and transport stress and different dietary treatment. (A-B) CIE L*, (C-D) chroma, (E-F) hue angle, (G-H) CIE a*, and (I-J) CIE b*. Results were displayed as means ± standard error.

Figure 4.4 continued

C)

D)

Figure 4.4 continued

E)

Hue angle

F)

Hue angle

Figure 4.4 continued

G)

CIE a*

■SPRING ⊘SUMMER

H)

CIE a*

Display days

▲ SPRING_LD △ SUMMER_LD
▲ SPRING_PM △ SUMMER_PM
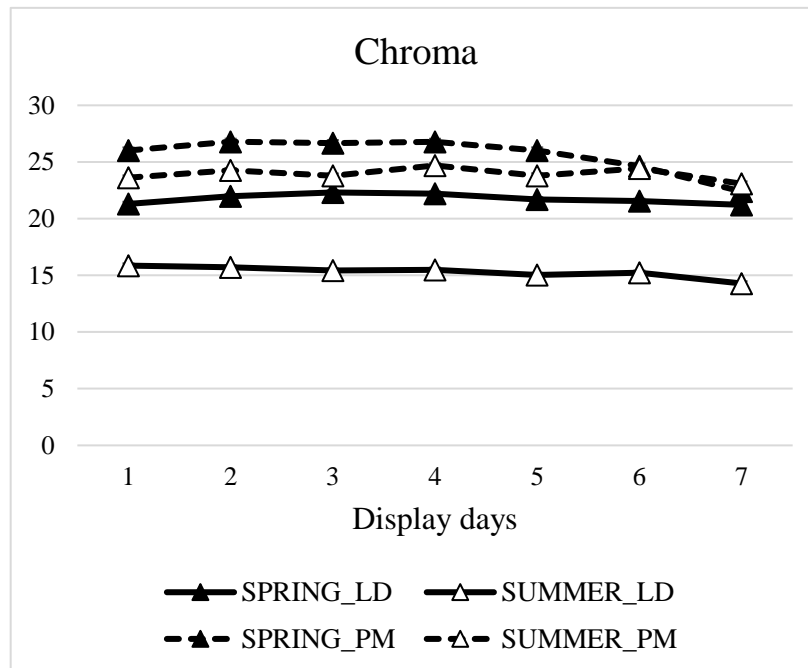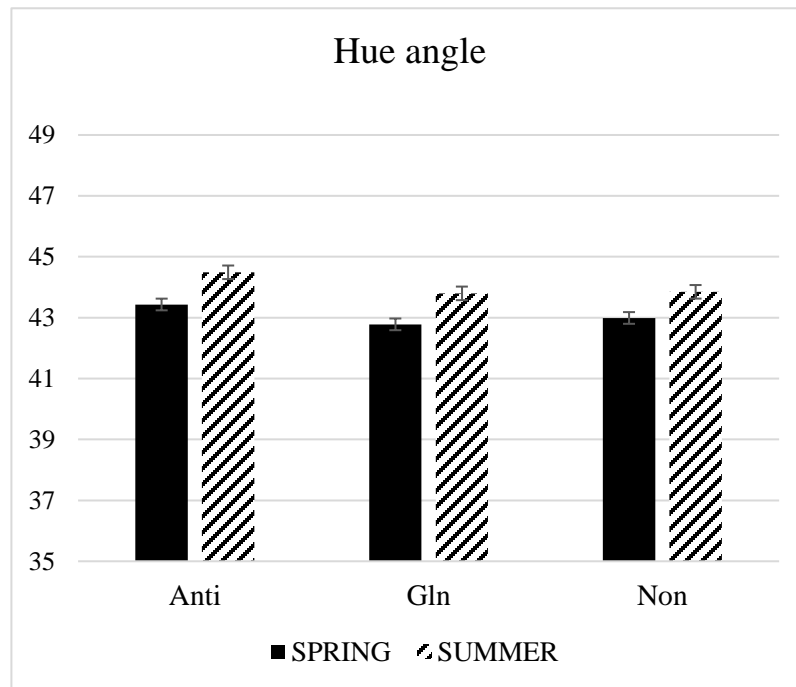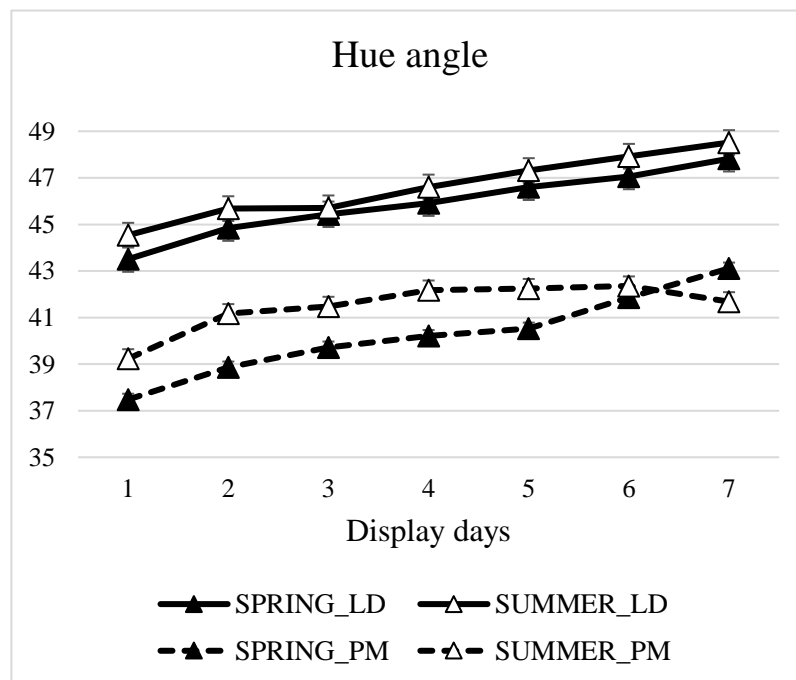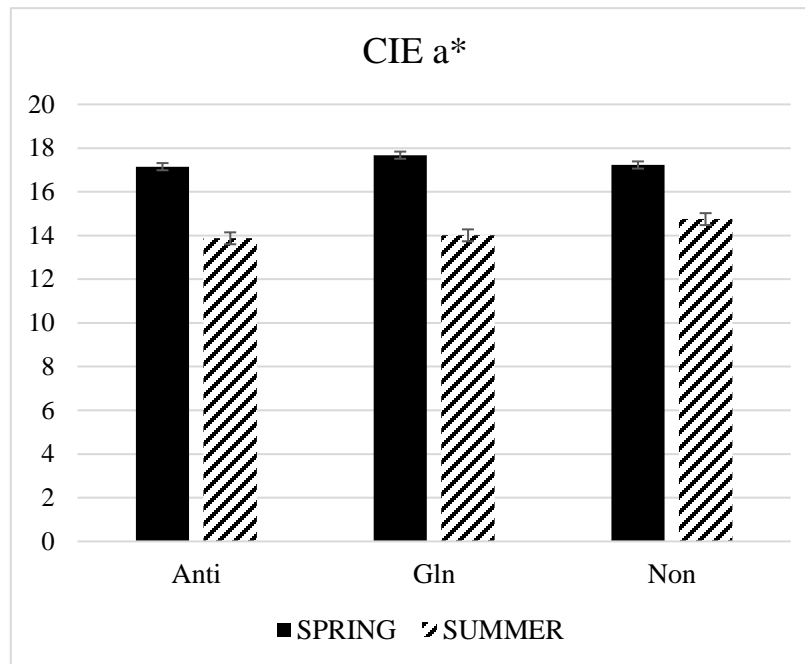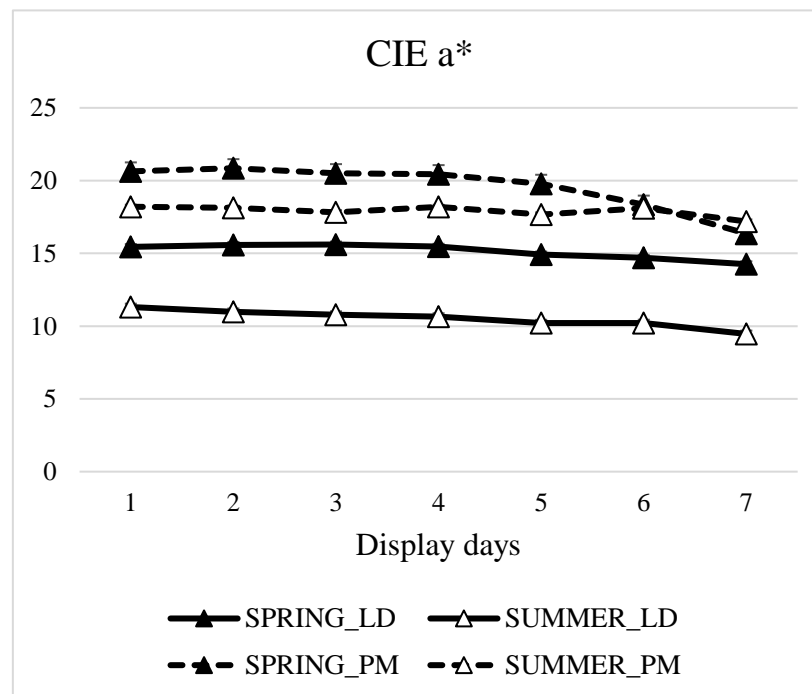
Figure 4.4 continued

I)

CIE b*

J)

CIE b*

Display days
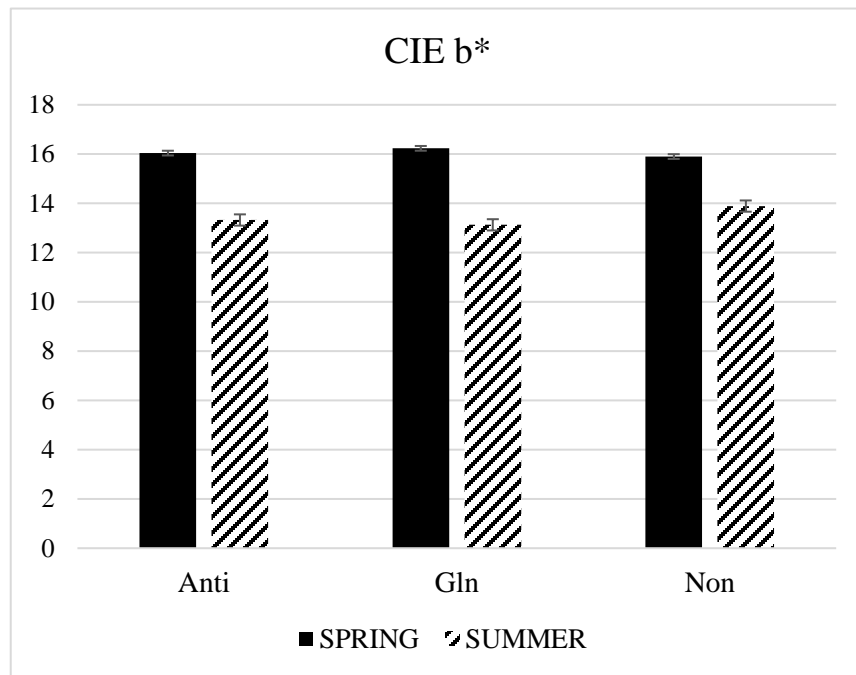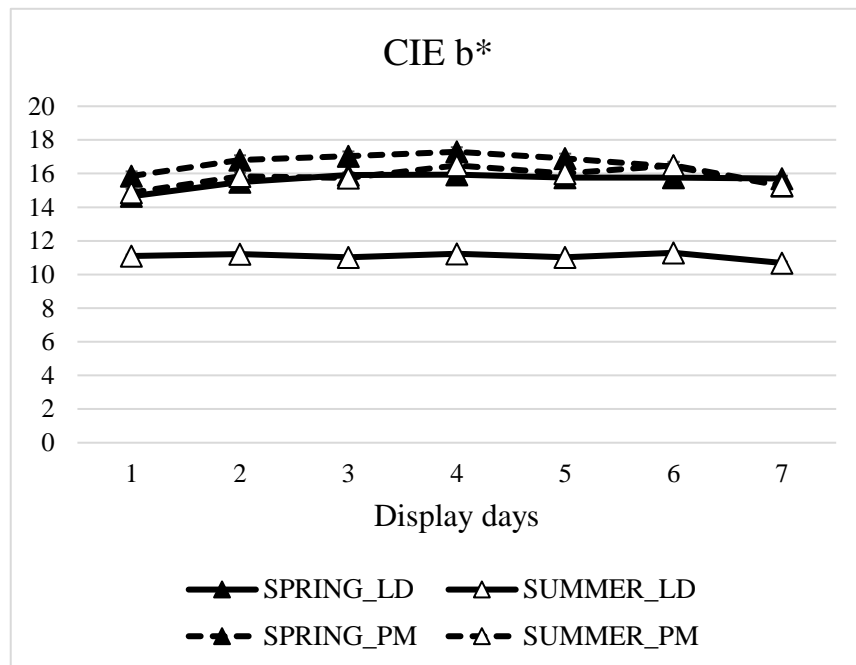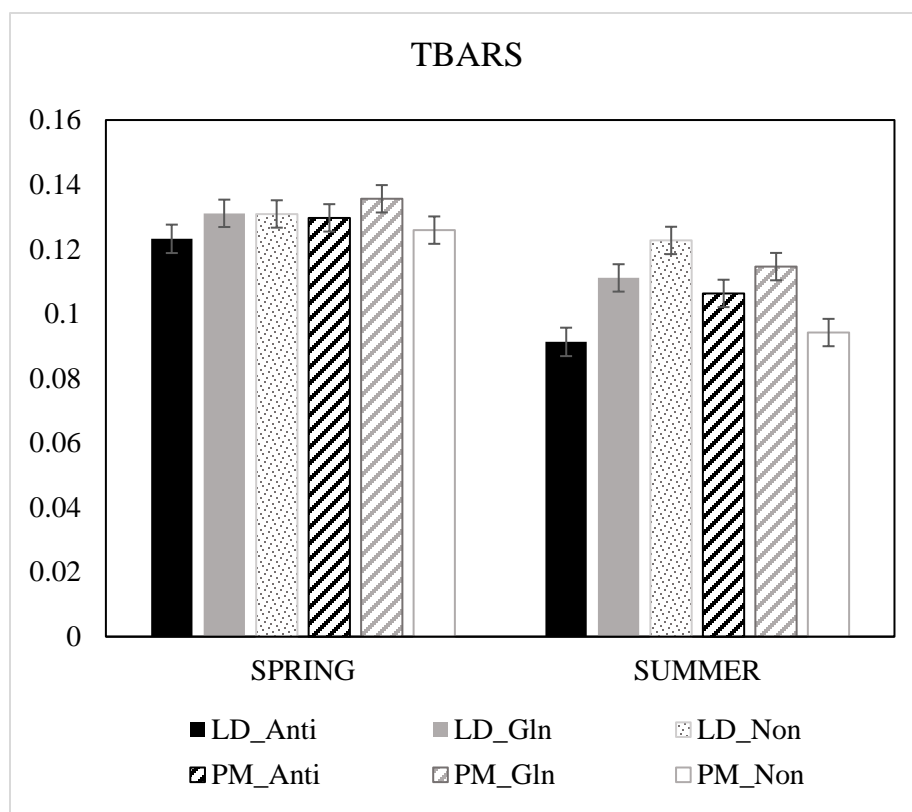
Figure 4.5 Effect of diet, muscle and trial interaction on TBARS of LD (longissimus dorsi) and PM (psoas major) muscles from pigs experienced weaning and transport stress and different dietary treatment. Results were expressed as means ± standard error

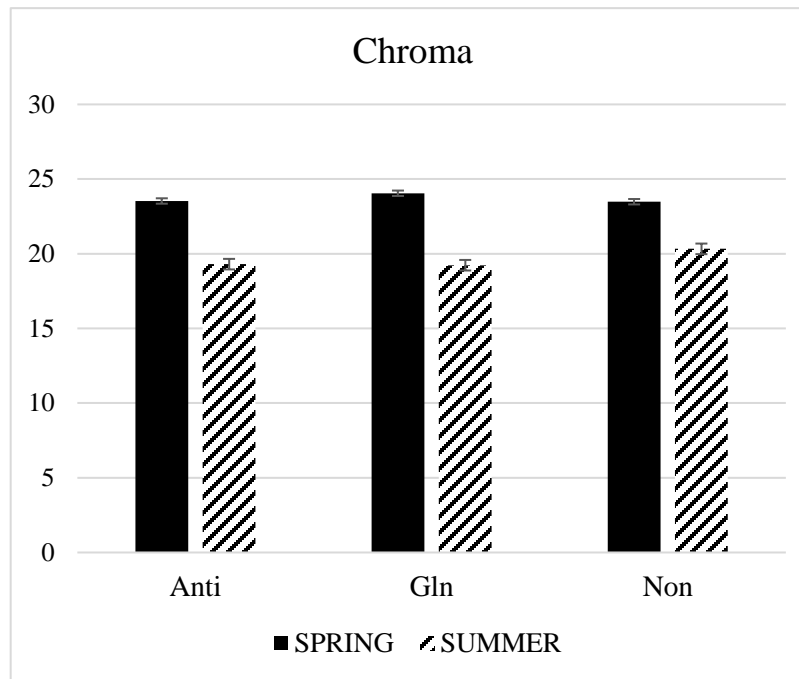# CHAPTER 5.   APOPTOTIC AND PROTEOLYTIC ATTRIBUTES AND METABOLOMICS CHANGES IN PORCINE MUSCLES FROM TWO PRODUCTION REPLICATES

5.1   Abstract

Apoptosis has been proposed as upstream event of postmortem proteolysis, possibly affected by antemortal metabolic activities. To evaluate apoptotic and proteolytic attributes and metabolomic changes in postmortem porcine muscles, two repetitions of pigs were raised in different seasonal cycles of the year, July-Jan (weaned in July, 2016 and harvested in January, 2017) and Apr-Sep (weaned in April, 2017 and harvested in September 2017) in north-central Indiana. Left- and right-sides of longissimus dorsi and psoas major muscles were collected at 1d and 7d postmortem. Proteolytic characteristics of structural proteins, small heat shock proteins and apoptotic factors including desmin, troponin T, calpain 1, HSP27, αβ-crystallin and cytochrome c were quantified using Western-blot assays. Mitochondria membrane permeability (MMP) was evaluated. Metabolome profiles of 1d samples were analyzed using the GC-TOF-MS platform. PM showed higher MMP than LD, but lower extent of calpain 1 autolysis ($P < 0.05$). Compared to July-Jan counterparts, Apr-Sep muscles showed concurrence of more extended apoptosis as indicated by higher MMP ($P < 0.05$). Further calpain 1 autolysis was observed in Apr-Sep regardless of muscle types. Troponin T degradation was higher in Apr-Sep, particularly in LD($P<0.05$). Small heat shock proteins were not affected by seasonal replicates, except HSP27 in LD was in July-Jan compared to Apr-Sep counterparts. Metabolomics profiling indicated Apr-Sep pigs showed increased carbohydrate and metabolic intermediates, branched chain amino acids, and free fatty acids. Antioxidant amino acids histidine and aspartic acid and ascorbic acid were higher in July-Jan, suggesting higher antioxidant stress-defending activity. Increased macronutrients in Apr-Sep could be related to higher cellular oxidative stress, favoring onset of apoptosis. As such, seasonal alternation of metabolism could render influential impact on meat quality development. The results of the present study provide initial evidence that extended apoptosis could promote proteolysis to a certain extent, whereas this is a muscle specific cross-talk. Early postmortem apoptosis might be facilitated by increased cellular macronutrient and oxidative stress, and

inhibited by antioxidant compounds, subsequentially impact proteolysis and meat quality development.

Keywords: Pork, Apoptosis, Metabolism, Proteolysis, Stress

5.2   Introduction

Postmortem aging is extensively practiced in meat industry as a value-adding process. Particularly, the action of endogenous protease system degrading myofibrillar structural proteins, also known as postmortem proteolysis, substantially enhance palatability attributes such as tenderness, juiciness, and flavor (Kim, Warner, & Rosenvold, 2014; Kristensen & Purslow, 2001; Lonergan, Zhang, & Lonergan, 2010; Spanier, Flores, Mcmillin, & Bidner, 1997). Although much effort has been made to decipher the role of muscle structure fragmentation in guaranteeing tenderness and / or water-holding capacity (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010; Kim et al., 2018), proteolysis can only explain limited portion of quality variations in pork (Carlson et al., 2017); hence, knowledge gaps still exist in explaining the exact mechanism by which postmortem aging governs palatability development. This is partially a result of isolating proteolysis from regulatory views of other associated biochemical factors and processes.

Apoptosis, or programmed cell death, referred to as a finely regulated and controlled process by which cells recruit innate enzyme systems to break down functional and/or structural compartment to terminate cellular life cycle (Kemp & Parr, 2012). Apoptosis was recently proposed as the very first phase in muscle -meat conversion process, and probably have active interaction with postmortem proteolysis (Kemp & Parr, 2012; Ouali et al., 2013; Ouali et al., 2006). Therefore, apoptotic impact on development of organoleptic and /or perceptional quality attributes has become a growing research interest. Recent evidence has identified apoptotic hallmarks upon muscle fibers receiving ante-mortal death stimuli, such as morphological change and anti- or pro-apoptotic factor expression (Cao et al., 2010; Chen, Zhang, Li, Gao, & Zhou, 2017; Guo et al., 2016; Huang et al., 2016). Particularly, mitochondria dysfunction due to damaged membrane system will lead to failure of respiration reactions, accumulation of reactive oxygen species, and release of pro-apoptotic factor release (Wang, Han, Ma, Yu, & Zhao, 2017; Wang et al., 2018). As mitochondria is the central organelle for energy metabolism, it is reasonable to postulate that

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postmortem anaerobic and/or aerobic respiration and apoptosis response should be interrelated. Skeletal muscles (or muscle fibers) are varied in respiration and contractile properties, ranging from glycolytic fast twitch (Type IIB) to oxidative slow twitch (Type I). Previous studies consider muscle fiber type as a key contributor of meat quality development (Lee, Joo, & Ryu, 2010). Moreover, oxidative muscles were reported to have inferior tenderization potential, accompanied with decreased protease activity (Ma & Kim, 2020). As such, postmortem metabolism features shall be profiled in different muscle types to establish linkage between postmortem metabolism, apoptosis, and subsequent proteolysis to shed lights on updated views of muscle to meat conversion.

Previous studies have shown the potential of metabolomics technology to elucidate molecular mechanism of meat quality development under various conditions (Ma et al., 2017; Subbaraj, Kim, Fraser, & Farouk, 2016). In the current study, we hypothesize that assorted key metabolism features could possibly involve in postmortem apoptotic and proteolytic process, leading to meat quality variation. In this regard, high-throughput metabolomics platform and various bioinformatics tools could be applied to identify metabolism fingerprints that were related to apoptosis, and establish linkages between postmortem energy metabolism, apoptosis, and proteolysis in a muscle-meat conversion prospective. Therefore, the objective of the current study was to evaluate apoptotic and proteolytic characteristics and metabolome changes of porcine muscles from two production season. Porcine muscles *longissimus dorsi* (LD) and *psoas major* (PM) were selected, because they were known to have distinct differences in fiber composition and metabolic features. This study is further elaboration of our recent study (Ma, Kim, Johnson, Duttlinger, & Guedes, 2019), where meat quality and oxidative stability results of porcine muscles were reported.

5.3   Materials and methods

5.3.1   Animals and muscle processing

The detailed information regarding live animal processing and performance was reported in previous study (Duttlinger et al., 2019). Two repetitions of pigs (barrows and gilts, N=480) were

weaned at 19 days of age and transported in a truck trailer for 12 hours in central Indiana, which were replicated during July of 2016 (SUMMER) and April of 2017 (SPRING). For 14 days post-transport, pigs were supplied with dietary antibiotics chlortetracycline (441 ppm) and tiamulin (38.6 ppm) to recover from wean and transport stress. Ten animals (age of 5-month, body weight 120.28±1.32 kg) were randomly chosen from SUMMER trial and SPRING trial, respectively, slaughtered in Purdue University Meat Lab. During carcass chilling, longissimus dorsi (LD) and psoas major (PM) muscles were sampled at both 1-day and 7-day postmortem, snap frozen and stored at -80 °C.

5.3.2 SDS-PAGE and western blot

Gel samples were prepared in accordance with Kim, Huff-Lonergan, Sebranek, and Lonergan (2010) with minor modifications (Carlson et al., 2017). One gram of muscle sample was homogenized in 10 mL of extraction buffer (10 mM phosphate, 2% w/v sodium dodecyl sulfate (SDS), pH 7.0 at 20 °C). After centrifugation at $1500 \times g$ for 15 minutes, supernatant was diluted to protein concentrate of 6.4 mg/ml, and mixed with 0.5 volume of tracking dye buffer (3 mM EDTA, 3% w/v SDS, 20% v/v glycerol, 0.003% w/v bromophenol blue, and 30 mM Tris-HCl; pH 8.0) and 0.1 volume of 2-mercaptoethanol, making the final protein concentrate 4.0 mg/ml. The mixture was incubated in 50 °C heat block for 20 min and stored at −80 °C.

The following loaded total proteins were applied to each of the targeted proteins: 20 µg for troponin T, 40 µg for desmin, µ-calpain, HSP27, αβ-crystallin, and cytochrome c. Gels were electrophoresed on a Hoefer SE260 unit at a constant voltage of 25 v for approximately 12 hours in running buffer (3.021% Tris, 14.4% glycine, 2% SDS, 0.058% EDTA). Proteins were then transferred to polyvinylidene fluoride membranes and were blocked for1 hour under 25 ℃ using PBS-Tween (PBST) solution (pH 7.0) containing 5% nonfat dry milk. Membranes were probed with primary antibody solutions at 4 °C for 8 hours overnight, which were prepared using PBST containing 3% nonfat dry milk. The following antibodies were used: 1:10,000 mouse monoclonal anti-desmin IgG (Sigma-Aldrich D1022), 1:20,000 anti-troponin T IgG (Sigma-Aldrich T6277), 1:10,000 mouse monoclonal anti-Mu-calpain IgG (ThermoFisher MA3-940), 1:3000 mouse monoclonal anti-HSP27 IgG (Abcam ab79868); 1:5000 rabbit polyclonal anti-αβ-crystallin IgG

(ThermoFisher PA1-16951); and 1:1000 rabbit polyclonal anti-cytochrome c IgG (Abcam ab90529); After washed 3 times for 10 min with PBST solution, membranes were incubated with the following secondary antibody preparations for one hour at room temperature: goat anti-mouse IgG (H + L) horseradish peroxidase conjugate (dilution of 1:20,000 for desmin, troponin T, and calpain 1; dilution of 1:5000 for HSP27; Bio-Rad) or goat anti-rabbit IgG peroxidase conjugated (dilution of 1:5000 for cytochrome c and αβ-crystallin, ThermoFisher Scientific). After three 10-min washes, ECL Western blotting reagents (ThermoFisher Scientific) were applied to visualize protein bands (UVP GelDoc-It). Intensity volume of each band was measured with UVP VisionworksLS Analysis Software (UVP, LLC; Upland, CA, USA) and were compared with an internal reference to normalize data and quantification.

5.3.3 Mitochondria membrane permeability

As mitochondrial morphological change is among the key features representing cellular apoptosis (Wang, Han, Ma, Yu, & Zhao, 2017), mitochondria membrane permeability (MMP) can be used to evaluate the extent of structural integrity loss of mitochondria membranes. Membrane integrity of the isolated mitochondria is indicated by measuring UV absorption peak at 540 nm of the mitochondria suspensions. A higher membrane integrity held more abundant membrane proteins, mainly TCA and ETC enzymes, leading to increased absorption peak. As such, higher numerical measurements indicated more integrated membrane structure, and therefore lower MMP.

Mitochondria isolation was in accordance with Cuillerier et al. (2017) with minor modifications. Muscle samples were minced and transferred to equal volume of pre-chilled isolation buffer (300 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.2). After 20 seconds homogenizing, the homogenate was centrifuged at 1000 ×g for 10 min at 4 °C for two times, supernatant was each collected. The resulting supernatant was then centrifuged at 8000 ×g for 10 min at 4 °C, and the precipitant pellet was kept, and re-suspended in pre-chilled suspension buffer (300 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.2). The suspension was centrifuged at 8000 ×g for 10 min at 4 °C, with the final pellet re-suspended in suspension buffer. The final mitochondria suspension was diluted to a protein concentration of 0.5 mg/mL and incubated for 3

min in water bath at 25 °C. Mitochondrial membrane permeability was determined by measuring absorbance value at 540 nm using the UV spectrophotometer.

5.3.4   Metabolomics sample preparation and extraction.

One-day aged LD and PM samples (1g in weight) were homogenized with 3mL of methanol for 30s. The homogenate was shake for 2 hours and centrifuged at 16000×g. Supernatant were collected and were evaporated to dryness with a vacuum centrifuge. Dried muscle extracts were oximated with 50 µL methoxyamine hydrochloride in pyridine at 30 °C for 90 min, and then added to 50 µL of the derivatizing agent, N-methyl-N-trimethylsilyl-trifluoroacetamide, and incubated at 37 °C for 30 min.

5.3.5   GC-TOF-MS Analysis.

The analysis used an Agilent 7890A GC system equipped with an Agilent 7693 autosampler coupled to a Pegasus TOF-MS detector. The separation used an Agilent HP-5MS capillary column, which has an internal diameter of 0.25 mm, a film thickness of 0.25 µm, and a length of 30 m. The carrier gas is chromatographic-grade helium with a constant flow of 1.0 mL/min. The oven temperature was held at 75 °C for 2 min, increased to 300 °C at a rate of 15 °C/min, then maintained at 300 °C for 3 min. A full scan was run at the electron impact mode set to 70 eV ionization energy. The injector line temperature was 250 °C and the transfer line temperature was 240 °C. The GC-TOF-MS used 1 microliter of reactant. A pooled quality control sample was analyzed at an interval of eight sample analysis.

The mass-spectrometry data files were converted to CDF format, processed using the metAlign software package to obtain a data matrix containing retention times, accurate masses, and then normalized peak intensities, using sample names and peak area information as variables. For multivariate statistical analysis, the resulting data metric was processed with SIMCA. Using SIMCA and PASW Statistics 18, Principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) modeling were performed. In PLS-DA, the discriminated variables were selected based on variable importance in the projection value and checked with *P-*

value from one-way ANOVA. Following multivariate analysis, the peaks corresponding to selected variables were confirmed in the original chromatograms and were positively or tentatively identified using either commercial standard compounds in comparison with the mass spectra and retention time or on the basis of the NIST mass spectral database, in-house library, and references for GC-TOF-MS.

5.3.6   Data analysis

The experimental design of this study was a complete randomized block design, with split plot factors. Seasonal trials were served as block factor, muscle type effect (LD and PM) as the whole plot, and aging time effect (1 and 7 days of aging) as a subplot. Animals were considered as a random effect. Apoptotic and proteolytic attributes were analyzed by the PROC MIXED procedure of SAS 9.4 software (SAS Institute Inc.). Least squares means were separated using the PDIFF option of LSMEANS. Significance level were set as $\alpha = 0.05$ or less.

5.4   Results

5.4.1   Calpain 1 and myofibrillar protein degradation.

Calpain 1 was visualized as three bands (80, 78, and 76 kDa) by western blot analysis (Figure 5.1). Overall, no significant interactions among seasonal trial, muscle or aging effects were observed on calpain 1 autolysis. Regardless of seasonal trial or muscle effects, intact calpain 1 (80 kDa) and partial autolyzed 78 kda subunits were decreased ($P < 0.05$, Table 5.1), while its 76 kDa subunits increased in abundance ($P < 0.005$; Table 5.1). Particularly, seasonal trial did affect protein abundance of all three bands (Table 5.1), where SPRING exhibited increased 76 kDa subunit ($P = 0.001$) and decreased 78 kDa ($P = 0.054$) and 80 kDa subunits ($P = 0.002$). Within both aging time point (1d vs. 7d postmortem), the LD from both SPRING and SUMMER were found to have lower abundance of un-autolysed calpain 1 80kDa bands ($P < 0.01$) and partial autolyzed 78 kDa bands ($P < 0.001$) and higher abundance of fully autolyzed 76 kDa product ($P < 0.0001$) than the PM counterparts of the same seasonal trial (Table 5.1).

The abundance of intact and degradation products of two myofibrillar proteins, desmin and troponin T, were analyzed (Figure 5.1 and Table 5.1). No 3-way interactions were found. Intact product of desmin was significantly affected by seasonal trials and aging time, but not muscle type. Intact desmin was decreased over aging (P < 0.001), which was found in overall greater abundance in SUMMER weaned pigs compared with SPRING trials (*P*=0.002; Table 5.1), regardless of aging time or muscle type. Conversely, muscle type effect was observed in desmin degradation, where LD muscles showed less desmin degradation products compared to the PM muscles (*P*=0.046). Intact product of troponin T were decreased over aging (P < 0.01). While troponin T intact product was not responsive to any of the treatment factors, degradation products, which were quantified at 35 kDa and 30 kDa, were decreased with aging (P < 0.05, Table 5.1). The 35 kDa bands were affected by trial × aging interaction: both seasonal trials showed similar levels at 1d postmortem aging, however, SPRING showed further extended degradation at 7d postmortem compared to SUMME samples (Figure 5.2, P < 0.05). Moreover, a muscle × aging interaction tended to affect troponin T degradation product at 30 kDa, where LD showed markedly higher troponin T degradation product than PM at 7d aging (Figure 5.2, P < 0.05).

5.4.2   Mitochondria membrane permeability (MMP) and cytochrome c.

Significant seasonal trial and muscle type effects on MMP were found (*P* < 0.0001, Table 5.1). SPRING muscles showed increased MMP compared to SUMMER trial, suggested higher mitochondrial membrane integrity in SUMMER (*P* < 0.001). In terms of muscle effect, LD had lower MMP than PM, meaning more integrated mitochondrial membrane compared to PM counterpart (*P* < 0.001).

Western blots were performed to evaluate cytochrome c abundance in LD and PM samples from different production trial and aging time. PM samples had higher cytochrome c abundance compared to LD (P < 0.0001, Table 5.1). However, no significant difference in cytochrome c abundance was found between production trials (Table 5.1).

5.4.3   Small heat shock proteins.

Western blots were performed to evaluate HSP27 and αβ-crystallin, no degradation products were found in the blots (Table 5.1, Figure 5.1). Neither aging time nor seasonal trial influenced αβ-crystallin abundance (Table 5.1). However, abundance of αβ-crystallin was higher in PM ($P = 0.029$, Table 5.1). HSP27 abundance, on the other hand, was affected by trial × muscle and muscle by aging interaction (Figure 5.3): LD had decreased HSP27 in SUMMER group, but PM did not change over the two replications; also, HSP27 decrease over aging in both LD and PM, with PM had further degradation compared to LD. Even though PM had further degradation, the overall abundance was higher in PM, regardless of aging time, which was corroborated with αβ-crystallin.

5.4.4   Metabolomics analysis.

Metabolomics profiling of LD and PM samples from both seasonal trials were obtained using GC-TOF-MS/MS platform. The PCA score plots indicated that all experimental groups were notably different from each other, as it showed separation of LD vs. PM muscles from SUMMER vs. SPRING groups (Figure 5.4). The samples primarily separated according to muscle type along the first principal component (PC1), which explained 25.9% of the total variance of the model. In addition, PC2 could explain 12.3 % of the total variance of the data set, which separated SUMMER and SPRING muscle samples (Figure 5.4).

While the PLS-DA score plot showed a pattern similar to PCA score plots, this model was used to identify metabolites that differentially presented between muscle and/or season groups (Figure 5.4). Overall, the first and second PLS component (PLS1 and PLS2) accounted for 25.6% and 12.3% of the possible variance, respectively (Figure 5.4). For the effect of muscle, 64 metabolites were differentially presented in LD vs. PM (VIP1 > 1, from PLS1); for the effect of trial, 84 metabolites were differentially presented SUMMER vs. SPRING (VIP2 > 1 from PLS2). For all the differentially presented metabolites, major groups can be categorized into carbohydrates, organic acids, free amino acids, fatty acids/lipids, and nucleotide–related metabolites (Table 5.2). In SPRING, higher abundance of macronutrients were found, including most carbohydrate species, lipids, and branched chain amino acids (BCAA). On the other hand, SUMMER muscles showed

elevated antioxidant compounds，such as assortments of redox regulating amino acids and ascorbic acids (Table 5.2). Further pathway analysis suggested that SPRING muscles went through further extent of cellular catabolism, including urea cycle and ATP degradation (Figure 5.5). These observations corroborated with discrepancies between trials in proteolytic and apoptotic features, with detailed biological interpretations elaborated in the following section.

5.5   Discussion

As the preceding chapter is conducted to determine the impacts of muscle type and trial effects on animal growth and productivity, carcass characteristics and pork quality attributes of the same pigs used in the current chapter. The previous chapter confirmed the trial discrepancies in animal growth and productivity and meat quality attributes: SUMMER pigs exhibited inferior productivity shown as decreased hot carcass weight and body fat. Moreover, LD and PM chops in SUMMER had higher Warner-Brazler shear force, thaw-purge loss, and cook loss. it should be noted that in the present study, seasonal climate change should not be considered as a major attribution to quality variations, because replications within each of the seasons was lacked. Rather, the SUMMER vs. SPRING animals were served as two equal repetitions in which relationships between apoptosis, proteolysis, and metabolomics in postmortem porcine muscles were investigated.

Although only a few research evidences were established in post-mortem skeletal muscles, the idea that apoptosis and proteolysis are coupling activities were well supported in various tissues in different model organisms. Calpain 1, the only protease that retains determinant proteolytic activity during postmortem aging, were among the universal key modulators of apoptosis process (Smith & Schnellmann, 2012). While calpain 1 autolysis was essential for the enzymatic activation of the critical apoptotic effector caspase-3 (Altznauer, Conus, Cavalli, Folkers, & Simon, 2004), activated caspase-3 cleaves calpastatin, the allosteric inhibitor of calpain 1, leading to further release of calpain 1 enzymatic activity (Wang et al., 1998). Moreover, calpain 1 induces endoplasmic reticulum stress, which facilitates cardiac muscle apoptosis under hypoxia conditions (Zheng, Wang, Li, Fan, & Peng, 2015). Although direct experimental evidence in postmortem skeletal muscles were yet insufficient (Kemp & Parr, 2012), degree of calpain 1 autolysis may to

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certain extent, depending on the on-set of apoptotic process. In this regard, we postulate that antemortal apoptosis may pose directly impact on muscle structural protein degradation during postmortem aging, through modulating enzyme activity of calpain 1.

In the current study, SPRING weaned pigs showed higher level of mitochondria membrane permeability, indicating a less integrated membrane system. While higher MMP in SPRING may suggest more extent of apoptosis, SPRING pigs also exhibited further calpain 1 autolysis, which was corresponded with increased troponin T and desmin degradation through-out the aging period. Overall, this indicated that extended proteolysis in SPRING muscles was concurrent with further apoptosis, which suggested a promoting role of apoptosis on downstream proteolysis events. In contrast, biochemical characteristics in SUMMER supported concurrent decrease in extent of proteolysis and apoptosis features. In this regard, current observations further supported a possible role of apoptosis facilitating postmortem proteolysis, subsequently resulting in improved meat quality.

Recently, Cramer et al. found up-regulation of anti-apoptotic HSP27 coincided with less cytochrome c and more inactivated pro-caspase 3 in m. longissimus muscle of callipyge lamb, a well-established animal model inherited with inferior proteolytic potential, refreshing the insights of apoptotic related proteolysis and meat tenderization with an emphasized role of small heat shock proteins (Cramer, Penick, Waddell, Bidwell, & Kim, 2018). The small heat shock proteins HSP27 and αβ-crystallin were well known anti-stress molecules retaining anti-apoptotic activities (Acunzo, Katsogiannou, & Rocchi, 2012), which were recently suggested as good indicators of the extent of proteolysis (Balan, Kim, & Blijenburg, 2014). In the current study, even though decrease of HSP27 during postmortem aging was further extended in PM compare to LD counterparts, PM muscles exhibited higher HSP27 and αβ-crystallin abundance regardless of aging treatment, corroborating with lower tenderization potential. From a stress-defending perspective, PM muscles as a red oxidative, slow twitch muscle, experienced higher level of oxidative stress (Ke et al., 2017). This may help to explain why higher sHSPs was observed in PM. In the current study, the seasonal impact on HSP27 was muscle type-specific: SPRING samples, which exhibited further degree of proteolysis and improved shear force, showed elevated HSP27 abundance in LD muscles, however, such change was not observed in PM counterpart.

Moreover, metabolomics analysis suggested that higher oxidative stress was concurrent with further extent of cell death in SPRING, whereas elevated stress defending response was found in SUMMER, adding power to anti-apoptotic activities. The GC-TOF-MS metabolomics showed that in general, SPRING had more abundant macronutrients including simple sugar and a variety of carbohydrate species, branched-chain amino acids, and 14-18 carbon saturated free fatty acids, whereas SUMMER was characterized with higher level of antioxidant or anti-stress molecules.

Fourteen lipids were found to be differentially presented between production seasons, including 6 free fatty acids, 3 glycolipids, 1 glycerophospholipids, and 1sterol lipids (Table 5.2). Particularly, except for oleamide, which was more abundant in SUMMER, all the differentially presented lipid compounds were elevated in SPRING muscles. Exposure of skeletal muscles to lipid surplus was recognized as a trigger of metabolic dysfunction (Koves et al., 2008). It was also suggested that saturated free fatty acids are pro-apoptotic compounds to the cells, primarily due to generation of intermediates with mitochondrial toxicity (Kusminski, Shetty, Orci, Unger, & Scherer, 2009; Unger & Orci, 2002). In the current study, 14:0, 16:0, 18:0 and 18:1 were identified with higher abundance in SPRING. Metabolism of excessive fatty acids in skeletal muscle involve in generation of signaling molecules such as diacylglycerol (DAG) and ceramide, leading to insulin resistance and hence promoting oxidative damage of the tissue and trigger apoptosis (Koves et al., 2008; Morino, Petersen, & Shulman, 2006; Turpin, Lancaster, Darby, Febbraio, & Watt, 2006). Therefore, we postulate that elevated lipid content may associated with early or extended on-set of apoptosis, resulting in improved proteolysis potential in SPRING weaned pigs.

Except for lipids, carbohydrates are essential targets in energy metabolism. The current study identified 31 carbohydrates, 8 of which were assigned to positive annotations, including fructose, glucose, lactose, maltose, gluconic acid, glyceric acid, glycerol, and myo-inositol. Cell respiration (glycolysis and TCA cycle) intermediates, including pyruvic acid, fumaric acid, malic acid, and succinic acid, were also identified as differentially presented metabolites in between seasons. Additionally, In the perspective of nitrogen metabolism, 19 associate metabolites have been found differentially presented in between production seasons, including 13 alpha amino acids. Since muscle is the largest reservoir of proteins, it plays key role in amino acid metabolism and protein turn-over. As such, a significant change in amino acids profile indicated shifted energy metabolism, protein homeostasis, and signaling transduction between the two seasonal trials.

Urea cycle are closely linked to TCA cycle; the primary modulating hinge includes glutamate (Katunuma, Okada, & Nishii, 1966). As urea cycle is responsible for ammonia detoxification and nitrogen excretion, elevated urea in SPRING could be an indicator of further amino acids catabolism, while elevated ornithine and Asp may suggest a more conserved nitrogen utilization in SUMMER pigs, allowing for more amino groups stayed in protein/ free amino acids pool rather than expelled out of the innate environment system (Figure 5.4). With increased amino groups disposed, elevated carbon skeleton should be processed via TCA cycle (Katunuma et al., 1966). For example, during catabolism of glutamate family molecules, Gln, Arg, His, Pro, and ornithine are first converted into Glu. Deamination of Glu results in α-ketoglutarate production, which could enter TCA cycle as a key intermediate, favoring respiratory flux and/or gluconeogenesis (Katunuma et al., 1966). Meanwhile, potentially enhanced gluconeogenesis in SPRING, which was suggested by increases in glucose, fructose, and lactose, could in turn favor glycolysis and TCA flux. Under various circumstance, the induction of TCA flux generates oxidative stress and inflammatory response (Fernie, Carrari, & Sweetlove, 2004; James, Collins, Logan, & Murphy, 2012). Considering the aforementioned increases of 6-carbon sugars and TCA intermediates in SPRING, these observations together suggested elevated cellular oxidative stress in SPRING porcine muscles, which could be favoring apoptosis settlement.

In the perspective of structural amino acid profile, Ile, Leu, Val, Met, Phe, Glu, Ser, and 5-oxoproline were more abundant in SPRING, whereas His, Asp, Gly, Pro, Thr, and creatinine, were elevated in SUMMER. It is established that Ile, Leu, and Val, also known as branched-chain amino acid (BCAA), are energy providing amino acids as well as major protein-turnover modulators in skeletal muscles (Yoshizawa, 2004). Energy-generating catabolism of BCAA produce reactive oxygen species, free radical molecules that cause oxidative stress, facilitating the onset of cell death process (Bridi et al., 2003; Holeček, 2018). Moreover, Met, Phe and 5-oxoproline are also among the amino acids related to potential cellular oxidative stress under both health and pathogenic conditions (Fernandes et al., 2010). Particularly, Met residuals in proteins are susceptible to oxidative attack. Met restriction prevents unwanted shift of amino acids metabolism, protecting DNA and cell structure integrity (Martínez et al., 2017). On the other hands, amino acids that are related to redox regulation and antioxidant activity, including His (Wade & Tucker, 1998), Gly (Chen et al., 2018), Thr (Min, Liu, Qu, Meng, & Gao, 2017), Asp (Duan et al., 2016;

Pi et al., 2014), Ala (Grosser et al., 2004), and Pro, were more abundant in SUMMER. Pharmaceutical dose or dietary supplementation of these amino acids could alleviate cellular stress under catabolic conditions. Therefore it is reasonable to speculate that elevated abundance of anti-stress amino acids could add power to anti-apoptotic activity during muscle to meat conversion.

Changes of purine/pyrimidine metabolites, consistently, showed that SPRING muscles underwent further degradation of cell structure integrity, in a perspective of adenosine triphosphate (ATP) breakdown. It was generally acknowledged that ATP can be catabolized into series of intermediates, including ADP/AMP, inosine mono-phosphate (IMP), inosine, and eventually hypoxanthine and uric acid (Grum, Simon, Dantzker, & Fox, 1985). In the current results, SUMMER muscles showed higher abundance of AMP, UMP, inosine, guanosine, and 5'-Methylthioadenosine, which were located in the upstream of ATP catabolism pathway, whereas hypoxanthine and 5-hydroxymethyluracil were more elevated in SPRING, which belong to further degradation product of ATP catabolism. It was well established that oxidative stress favors ATP catabolism, consequentially increased hypoxanthine (Buonocore et al., 2002). As such, elevated hypoxanthine level in SPRING could be an indicator of further degradation of ATP, consistent with extended tendency of apoptosis and proteolysis activities.

In conclusion, changes in global metabolomics profiling between production trials supported a postulation that SPRING muscles retained higher level of oxidative stress-associated macronutrients, including 6 carbon sugars, 16-18 carbon fatty acids, and BCAA, therefore possibly making muscle cells more prone to antemortal apoptotic process. On the other hand, SUMMER muscle samples showed increases in stress defending compounds—His, Asp, Gly, Pro, Thr, ascorbic acid, inosine, and guanosine—which may retain anti-apoptotic activities during muscle to meat conversion, impeding the programmed cell death, subsequently render adverse impact on proteolytic potential of postmortem muscles.

5.6   Conclusion

In conclusion, changes in global metabolomics profiling between production trials supported a postulation that SPRING muscles retained higher level of oxidative stress-associated macronutrients, including 6 carbon sugars, 16-18 carbon fatty acids, and BCAA, therefore possibly

making muscle cells more prone to antemortal apoptotic process. On the other hand, SUMMER muscle samples showed increases in stress-defending compounds—His, Asp, Gly, Pro, Thr, ascorbic acid, inosine, and guanosine—which may retain anti-apoptotic activities during muscle to meat conversion, impeding the programmed cell death, subsequently render adverse impact on proteolytic potential of postmortem muscles.

5.7   Literatures cited

Acunzo, J., Katsogiannou, M., & Rocchi, P. (2012). Small heat shock proteins HSP27 (HspB1), αB-crystallin (HspB5) and HSP22 (HspB8) as regulators of cell death. *The international journal of biochemistry & cell biology, 44* (10), 1622-1631.

Altznauer, F., Conus, S., Cavalli, A., Folkers, G., & Simon, H.-U. (2004). Calpain-1 regulates Bax and subsequent Smac-dependent caspase-3 activation in neutrophil apoptosis. *Journal of Biological Chemistry, 279* (7), 5947-5957.

Balan, P., Kim, Y. H. B., & Blijenburg, R. (2014). Small heat shock protein degradation could be an indicator of the extent of myofibrillar protein degradation. *Meat Science, 97* (2), 220-222.

Bridi, R., Araldi, J. n., Sgarbi, M. B., Testa, C. G., Durigon, K., Wajner, M., & Dutra-Filho, C. S. (2003). Induction of oxidative stress in rat brain by the metabolites accumulating in maple syrup urine disease. *International Journal of Developmental Neuroscience, 21* (6), 327-332.

Buonocore, G., Perrone, S., Longini, M., Vezzosi, P., Marzocchi, B., Paffetti, P., & Bracci, R. (2002). Oxidative stress in preterm neonates at birth and on the seventh day of life. *Pediatric Research, 52* (1), 46.

Cao, J., Sun, W., Zhou, G., Xu, X., Peng, Z., & Hu, Z. (2010). Morphological and biochemical assessment of apoptosis in different skeletal muscles of bulls during conditioning. *Journal of animal science, 88* (10), 3439-3444.

Carlson, K., Prusa, K., Fedler, C., Steadham, E., Outhouse, A., King, D.,... Lonergan, S. (2017). Postmortem protein degradation is a key contributor to fresh pork loin tenderness. *Journal of animal science, 95* (4), 1574-1586.

Chen, L., Zhang, J., Li, C., Wang, Z., Li, J., Zhao, D.,... Guo, X. (2018). Glycine Transporter-1 and glycine receptor mediate the antioxidant effect of glycine in diabetic rat islets and INS-1 cells. *Free Radical Biology and Medicine, 123*, 53-61.

Chen, X., Zhang, L., Li, J., Gao, F., & Zhou, G. (2017). Hydrogen peroxide-induced change in meat quality of the breast muscle of broilers is mediated by ROS generation, apoptosis, and autophagy in the NF-κB signal pathway. *Journal of agricultural and food chemistry, 65* (19), 3986-3994.

Cramer, T., Penick, M. L., Waddell, J. N., Bidwell, C. A., & Kim, Y. H. B. (2018). A new insight into meat toughness of callipyge lamb loins-The relevance of anti-apoptotic systems to decreased proteolysis. *Meat science, 140*, 66-71.

Cuillerier, A., Honarmand, S., Cadete, V. J., Ruiz, M., Forest, A., Deschênes, S.,... Rioux, J. D. (2017). Loss of hepatic LRPPRC alters mitochondrial bioenergetics, regulation of permeability transition and trans-membrane ROS diffusion. *Human molecular genetics, 26* (16), 3186-3201.

Duan, J., Yin, J., Ren, W., Liu, T., Cui, Z., Huang, X.,... Wu, X. (2016). Dietary supplementation with L-glutamate and L-aspartate alleviates oxidative stress in weaned piglets challenged with hydrogen peroxide. *Amino Acids, 48* (1), 53-64.

Duttlinger, A. W., Kpodo, K. R., Lay Jr, D. C., Richert, B. T., & Johnson, J. S. (2019). Replacing dietary antibiotics with 0.20% l-glutamine in swine nursery diets: impact on health and productivity of pigs following weaning and transport. *Journal of animal science, 97* (5), 2035-2052.

Fernandes, C. G., Leipnitz, G., Seminotti, B., Amaral, A. U., Zanatta, Â., Vargas, C. R.,... Wajner, M. (2010). Experimental evidence that phenylalanine provokes oxidative stress in hippocampus and cerebral cortex of developing rats. *Cellular and molecular neurobiology, 30* (2), 317-326.

Fernie, A. R., Carrari, F., & Sweetlove, L. J. (2004). Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Current opinion in plant biology, 7* (3), 254-261.

Grosser, N., Oberle, S., Berndt, G., Erdmann, K., Hemmerle, A., & Schröder, H. (2004). Antioxidant action of L-alanine: heme oxygenase-1 and ferritin as possible mediators. *Biochemical and biophysical research communications, 314* (2), 351-355.

Grum, C. M., Simon, R. H., Dantzker, D. R., & Fox, I. H. (1985). Evidence for adenosine triphosphate degradation in critically-ill patients. *Chest, 88* (5), 763-767.

Guo, B., Zhang, W., Tume, R. K., Hudson, N. J., Huang, F., Yin, Y., & Zhou, G. (2016). Disorder of endoplasmic reticulum calcium channel components is associated with the increased apoptotic potential in pale, soft, exudative pork. *Meat science, 115*, 34-40.

Holeček, M. (2018). Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. *Nutrition & metabolism, 15* (1), 33.

Huang, F., Huang, M., Zhang, H., Zhang, C., Zhang, D., & Zhou, G. (2016). Changes in apoptotic factors and caspase activation pathways during the postmortem aging of beef muscle. *Food chemistry, 190*, 110-114.

James, A. M., Collins, Y., Logan, A., & Murphy, M. P. (2012). Mitochondrial oxidative stress and the metabolic syndrome. *Trends in endocrinology & metabolism, 23* (9), 429-434.

Katunuma, N., Okada, M., & Nishii, Y. (1966). Regulation of the urea cycle and TCA cycle by ammonia. *Advances in enzyme regulation, 4*, 317-335.

Ke, Y., Mitacek, R. M., Abraham, A., Mafi, G. G., VanOverbeke, D. L., DeSilva, U., & Ramanathan, R. (2017). Effects of Muscle-Specific Oxidative Stress on Cytochrome c Release and Oxidation–Reduction Potential Properties. *Journal of agricultural and food chemistry, 65* (35), 7749-7755.

Kemp, C. M., & Parr, T. (2012). Advances in apoptotic mediated proteolysis in meat tenderisation. *Meat Science, 92* (3), 252-259.

Kemp, C. M., Sensky, P. L., Bardsley, R. G., Buttery, P. J., & Parr, T. (2010). Tenderness – An enzymatic view. *Meat Science, 84* (2), 248-256. doi:

Kim, Y. H. B., Ma, D., Setyabrata, D., Farouk, M. M., Lonergan, S. M., Huff-Lonergan, E., & Hunt, M. C. (2018). Understanding postmortem biochemical processes and post-harvest aging factors to develop novel smart-aging strategies. *Meat Science, 144*, 74-90. doi:

Kim, Y. H. B., Warner, R. D., & Rosenvold, K. (2014). Influence of high pre-rigor temperature and fast pH fall on muscle proteins and meat quality: a review. *Animal Production Science, 54* (4), 375-395.

Koves, T. R., Ussher, J. R., Noland, R. C., Slentz, D., Mosedale, M., Ilkayeva, O.,... Newgard, C. B. (2008). Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell metabolism, 7* (1), 45-56.

Kristensen, L., & Purslow, P. P. (2001). The effect of ageing on the water-holding capacity of pork: role of cytoskeletal proteins. *Meat science, 58* (1), 17-23.

Kusminski, C. M., Shetty, S., Orci, L., Unger, R. H., & Scherer, P. E. (2009). Diabetes and apoptosis: lipotoxicity. *Apoptosis, 14* (12), 1484-1495.

Lee, S., Joo, S., & Ryu, Y. (2010). Skeletal muscle fiber type and myofibrillar proteins in relation to meat quality. *Meat science, 86* (1), 166-170.

Lonergan, E. H., Zhang, W., & Lonergan, S. M. (2010). Biochemistry of postmortem muscle— Lessons on mechanisms of meat tenderization. *Meat science, 86* (1), 184-195.

Ma, D., & Kim, Y. H. B. (2020). Proteolytic changes of myofibrillar and small heat shock proteins in different bovine muscles during aging: Their relevance to tenderness and water-holding capacity. *Meat Science*, 108090.

Ma, D., Kim, Y. H. B., Cooper, B., Oh, J.-H., Chun, H., Choe, J.-H.,... Min, B. (2017). Metabolomics profiling to determine the effect of postmortem aging on color and lipid oxidative stabilities of different bovine muscles. *Journal of Agricultural and Food Chemistry, 65* (31), 6708-6716.

Ma, D., Kim, Y. H. B., Johnson, J. S., Duttlinger, A., & Guedes, J. M. (2019). Effect of L-Glutamine Supplementation in Replacement of Antibiotics on Meat Quality Attributes of Pigs Exposed To Transport and Weaning Stress during Different Seasons. *Meat and Muscle Biology*, *2* (2), 89-89.

Martínez, Y., Li, X., Liu, G., Bin, P., Yan, W., Más, D.,... Yin, Y. (2017). The role of methionine on metabolism, oxidative stress, and diseases. *Amino Acids, 49* (12), 2091-2098.

Min, Y., Liu, S., Qu, Z., Meng, G., & Gao, Y. (2017). Effects of dietary threonine levels on growth performance, serum biochemical indexes, antioxidant capacities, and gut morphology in broiler chickens. *Poultry science, 96* (5), 1290-1297.

Morino, K., Petersen, K. F., & Shulman, G. I. (2006). Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction: Am Diabetes Assoc.

Ouali, A., Gagaoua, M., Boudida, Y., Becila, S., Boudjellal, A., Herrera-Mendez, C. H., & Sentandreu, M. A. (2013). Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved. *Meat Science, 95* (4), 854-870. doi: https://doi.org/10.1016/j.meatsci.2013.05.010

Ouali, A., Herrera-Mendez, C. H., Coulis, G., Becila, S., Boudjellal, A., Aubry, L., & Sentandreu, M. A. (2006). Revisiting the conversion of muscle into meat and the underlying mechanisms. *Meat science, 74* (1), 44-58.

Pi, D., Liu, Y., Shi, H., Li, S., Odle, J., Lin, X.,... Leng, W. (2014). Dietary supplementation of aspartate enhances intestinal integrity and energy status in weanling piglets after lipopolysaccharide challenge. *The Journal of nutritional biochemistry, 25* (4), 456-462.

Smith, M. A., & Schnellmann, R. G. (2012). Calpains, mitochondria, and apoptosis. *Cardiovascular research, 96* (1), 32-37.

Spanier, A., Flores, M., McMillin, K., & Bidner, T. (1997). The effect of post-mortem aging on meat flavor quality in Brangus beef. Correlation of treatments, sensory, instrumental and chemical descriptors. *Food Chemistry, 59* (4), 531-538.

Subbaraj, A. K., Kim, Y. H. B., Fraser, K., & Farouk, M. M. (2016). A hydrophilic interaction liquid chromatography–mass spectrometry (HILIC–MS) based metabolomics study on colour stability of ovine meat. *Meat science, 117*, 163-172.

Turpin, S. M., Lancaster, G. I., Darby, I., Febbraio, M. A., & Watt, M. J. (2006). Apoptosis in skeletal muscle myotubes is induced by ceramides and is positively related to insulin resistance. *American Journal of Physiology-Endocrinology and Metabolism, 291* (6), E1341-E1350.

Unger, R. H., & Orci, L. (2002). Lipoapoptosis: its mechanism and its diseases. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1585* (2-3), 202-212.

Wade, A. M., & Tucker, H. N. (1998). Antioxidant characteristics of L-histidine. *The Journal of Nutritional Biochemistry, 9* (6), 308-315.

Wang, K. K., Posmantur, R., Nadimpalli, R., Nath, R., Mohan, P., Nixon, R. A.,... Allen, H. (1998). Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. *Archives of biochemistry and biophysics, 356* (2), 187-196.

Wang, L.-L., Han, L., Ma, X.-L., Yu, Q.-L., & Zhao, S.-N. (2017). Effect of mitochondrial apoptotic activation through the mitochondrial membrane permeability transition pore on yak meat tenderness during postmortem aging. *Food chemistry, 234*, 323-331.

Wang, L.-L., Yu, Q.-L., Han, L., Ma, X.-L., Song, R.-D., Zhao, S.-N., & Zhang, W.-H. (2018). Study on the effect of reactive oxygen species-mediated oxidative stress on the activation of mitochondrial apoptosis and the tenderness of yak meat. *Food chemistry, 244*, 394-402.

Yoshizawa, F. (2004). Regulation of protein synthesis by branched-chain amino acids in vivo. *Biochemical and biophysical research communications, 313* (2), 417-422.

Zheng, D., Wang, G., Li, S., Fan, G.-C., & Peng, T. (2015). Calpain-1 induces endoplasmic reticulum stress in promoting cardiomyocyte apoptosis following hypoxia/reoxygenation. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1852* (5), 882-892.

Table 5.1 Effect of different production season and muscle type on proteolysis and apoptosis features of porcine skeletal muscles over 7-day postmortem aging

Parameters[1,2,3]	Trial[4]		Muscle[5]		Aging		S.E.M[6]	P-value		
	SPRING	SUMMER	LD	PM	1 d	7 d		T	M	A
MMP	0.082	0.092	0.092	0.081	0.094	0.079	0.002	<.001	<.001	<.0001
Cytochrom c	1.76	1.91	0.79	2.88	1.99	1.68	0.22	0.20	<.0001	0.003
HSP27	0.90	0.78	0.61	1.07	0.98	0.70	0.08	0.018	<.0001	<.0001
Aβ-crystallin	1.75	1.42	1.39	1.78	1.74	1.43	0.30	0.071	0.029	0.092
Calpain 1 80kDa	0.16	0.20	0.16	0.20	0.19	0.17	0.02	0.002	0.004	0.025
Calpain 1 78kDa	0.38	0.41	0.35	0.44	0.41	0.38	0.02	0.054	<.0001	0.050
Calpain 1 76kDa	0.46	0.39	0.49	0.36	0.40	0.45	0.03	0.001	<.0001	0.005
Troponin T intact	1.72	1.73	2.14	1.32	2.71	0.75	0.48	0.98	0.071	0.002
Troponin T Degradation 35kDa	1.04	0.68	0.95	0.78	0.44	1.29	0.19	0.038	0.17	<.001
Troponin T Degradation 30kDa	0.92	0.43	1.05	0.30	0.32	1.03	0.40	0.10	0.039	0.037
Desmin intact	1.21	1.59	1.22	1.58	1.58	1.22	0.17	0.002	0.094	<.001
Desmin degradation 39 kDa	1.02	1.21	0.99	1.24	1.08	1.15	0.12	0.085	0.046	0.22

[1]MMP (mitochondrial membrane permeability) was obtained by measuring UV absorption peak at 540 nm of the isolated mitochondria suspensions, higher number indicates lower MMP

[2]Protein abundance except calpain 1 was expressed as relative ratio of band intensity compared to the corresponding bands of the reference samples

[3]Three bands of 80, 78, and 76 kDa of calpain 1 were quantified and expressed as the ratio of each band that relative to the total intensity

[4]Trial: SUMMER, pigs weaned in July 2016 and harvested in Janurary 2017; SPRING: pigs weaned in April 2017 and harvested in September 2017

[5]Muscle: LD, *longissimus dorsi*; PM, *psoas major*

[6]Pooled standard errors of means

Table 5.2 Differentially presented metabolites in LD vs PM with two production seasons.

Peak Nr	RT	Mass	VIP[1] (muscle)	VIP[2] (season)	Metabolites	higher in which muscle	higher in which season
Amino acids							
15307	9.54	156	1.92	1.47	5-Oxoproline	PM	Spring
17577	8.68	174	0.84	1.68	Alanine	LD	Summer
22436	9.08	218	1.31	1.05	Aminomalonic acid	LD	Summer
23873	9.1	232	1.32	1.13	Aspartic acid	PM	Summer
29928	9.83	329	0.07	1.07	Creatinine		Summer
17586	9.56	174	0.25	1.22	GABA		Spring
24982	10.26	246	1.62	1.45	Glutamic acid	PM	Spring
17567	7.61	174	1.57	1.41	Glycine	PM	Summer
15821	15.97	159	0.6	1.09	Histidine		Summer
15580	7.48	158	1.28	1.08	Isoleucine	PM	Spring
19100	6.06	188	0.23	1.09	Leucine		Spring
17847	9.48	176	1.48	1.33	Methionine	PM	Spring
17607	11.75	174	0.42	1.04	Ornithine		Summer
22450	10.37	218	1.4	1.13	Phenylalanine	PM	Spring
22181	7.53	216	1.25	0.94	Proline	PM	Summer
20838	8.09	204	1.67	1.25	Serine	PM	Spring
22581	8.34	219	1.72	1.23	Threonine	PM	Summer
22416	6.7	218	1.45	1.14	Valine	PM	Spring
Carbonhydrates							
28872	12.28	307	1.99	1.43	Fructose	LD	Spring
8137	12.21	103	2	1.43	Fructose	LD	Spring
30112	13.1	333	1.4	1.19	Gluconic acid	LD	Spring
15927	12.41	160	1.83	1.36	Glucose	LD	Spring
15928	12.53	160	1.85	1.39	Glucose	LD	Spring
15926	12.31	160	1.96	1.43	Glucose	LD	Spring
19223	7.82	189	0.2	1.01	Glyceric acid		Spring
20990	7.3	205	1.34	1.05	Glycerol	PM	Summer

Table 5.2 continued

Peak Nr	RT	Mass	VIP[1] (muscle)	VIP[2] (season)	Metabolites	higher in which muscle	higher in which season
Carbohydrates, continued							
20940	17.1	204	1.31	1.03	Lactose	LD	Spring
20938	16.91	204	1.32	1.03	Lactose	LD	Spring
31193	17.41	361	1.38	1.08	Maltose	LD	Spring
31191	17.27	361	1.44	1.1	Maltose	LD	Spring
19582	13.66	191	1.97	1.58	Myo-inositol	PM	Spring
24985	11	246	0.07	1.48	Carbohydrate 1		Spring
20891	12.9	204	1.64	1.18	Carbohydrate 10	LD	Spring
29484	13.25	318	2.01	1.51	Carbohydrate 11	PM	Spring
29289	14.62	315	1.82	1.35	Carbohydrate 12	LD	Summer
20913	14.75	204	1.65	1.26	Carbohydrate 13	LD	Summer
29293	14.97	315	2	1.44	Carbohydrate 15	LD	Spring
31904	14.98	387	2.01	1.44	Carbohydrate 16	LD	Spring
15954	15.05	160	1.98	1.42	Carbohydrate 17	LD	Spring
31906	15.15	387	1.97	1.41	Carbohydrate 18	LD	Spring
20918	15.22	204	1.63	1.27	Carbohydrate 19	LD	Summer
20878	11.74	204	1.63	1.25	Carbohydrate 2	LD	Spring
31908	15.34	387	1.85	1.34	Carbohydrate 21	LD	Spring
22317	11.77	217	1.73	1.24	Carbohydrate 3	LD	Summer
20879	11.85	204	1.76	1.32	Carbohydrate 4	LD	Spring
22319	11.98	217	1.16	1.1	Carbohydrate 5	LD	Spring
20886	12.34	204	1.68	1.21	Carbohydrate 7	LD	Spring
29546	12.66	319	1.28	1.25	Carbohydrate 8	PM	Spring
22330	12.9	217	1.47	1.09	Carbohydrate 9	LD	Spring

Table 5.2 continued

Peak Nr	RT	Mass	VIP[1] (muscle)	VIP[2] (season)	Metabolites	higher in which muscle	higher in which season
Lipids							
21088	15.7	205	0.65	1.12	1-O-hexadecylglycerol		Spring
30958	11.39	357	0.65	1.01	alpha-Glycerophosphoric acid		Spring
10212	15.12	117	1.49	1.2	Arachidonic acid		Spring
24702	11.13	243	0.98	1.83	beta-Glycerophosphoric acid		Spring
32192	17.2	399	0.38	1.18	Glycerol monostearate		?
22516	16.07	218	0.66	1.2	Glyceryl 2-palmitate		Spring
22527	17.02	218	0.16	1.1	Glyceryl 2-stearate		?
30253	14.2	337	0.42	1.89	Linoleic acid		Spring
30479	15.24	343	1.03	0.82	Myristic acid	?	?
11546	15.34	128	0.51	1.15	Oleamide		Summer
20315	14.22	199	1.07	1.98	Oleic acid		Spring
10190	13.17	117	0.54	1.99	Palmitic acid		Spring
28308	11.54	299	1.52	1.12	Phosphoethanolamine		Spring
30364	14.35	341	0.85	1.14	Stearic acid		Spring
Purine/pyrimidine							
16581	6.54	166	1.41	1.07	3-Hydroxy-6-methylpyridine	LD	
24540	7.9	241	1.55	1.55	5-Hydroxymethyluracil	PM	Spring
24222	17.42	236	0.86	1.6	5'-Methylthioadenosine		Summer
16985	19.13	169	1.19	1.44	Adenosine 5'-monophosphate	PM	Summer
29824	17.3	324	1.66	1.24	Guanosine	PM	Summer
26275	11.74	265	1.72	1.37	Hypoxanthine	PM	Spring
23715	16.32	230	1.44	1.08	Inosine	PM	Summer
16983	18.65	169	1.27	1.27	Uridine 5′-monophosphate	PM	Summer

Table 5.2 continued

| Peak Nr	RT	Mass	VIP[1] (muscle)	VIP[2] (season)	Metabolites	higher in which muscle	higher in which season
Organic acids							
10112	6.11	117	1.3	1.07	2-Hydroxybutyric acid	PM	Summer
25068	9.68	247	1.67	1.49	2-Hydroxyglutaric acid	PM	Spring
11934	6.62	131	0.99	1.06	3-Hydroxy-3-methylbutyric acid	PM	Spring
23952	6.86	233	1.65	1.33	4-Hydroxybutyric acid	PM	Spring
24885	7.91	245	1.55	1.37	Fumaric acid	PM	Spring
31167	12.76	361	1.08	0.99	Galactonic acid 1,4-lactone	LD	Summer
17917	5.23	177	1.43	1.05	Glycolic acid	PM	
22434	8.88	218	0.86	1.52	Homoserine		Summer
23968	9.21	233	1.69	1.65	Malic acid	PM	Spring
22256	5.36	217	1.32	1.26	Pyruvic acid	PM	Spring
25049	7.63	247	1.68	1.33	Succinic acid	PM	Summer
19215	7.14	189	0.54	1.62	Urea		Spring
30055	12.7	332	0.27	1.59	Ascorbic acid		Summer
Others							
29906	11.65	328	0.93	1.11	Ammeline		Spring
25586	10.23	255	1.18	0.99	Melamine	PM	
10159	10.44	117	0.53	1.18	N.I 5		
28720	12.52	305	1.6	1.25	N.I 6		
27854	12.95	291	1.27	0.94	Pantothenic acid	PM	
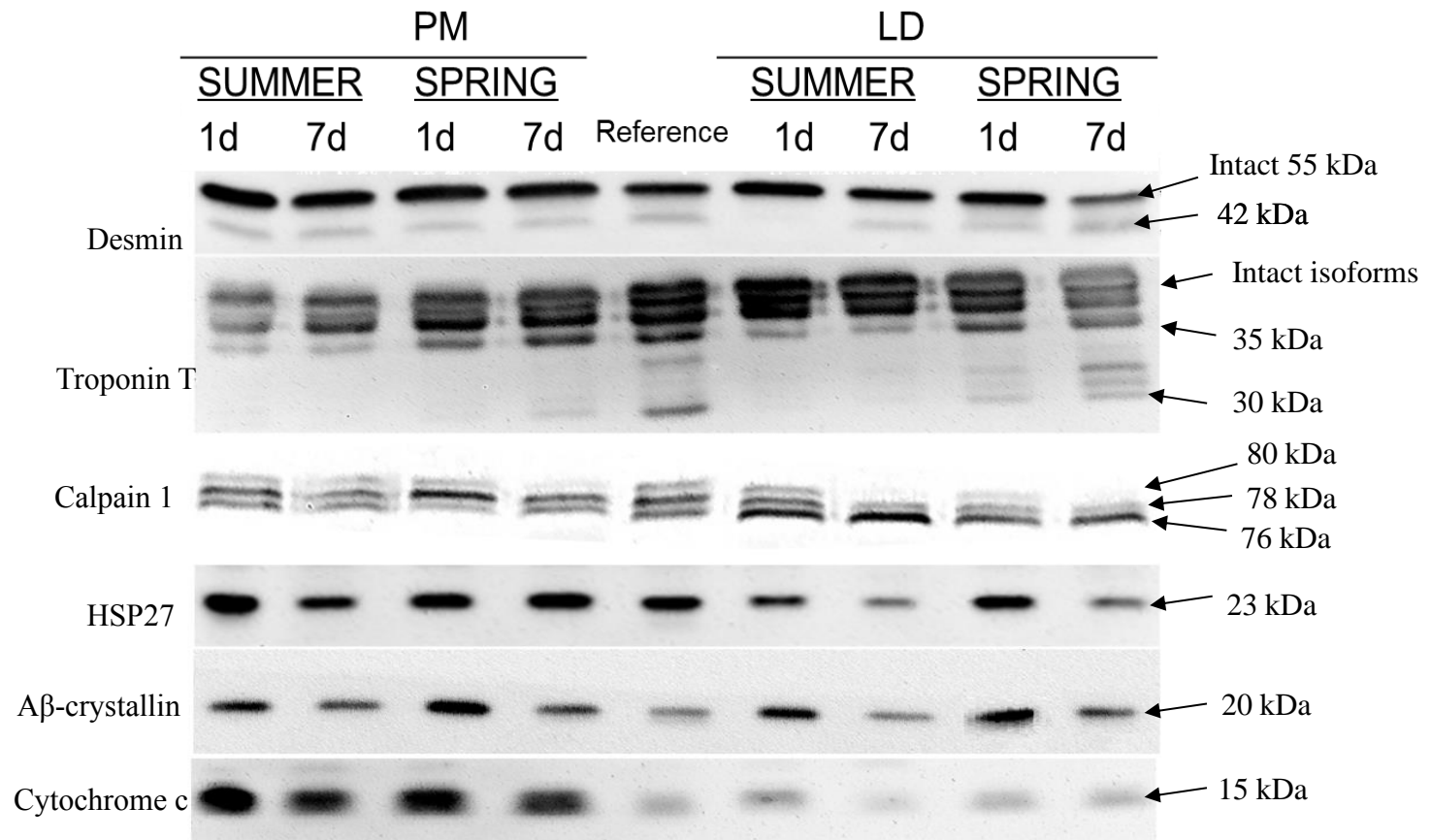
Figure 5.1 A representative set of western blot images showing 1d vs. 7d postmortem change of desmin, troponinT, calpain 1, HSP27, αβ-crystallin and cytochrome c of porcine LD (*longissimus dorsi*) and PM (*psoas major*) muscles with two production trials. Reference: consistently use a 7d postmortem LD for desmin and troponin T and a 45min postmortem LD for calpain 1, HSP27, αβ-crystallin and cytochrome c. SUMMER: Pigs weaned in July 2016 and harvested in January 2017; SPRING: Pigs weaned in April 2017 and harvested in September 2017
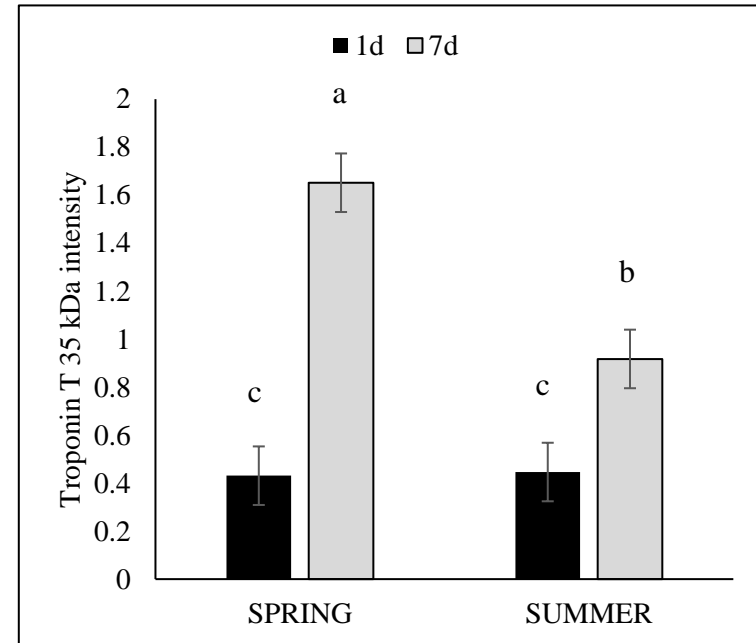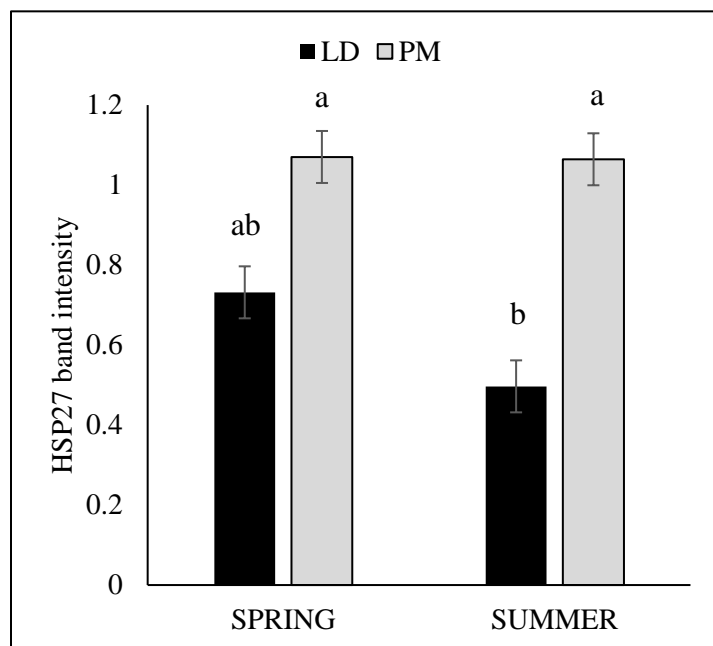
A)
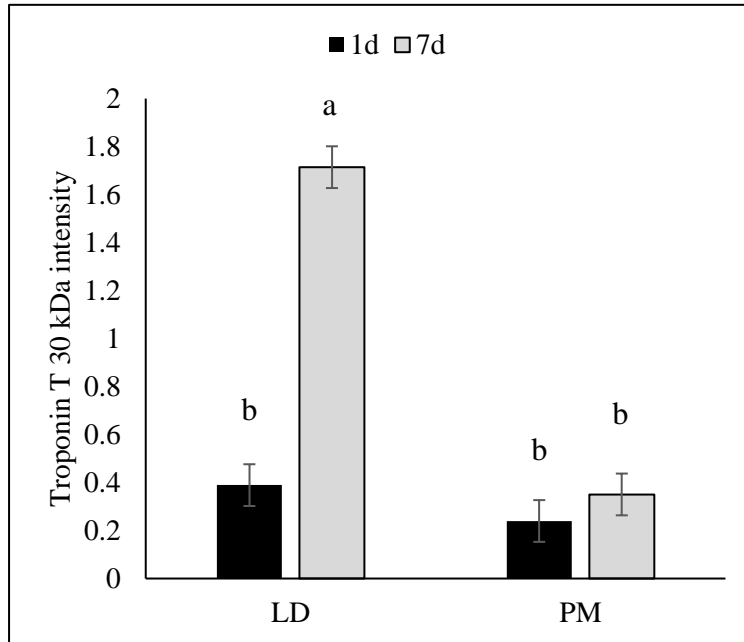
B)

Figure 5.2 Densitometric analysis of troponin T degradation of porcine LD (longissimus dorsi) and PM (psoas major) muscles with two production trials. A. Muscle by aging interaction (P = 0.02) on troponin T degradation product intensity at 30 kDa; B. Trial by aging interaction tendency (P = 0.054) on troponin T degradation product intensity at 35 kDa; Results were displayed as means ± standard error. (a-c) Means with different number are significantly different (P<0.05). SUMMER: Pigs weaned in July 2016 and harvested in January 2017; SPRING: Pigs weaned in April 2017 and harvested in September 2017
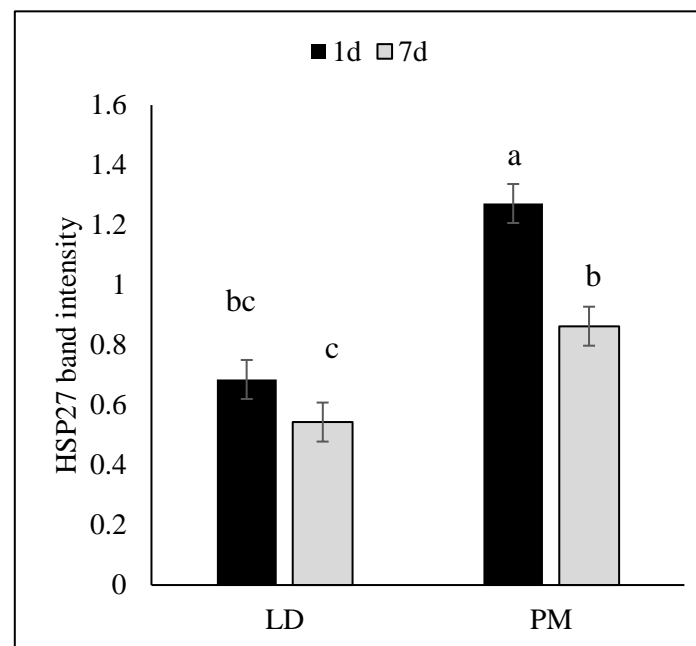
A)

B)

Figure 5.3 Densitometric analysis of HSP27 of porcine LD (longissimus dorsi) and PM (psoas major) muscles with two production trials. A. Muscle by trial interaction (P = 0.024); B. Muscle by aging interaction (P = 0.009); Results were displayed as means ± standard error. (a-c) Means with different number are significantly different (P<0.05). SUMMER: Pigs weaned in July 2016 and harvested in January 2017; SPRING: Pigs weaned in April 2017 and harvested in September 2017
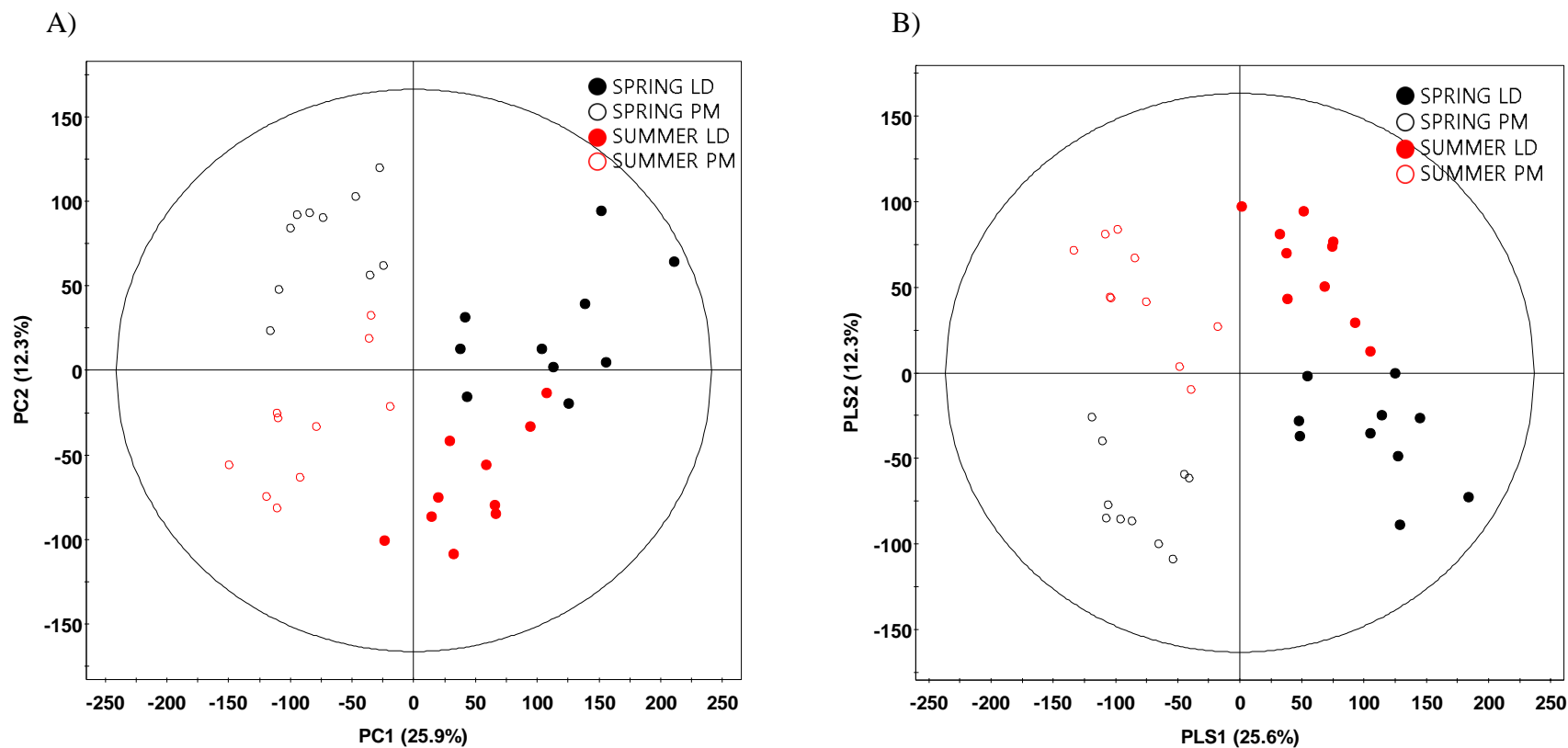
Figure 5.4 PCA and PLS-DA score plots of metabolome profiling porcine LD (longissimus dorsi) and PM (psoas major) muscles from two production trials; A. PCA score plot, R2X=0.49, Q2=0.336; B. PLS-DA score plot, R2X=0.559, R2Y=0.981, Q2=0.835, P < 0.05. SUMMER: Pigs weaned in July 2016 and harvested in Janurary 2017; SPRING: Pigs weaned in April 2017 and harvested in September 2017
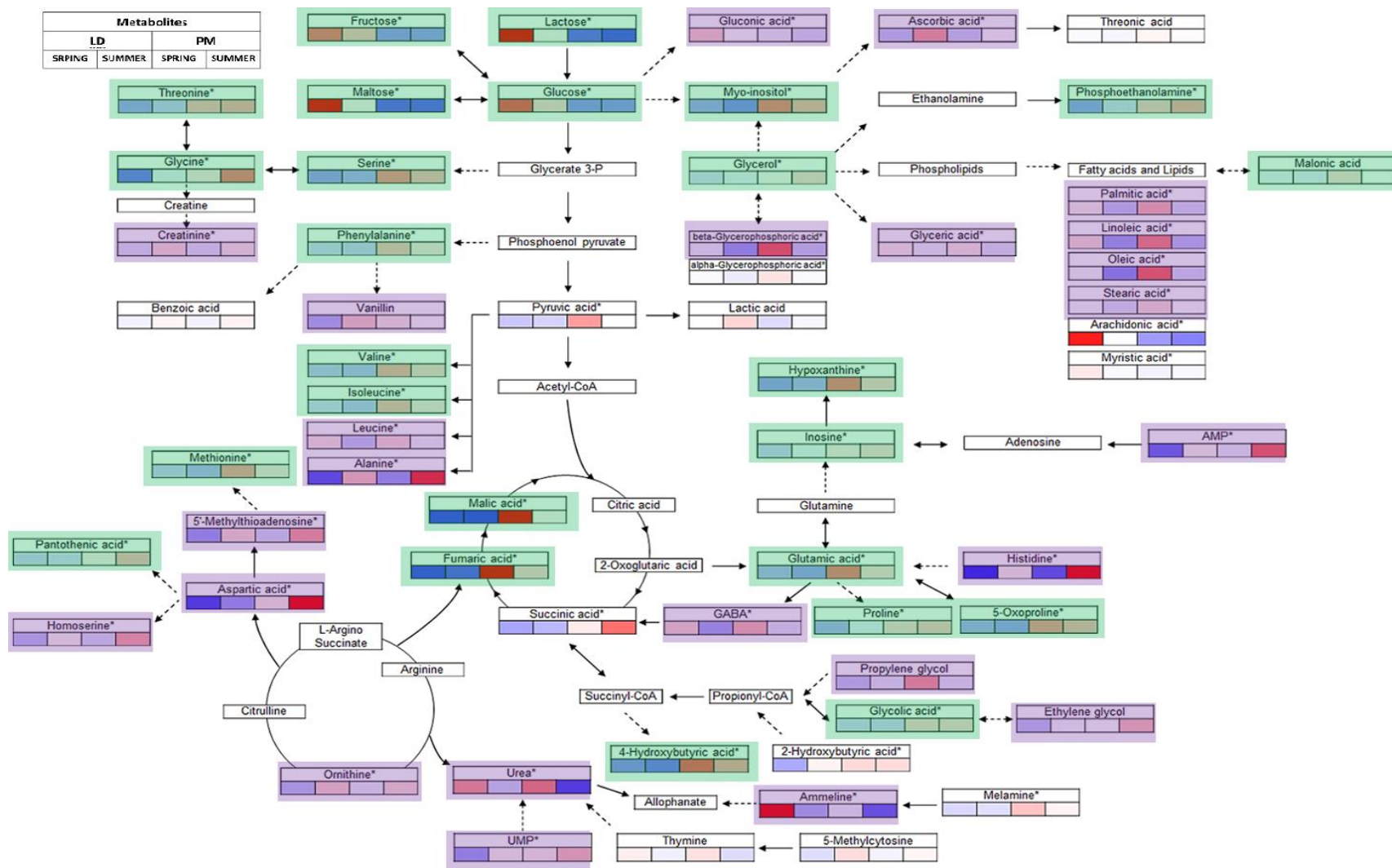
Figure 5.5 Pathway analysis of differentially affected metabolites and pathways in porcine LD (longissimus dorsi) and PM (psoas major) muscles from two production trials. SUMMER: Pigs weaned in July 2016 and harvested in Janurary 2017; SPRING: Pigs weaned in April 2017 and harvested in September 2017

CHAPTER 6.   CONCLUSION AND IMPLECATIONS

6.1   Overall summary

To shed lights on regulatory mechanism of postmortem apoptosis-proteolysis cross talk and consequential impact on meat quality development, the primary study objective of this dissertation was to evaluate postmortem changes of small heat shock proteins, apoptosis features, myofibrillar proteolysis, and metabolic characteristics in postmortem skeletal muscles under different metabolic, genetic, or management conditions across species. Specifically, Chapter 2 utilized three bovine muscles with different anatomic and metabolic properties, namely LL (glycolytic), SM (intermediate) and PM (oxidative). The results indicate that these muscles with different metabolic backgrounds showed different rate of aging potential, which was coincided with muscle specific HSP27 degradation. LL exhibited an increase in tenderization potential and higher WHC during postmortem aging compared to other muscles, particularly PM, which was concurrent with extended calpain 1 autolysis, troponin T degradation, and HSP27 degradation. LL showed more extensive HSP27 degradation and an early onset of caspase 3 activation, as HSP27 degradation could indicate loss of protective/anti-apoptotic activity from caspase cascades activation, delaying myofibrillar protein degradation.

In Chapter 3, proteomic profile of *M. longissimus* in callipyge sheep confirmed an overall increased glycolytic metabolism features as indicated by less cytochrome c, higher abundance in eight glycolysis enzymes, and unique myosin heavy chain isoform profile in *+/C* groups, which was coupled with alteration in apoptotic and anti-stress protein profiles. Particularly, compromised proteolytic aging potential in postmortem muscles from callipyge lamb carcasses would be partly attributed to more elevated anti-apoptotic features compared to muscles from non-callipyge counterparts. These results demonstrated that variations in proteolytic aging potential was concurrent with alternations in apoptotic response in muscles with distinctive metabolic backgrounds; elevated anti-apoptotic systems were associated with restrained aging response.

In Chapter 4, two groups of pigs were weaned in July 2016 and harvest in January 2017 (SUMMER) and weaned in April 2017 and harvest in September 2017 (SPRING), respectively. The early-life weaning and transporting stress were intervened using dietary antibiotics and L-

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glutamine supplementation to evaluate the possibility of using L-glutamine as an antibiotics replacement in post-weaning nursery pigs from a meat quality perspective. L-glutamine supplementation showed no difference (or very marginal improvement based on statistical analysis) in carcass characteristics, proximate composition, tenderness, water-holding capacity, display color and TBARS compared to dietary antibiotics treatment. However, the two seasonal replicates showed marked difference in carcass productivity and meat quality. SPRING replicates showed improvements in growth as indicated by higher body weight and more intra-muscular fat and superior pork quality as indicated by lower shear force values, thaw-purge loss, and cooking loss.

Chapter 5 further evaluated metabolomics profiles and proteolytic changes of the above-mentioned porcine muscles, because Chapter 4 give rise to a further research question whether the differentiated productivity and meat quality can be linked to alternations in metabolic, small het shock proteins and apoptotic response. The metabolomics profiles confirmed metabolic alternations between the production trials, where LD and PM muscles of SPRING retained higher level of macronutrients, including 6 carbon sugar species, 16-18 carbon fatty acids, and branched-chain amino acids, all of which are associated with cellular oxidative stress under certain circumstances. Further extended proteolysis and apoptosis characteristics and ATP degradation products were also found in SPRING. On the contrary, SUMMER showed elevated stress-defending compounds such as antioxidant amino acid, ascorbic acid, inosine, and decreased inhibitory neuro-transmitter GABA, possibly suggested that anti-stress system could be more activated in SUMMER, impeding postmortem apoptosis and render adverse impact on proteolytic potential of postmortem muscles.

6.2   Future research

As demonstrated by Chapter 3 and 5, system biology tools, particularly mass-spectrometry based *omics* technologies, provides intense information regarding postmortem biochemical changes, confirming well established knowledge as well as generating further hypothesis and insights of chemical and protein networks in meat system, which may offer opportunities to identify key proteins, pathways and reactions that involve in mediating and regulating postmortem proteolysis. With the development of more advanced computational algorithms and tools, high-throughput *omics* platforms will provide more accurate analysis to elucidate interactions among early postmortem biochemical processes - energy metabolism, apoptosis response, small HSPs,

calpain mediated proteolysis. However, it should be noted that *omics* profiling studies are hypothesis-generating rather than hypothesis-testing studies; therefore, novel factors (both cellular metabolites and proteins) identified in current chapters (and any future ones) need further validations to decipher specific roles in regulating meat quality development.

In Chapter 5, although we suggest that concurrent improvements in tenderness/proteolysis characteristics can be partially attributed to more advanced apoptosis, a direct causal relationship needs to be further established. The current results also corroborate with the notion that nutrition status is among key pre-slaughter factors affecting meat quality development. Previous publications confirmed that high-fat low-protein diet in pigs generating mor tender and juicer pork primarily due to elevated intra-muscular fat (Pettigrew & Esnaola, 2001). Taken together, future researches are needed focusing on elucidating the role of nutrition manipulation and insulin resistance (Martins et al., 2012) in mediating muscle cell apoptosis and pork quality: pigs are well established large animal model to study human metabolic disorders such as diabetes; the previous research outcomes indicated that insulin resistance in pigs are closely associated to increased cellular oxidative stress, apoptosis and other forms of cell and tissue degeneration (Liu et al., 2012). From an animal farming perspective, in finishing pigs, if dietary manipulation in inducing insulin resistance, cellular oxidative stress and apoptosis is practically and economically feasible, novel feeding regimes can be further developed aiming for producing pork with premium tenderness.

Similarly, different feeding regimes (grass-fed vs. concentrated diet) in beef cattle may also induce different levels of cellular oxidative stress and therefore impact tenderness development. Despite its fast growth driven by consumer demand, grass-fed beef is actually facing some draw backs in meat quality attributes, primarily due to lower marbling content and unique fatty acids profile (Berger et al., 2018). Moreover, grass-fed beef retains higher level of polyphenols and other antioxidant secondary metabolites, which are derived from forage-based diets (Daley, Abbott, Doyle, Nader, & Larson, 2010). Further studies are recommended to determine the impacts of 1. low energy density diet and 2. plant -derived bioactive matter deposition on metabolic, anti-stress and anti-apoptotic responses in postmortem beef muscles, which outcomes may help industry to cope with the inherent palatability challenges of grass-fed beef.

Another on-farm factor possibly influencing meat quality via apoptosis mechanisms, as suggested by Chapter 3, is the genetic background of the farm animals. Through a proteomic approach, Chapter 3 demonstrated elevated anti-stress and anti-apoptotic proteins in callipyge

genotype. The callipyge phenotype is due to a single gene mutation leading to calpastatin overexpression, while more genetic selection programs are based on multiple gene controlled quantitative traits. For example, residual feed intake (RFI) is an index widely used in cattle and swine production to evaluate feed efficiency (low RFI indicates high efficiency). Comparing pigs selected for high vs. low RFI, previous studies have found differentially expressed genes in regulating mitochondria function, suggesting that low RFI porcine muscles may be less prone to oxidative stress (Louveau, Vincent, Tacher, Gilbert, & Gondret, 2016; Merlot, Gilbert, & Le Floc'h, 2016; Outhouse et al., 2018). Future studies can utilize porcine muscles from high vs. low RFI genetic lines as a unique platform to determine anti-mortal metabolism and apoptosis characteristics in affecting postmortem aging proteolysis.

While on-farm management and genetic selection provide multiple control point to possibly optimize meat quality outcome via manipulating muscle metabolism and apoptosis, opportunities also exist in animal slaughter and postmortem processing practices. For example, electrical simulation (ES) of carcasses is widely accepted practice to improve meat tenderness, which relies on its ability to accelerate glycolysis to prevent shortening and to induce Ca^{++} release (from sarcoplasmic reticulum) to escalate calpain activity (Hwang, Devine, & Hopkins, 2003). It is reasonable to postulate that electrical current can interfere polarity of the cellular membrane system including sarcoplasmic reticulum and mitochondria, possibly releasing pro-apoptotic signals to the cytosol. Future mechanistic studies are recommended to determine the role of antimoral apoptosis in proteolysis of ES treated skeletal meats. The proposed research will help industry to establish robust ES protocols.

In conclusion, this dissertation provides initial evidence that anti-apoptotic systems and apoptotic response evolved in postmortem proteolysis and meat quality development. When internal or external factors affected metabolic properties of skeletal muscles in farm mammals, synergistic alternations of small heat shock proteins, apoptosis related characteristics, and protease systems were consistently observed, render consequential impacts on fresh meat development. Future studies should be focusing on explaining the fundamental mechanisms of apoptosis-proteolysis cross talk while supporting the wholesomeness of red meat production.

6.3 Literatures cited

Berger, J., Kim, Y. H. B., Legako, J. F., Martini, S., Lee, J., Ebner, P., & Zuelly, S. M. S. (2018). Dry-aging improves meat quality attributes of grass-fed beef loins. *Meat science, 145*, 285-291.

Daley, C. A., Abbott, A., Doyle, P. S., Nader, G. A., & Larson, S. (2010). A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. *Nutrition journal, 9*(1), 10.

Hwang, I., Devine, C., & Hopkins, D. (2003). The biochemical and physical effects of electrical stimulation on beef and sheep meat tenderness. *Meat Science, 65*(2), 677-691.

Liu, J., Chen, D., Yao, Y., Yu, B., Mao, X., He, J., . . . Zheng, P. (2012). Intrauterine growth retardation increases the susceptibility of pigs to high-fat diet-induced mitochondrial dysfunction in skeletal muscle. *PloS one, 7*(4).

Louveau, I., Vincent, A., Tacher, S., Gilbert, H., & Gondret, F. (2016). Increased expressions of genes and proteins involved in mitochondrial oxidation and antioxidant pathway in adipose tissue of pigs selected for a low residual feed intake. *Journal of animal science, 94*(12), 5042-5054.

Martins, A. R., Nachbar, R. T., Gorjao, R., Vinolo, M. A., Festuccia, W. T., Lambertucci, R. H., . . . Hirabara, S. M. (2012). Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function. *Lipids in health and disease, 11*(1), 30.

Merlot, E., Gilbert, H., & Le Floc'h, N. (2016). Metabolic response to an inflammatory challenge in pigs divergently selected for residual feed intake. *Journal of animal science, 94*(2), 563-573.

Outhouse, A., Patterson, B., Huff-Lonergan, E., Helm, E., Dekkers, J., Schwartz, K., . . . Lonergan, S. (2018). Novel Observations of Peroxiredoxin-2 Profile and Protein Oxidation in Skeletal Muscle from Pigs that Differ In Residual Feed Intake and Health Status. *Meat and Muscle Biology, 2*(2).

Pettigrew, J., & Esnaola, M. (2001). Swine nutrition and pork quality: A review. *Journal of Animal Science, 79*(suppl_E), E316-E342.