Meat and Muscle Biology™

Investigation of the Sarcoplasmic Proteome Contribution to the Development of Pork Loin Tenderness

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Abstract: The study objectives were to determine the extent to which the sarcoplasmic proteome explains variations in aged pork loin star probe value. Pork loins ($n = 12$) were categorized by differences in star probe at 21 d post mortem from a larger set of loins. Loins were categorized into low star probe (LSP) group ($n = 6$; star probe < 5.80 kg) and high star probe (HSP) group ($n = 6$; star probe > 7.00 kg) based on 21-d star probe value with inclusion criteria of marbling score (1.0–3.0) and 24-h pH (5.69–5.98). Quality traits were measured at 1-, 8-, 14-, and 21-d aging. Desmin and troponin-T degradation, peroxiredoxin-2 abundance, calpain-1 autolysis, and sarcomere length were determined. Two-dimensional difference gel electrophoresis and mass spectrometry were used to identify proteins that differed in abundance due to category. Star probe values were lower ($P < 0.01$) in LSP at each day of aging compared with HSP. Greater pH values were observed ($P < 0.05$) in LSP compared with HSP at each day of aging. Marbling score was greater ($P < 0.05$) in LSP compared with HSP at each day of aging. Greater ($P < 0.05$) desmin and troponin-T degradation was detected in LSP chops at 14- and 21-d aging and 8-, 14-, and 21-d aging, respectively. Greater ($P < 0.05$) sarcomere length was determined in LSP compared with HSP at 1-, 8-, and 21-d aging. Sarcoplasmic proteins from HSP chops had greater abundance ($P < 0.10$) of metabolic and regulatory proteins, whereas the LSP chops had greater abundance ($P < 0.10$) of stress response proteins. Star probe values were affected by pH, marbling score, protein degradation, sarcomere length, and sarcoplasmic proteome.

Key words: glycolytic proteins, pork quality, proteolysis, stress response proteins
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Introduction

In the current retail case, large variations in pork quality exist (Bachmeier et al., 2016), and consumers in the United Sates are willing to pay a premium for high-quality, third-party-quality grade-assigned pork cuts (Lusk et al., 2018). Tenderness is a fundamental aspect of pork quality (Moeller et al., 2010). Internationally, customers place greatest value on eating quality attributes such as tenderness and pork flavor in fresh pork products (Murphy et al., 2015). However, predicting pork tenderness is challenging due to the variety of quality factors that influence tenderness. There have been many efforts to predict pork quality, but a robust system has not been defined, demonstrating that the problem is multifactorial and complex. Consumers desire to purchase high-quality pork products, but the ability to predict pork quality differences is not currently available. One approach to accomplish this is through a greater understanding of how development of pork tenderness occurs post mortem.

Star probe is an instrumental tenderness method that determines the force needed to compress the product to 20% of its original height. This method uses a 5-point star probe attachment on an Instron (Instron, Norwood, MA) to compress the product

similar to the act of chewing (Huff-Lonergan et al., 2002). This method has been shown to have a moderate correlation ($r = -0.54$; $P < 0.01$) with sensory tenderness (Huff-Lonergan et al., 2002) and has more recently been shown to have a high correlation ($r = 0.84$; $P < 0.01$) with another instrumental tenderness method, Warner-Bratzler shear force (Schulte et al., 2019).

Postmortem tenderness development is influenced by a multitude of factors, including pH (Melody et al., 2004; Lonergan et al., 2007), collagen content (Wheeler et al., 2000; Nishimura et al., 2009), sarcomere length (Wheeler et al., 2000), and protein degradation (Huff-Lonergan et al., 1996; Wheeler et al., 2000; Melody et al., 2004; Carlson et al., 2017b). Carlson et al. (2017a) demonstrated that abundance of a desmin degradation product and peroxiredoxin-2 in aged pork loin sarcoplasmic proteome explained variations in star probe values. What remains undefined is how the sarcoplasmic proteome in early postmortem pork can be used to predict fresh pork loin quality after aging. It was hypothesized that star probe groups would have differences in pork quality attributes and sarcoplasmic protein profiles at each day of aging. Therefore, the objective of this study was to document protein profile differences of pork loins aged 1 d post mortem based on high star probe (HSP) and low star probe (LSP) values at 21 d post mortem. Identifying early postmortem tenderness biomarkers is critical for quality-based pork marketing.

Materials and Methods

Twenty pork loins, previously described (Schulte et al., 2019), were sorted based on 21-d aged pork loin star probe values to obtain HSP and LSP groups. Previously, pairs of loins were collected from a commercial harvest facility at 1 d post mortem and transported to the Iowa State University Meat Laboratory for fabrication (Schulte et al., 2019). Chops were aged 1, 8, 14, or 21 d, and quality data were collected as previously described (Schulte et al., 2019). Loins with HSP (>7.0 kg) and LSP (<5.8 kg) values at 21 d post mortem were chosen to represent extreme differences in star probe value. Marbling score and pH value parameters were set as inclusion criteria to further identify sample experimental groups. Loin marbling scores at 21-d aging ranged from 1.0 to 3.0. Loin pH at 21-d aging ranged from 5.69 to 5.93. This 21-d aging classification criteria narrowed the sample set to a balanced experiment of HSP ($n = 6$) and LSP ($n = 6$) categories.

Whole-muscle protein extraction

Frozen meat containing only the *longissimus dorsi* (100 g) was homogenized in liquid nitrogen. Samples from each aging time (0.5 g) were homogenized, and whole-muscle protein extracts were completed using 10 mM sodium phosphate (pH 7.0) and 2% sodium dodecyl sulfate (SDS) (weight/volume) as described by Carlson et al. (2017b).

Sarcoplasmic protein extraction

Frozen meat containing only the *longissimus dorsi* (100 g) was homogenized in liquid nitrogen. Samples from each aging time (3 g) were homogenized, and sarcoplasmic proteins were extracted (4°C; 50 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid [pH 8.0]) as described by Carlson et al. (2017a). Samples to be used for two-dimensional difference gel electrophoresis (2D-DIGE) were diluted to 10 mg/mL using cold sarcoplasmic extraction buffer (4°C; 50 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid [pH 8.0]) and were frozen.

Running conditions

Desmin and troponin-T degradation in the whole-muscle protein extracts (1-, 8-, 14-, and 21-d aged) and sarcoplasmic peroxiredoxin-2 abundance (1-, 8-, 14-, and 21-d aged) as well as calpain-1 autolysis at 1 d post mortem were determined using one-dimensional SDS-polyacrylamide gel electrophoresis as described by Carlson et al. (2017a, 2017b). Protein was extracted from porcine *longissimus dorsi* (aged 0, 1, and/or 7 d) with the identical protocol (Carlson et al. 2017a, 2017b) to generate a reference sample (4 mg/mL of protein) that was included in 1 well on each gel. A reference (*longissimus* muscle, 0-d aging) was used for desmin analysis, a 0-d/7-d mixed reference sample was used for troponin-T analysis, and a 1-d reference sample was used for peroxiredoxin-2 analysis. SE 260 Hoefer Mighty Small II electrophoresis units (Hoefer, Inc., Holliston, MA) were used to run 15% gels for desmin, troponin-T, and peroxiredoxin-2 analysis and 10% gels for calpain-1 autolysis.

Transferring conditions and western blot analysis

At completion of running the SDS-polyacrylamide gel electrophoresis, the gels were transferred to polyvinylidene difluoride membranes with pore sizes of 0.2 µm as described by Carlson et al. (2017b). Western blot analysis was conducted as described by

Carlson et al. (2017a, 2017b). The following primary antibody concentrations were diluted with phosphate-buffered saline (PBS)-Tween and added to separate blots: desmin (1:40,000) using polyclonal rabbit anti-desmin antibody produced at Iowa State University (Huff-Lonergan et al., 1996; Carlson et al., 2017a, 2017b), troponin-T (1:10,000) using monoclonal mouse anti–troponin-T primary antibody (T6277, JLT-12; Sigma Aldrich, St. Louis, MO), peroxiredoxin-2 (1:20,000) using monoclonal rabbit anti–peroxiredoxin-2 antibody (ab109367; ABCam, Cambridge, UK), and calpain-1 (1:5,000) using monoclonal mouse anti–calpain-1 (MA3-940; Thermo Scientific, Rockford, IL). Desmin, troponin-T, and peroxiredoxin-2 secondary antibodies were diluted with PBS-Tween, and calpain-1 secondary antibodies were incubated in PBS-Tween mixed with 5% nonfat dry milk. All blots were incubated for 1 h at 22°C. Secondary antibodies were diluted with PBS-Tween and added to separate blots following washes at the following concentrations: desmin (1:20,000): goat anti-rabbit-horseradish peroxidase (HRP) antibody (32430; Thermo Scientific); troponin-T (1:5,000): goat anti-mouse HRP antibody (32430; Pierce); peroxiredoxin-2 (1:5,000): goat anti-rabbit-HRP (31460; Thermo Scientific); and calpain-1 (1:10,000): goat anti-mouse-HRP antibody (A2554; Sigma Aldrich, St. Louis, MO). Following incubation with secondary antibodies, desmin, troponin-T, and peroxiredoxin-2 blots were washed with PBS-Tween 3 times for 10 min, and calpain-1 blots were washed 5 times for 10 min. Blots were analyzed as described by Carlson et al. (2017a, 2017b). Using the internal reference sample on each blot, the intensity of the 55-kDa intact desmin band, 30-kDa troponin-T degradation product, and 22-kDa intact peroxiredoxin-2 band was quantified as a comparative ratio of the sample protein band to the same internal reference protein band (40 µg of protein per lane) on each gel. Calpain-1 autolysis was analyzed as a percentage of the 80-, 78-, or 76-kDa band within each sample. All western blots were completed in at least duplicate with a coefficient of variance less than 20%.

Sarcomere length

Frozen meat containing only the *longissimus dorsi* (20 g) was homogenized in liquid nitrogen. Sarcomere length determination was made at all-day aging using the helium-neon laser diffraction method as described by Cross et al. (1981).

2D-DIGE

Sarcoplasmic protein (50 µg) extracts from each sample of each experimental group were labeled alternatively with CyDye3 and CyDye5 (Carlson et al., 2017a) according to the manufacturer's directions (GE Healthcare, Piscataway, NJ). A pooled reference sample containing equal amounts of all samples ($n = 12$ total) was used for identification and pick gels. Three aliquots of the pooled reference sample (100 µg) were labeled with CyDye2. The final protein concentration of samples was 7.14 mg/mL. Samples were stored at −80°C until use to complete experiments at corresponding pH ranges.

Labeled samples were prepared for running on 11-centimeter pH 3–10 or pH 4–7 immobilized pH gradient strips (GE Healthcare, Piscataway, NJ) as described previously (Cruzen et al., 2015) with minor adjustments. Fifteen µg of sample from both experimental groups and 15 µg of protein from the pooled reference sample were combined for a total of 45 µg protein for each strip (Carlson et al., 2017a). Rehydration solution (DeStreak; GE Healthcare, Piscataway, NJ) was adjusted to 20 mM 1,4-Dithiolthreitol and 2% immobilized pH gradient buffer (either pH 3–10 [17-6000-87; GE Healthcare] or pH 4–7 [17-6000-86; GE Healthcare]). The rehydration solution was mixed with the prepared proteins and placed in an individual well of a humidified rehydration chamber. Strips rehydrated overnight (approximately 17 h) in the dark at room temperature (23°C). Strips were run in the first dimension for a total of 14,000 V-h, stored, equilibrated, loaded, run, imaged, and analyzed as described by Carlson et al. (2017a). Figure 1 is a representative 11-cm, pH 3–10 2D-DIGE gel. Figure 2 is a representative 11-cm, pH 4–7 2D-DIGE gel.

Spot identification

Spots of interest between experimental groups were chosen for identification, and the pooled reference sample was used for identification. For spot identification, gels were run as described earlier with the corresponding pH range to isolate spots of interest. Picked spots were excised from gels, sent to the Iowa State University Protein Facility, and digested with trypsin using a Genomic Solutions Investigator ProGest automated digester (Genomic Solutions Inc., Ann Arbor, MI). After digestion, the solution was dried down and reconstituted in 25 µL water containing 0.1% formic acid. Spots were then separated through liquid chromatography (Thermo Scientific

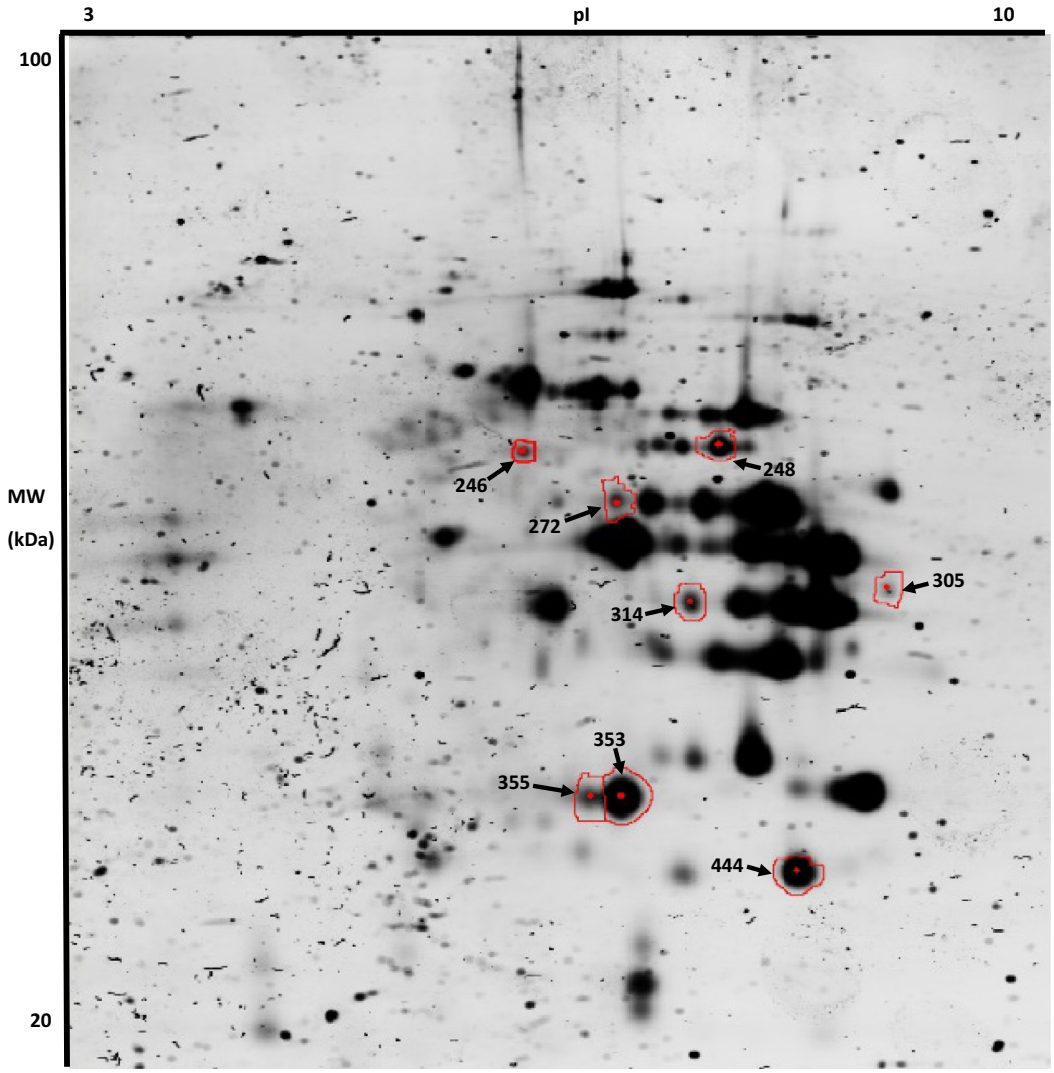


Figure 1. Representative two-dimensional difference gel electrophoresis (2D-DIGE) gel from the sarcoplasmic fraction of aged pork *longissimus dorsi* showing identified proteins. Immobilized pH gradient strips (11 cm, pH 3–10) were loaded with 45 µg of CyDye labeled protein (15 µg each of CyDye 2, 3, and 5), and strips were run on 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel. Proteins labeled with CyDye2 are shown.

EASY nLC-1200 coupled to a Thermo Scientific Nanospray FlexIon source) using a pulled glass emitter 75 µm × 20 cm (Agilent capillary, Part Number 16-2644-5), with the tip packed with Agilent SB-C18 Zorbax 5-µm packing material (Part Number 820966-922) and the remaining emitter packed with nanoLCMS Solutions UChrom C18 3-µm packing material (Part Number 80002). Samples were analyzed by tandem mass spectrometry using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA). Thermo Scientific's Proteome Discoverer Software (Waltham, MA) was used to analyze the raw data for identification of matched proteins and peptides against publicly available or user-provided databases. Peptide fragments were compared to a known database program using Mascot (London, UK) and Sequest HT against Sus Scrofa to identify proteins.

Statistical analysis

All quality data (cook loss; pH; purge; subjective color and marbling; Hunter L, a, and b; and star probe value) were analyzed using the "MIXED" procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). Fixed effects included days aged and category (HSP or LSP). Loin was used as a random effect in all models. Whole-muscle intact desmin and troponin-T degradation product, sarcoplasmic peroxiredoxin-2 abundance, and autolyzed sarcoplasmic calpain-1 data were analyzed using the "MIXED" procedure of SAS version 9.4 (SAS Institute Inc.) with fixed

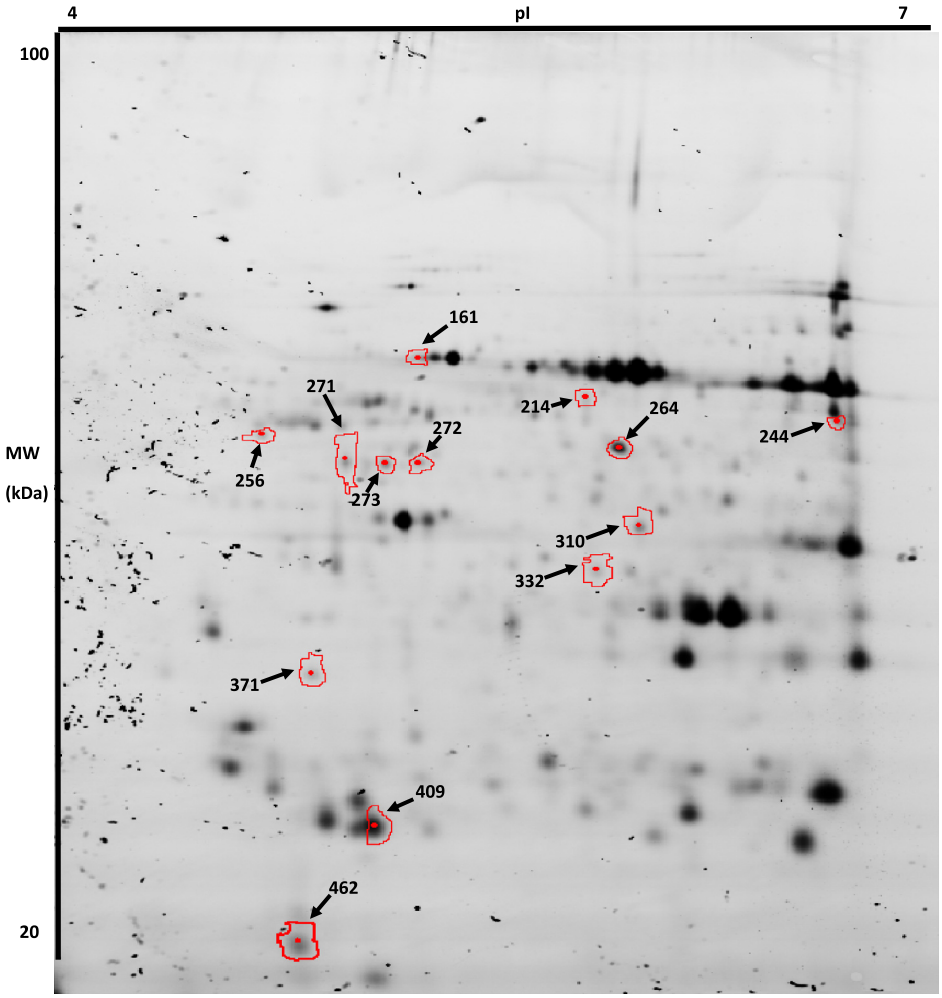


Figure 2. Representative two-dimensional difference gel electrophoresis (2D-DIGE) gel from the sarcoplasmic fraction of aged pork *longissimus dorsi* showing identified proteins. Immobilized pH gradient strips (11 cm, pH 4–7) were loaded with 45 µg of CyDye labeled protein (15 µg each of CyDye 2, 3, and 5), and strips were run on 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel. Proteins labeled with CyDye2 shown.

effects of days aged and category. Gel was used as a random effect in the model. Sarcomere length measurements were analyzed using the "MIXED" procedure of SAS version 9.4 (SAS Institute Inc.) with fixed effects of days aged and category. Carcass was used as a random effect in all models. Least-square means and standard errors were reported for all measured attributes. Least-square means were separated using the "pdiff" procedure of SAS version 9.4 (SAS Institute Inc.). Significance levels were denoted with $P \leq 0.05$.

2D-DIGE gel images were analyzed using DeCyder 2D software version 6.5 (GE Healthcare, Piscataway, NJ) using Student paired *t* test to determine differences in protein spot relative abundance between treatment groups. Significance was determined at $P \leq 0.10$ and trending at $0.10 < P \leq 0.15$.

Results and Discussion

Fresh quality attribute comparison

Fresh pork characteristics of both experimental groups are summarized in Table 1. Star probe values were significantly greater in the HSP group compared with the LSP group at each day of aging ($P < 0.01$). Pork loin star probe values at 21-d aging ranged from 4.76 to 5.77 kg for LSP and from 7.05 to 9.35 kg for HSP. Star probe groups had a 2.51-kg difference in average star probe value at 1-d aged and a 2.94-kg difference in average star probe at 21-d aging. The LSP probe group demonstrated a 25% decrease in star probe value from 1- to 21-d aging, while the HSP group only had a 15% decrease in star probe value during the aging period. Star probe values have been shown to

Table 1. Summary of fresh pork loin quality attributes, proteolysis of whole-muscle protein fraction desmin and troponin-T, sarcoplasmic protein fraction calpain-1 autolysis, and peroxiredoxin-2 in pork LM of selected star probe groups

	Low Star Probe Group ($n = 6$)					High Star Probe Group ($n = 6$)					*P* Value		
Item	1-Day Aged	8-Day Aged	14-Day Aged	21-Day Aged	SEM	1-Day Aged	8-Day Aged	14-Day Aged	21-Day Aged	SEM	Days Aged	Category	Days Aged × Category
Star Probe (kg)[1]	7.64^{ax}	5.50^{bx}	5.86^{bx}	5.72^{bx}	0.57	10.26^{ay}	8.59^{by}	8.30^{by}	8.76^{by}	0.60	<0.01	<0.01	0.90
Purge (%)[2]	0.14^{c}	1.32^{b}	1.83^{abx}	2.41^{ax}	0.35	0.16^{d}	1.64^{c}	3.27^{by}	4.54^{ay}	0.35	<0.01	<0.01	0.02
pH[3]	5.82^{abx}	5.79^{b}	5.86^{ax}	5.86^{ax}	0.02	5.76^{ay}	5.73^{a}	5.78^{ay}	5.76^{ay}	0.02	0.06	<0.01	0.80
Fabrication pH[4]	5.94	-	-	-	0.02	5.96	-	-	-	0.02	-	0.18	-
Color Score[5]	3.1	2.8	2.5	2.5	0.2	3.1	2.7	2.6	2.6	0.2	0.02	0.88	0.99
Marbling Score[6]	2.0^{bx}	2.3^{abx}	2.0^{bx}	2.5^{ax}	0.2	1.3^{y}	1.5^{y}	1.4^{y}	1.7^{y}	0.2	0.02	<0.01	0.86
Cook Loss (%)[7]	22.40^{a}	17.32^{cy}	20.47^{ab}	18.71^{bcy}	1.00	19.71	20.49^{y}	22.49	22.22^{y}	1.00	0.07	0.04	0.01
Hunter L Value[8]	44.96^{b}	49.79^{a}	49.96^{a}	50.55^{a}	0.76	44.34^{b}	49.23^{a}	48.44^{a}	50.25^{a}	0.76	<0.01	0.17	0.87
Hunter a Value[8]	11.81^{b}	13.91^{a}	13.87^{a}	13.79^{a}	0.26	11.97^{b}	13.19^{a}	13.93^{a}	13.58^{a}	0.26	<0.01	0.35	0.35
Hunter b Value[8]	2.86^{b}	4.24^{a}	4.23^{a}	3.92^{a}	0.19	2.71^{b}	3.89^{a}	3.82^{a}	3.77^{a}	0.20	<0.01	0.04	0.82
Intact Desmin[9]	1.16^{a}	0.58^{b}	0.47^{bx}	0.38^{bx}	0.12	1.32^{a}	0.81^{b}	0.81^{by}	0.93^{by}	0.13	<0.01	<0.01	0.33
Degraded Troponin-T[10]	-	0.60^{cx}	1.04^{bx}	1.53^{ax}	0.13	-	0.05^{b}	0.33^{ab}	0.50^{a}	0.14	<0.01	<0.01	<0.01
Peroxiredoxin-2[11]	1.01^{a}	0.65^{bx}	0.73^{b}	0.58^{bx}	0.06	1.00^{a}	0.95^{aby}	0.78^{bc}	0.77^{cy}	0.06	<0.01	<0.01	0.06
Autolyzed Calpain-1 (%)[12]	53.67	-	-	-	9.14	57.83	-	-	-	9.14	-	0.75	-
Sarcomere Length, μm[13]	1.92^{ax}	1.89^{ax}	1.86^{ab}	1.89^{ax}	0.04	1.78^{by}	1.76^{by}	1.79^{b}	1.78^{by}	0.04	0.86	<0.01	0.77

[1]A 5-point star probe attachment fitted with an Instron was used to assess force needed to compress a chop to 20% of its original height (Carlson et al., 2017b).

[2]Percent chop purge = [(weight of package with purge – weight of package without purge) ÷ chop weight] × 100.

[3]pH measurements were taken at the center of each chop.

[4]pH measurements taken at the commercial processing plant at approximately 20 h post mortem.

[5]National Pork Board standards, 6-point scale (1 = pale pinkish gray/white; 6 = dark purplish red).

[6]National Pork Board standards, 10-point scale (1 = 1% intramuscular fat; 10 = 10% intramuscular fat).

[7]Chops were cooked to an internal temperature of 68°C on clamshell grills. Percent cook loss = [(raw chop weight – cooked chop weight) ÷ raw chop weight] × 100.

[8]Hunter L, a, and b determined with Minolta Chroma Meter with D65 light source, 50-mm aperture, and 0° observer.

[9]Ratio of the densitometry units of the intact 55-kDa band of the sample compared to the 55-kDa band of the reference sample.

[10]Ratio of the densitometry units of the degraded 30-kDa band of the sample compared to the 30-kDa band of the reference sample.

[11]Ratio of the densitometry units of the intact 22-kDa band of the sample compared to the 22-kDa band of the reference sample.

[12]Percentage indicates the percent of autolyzed calpain-1 as a total of calpain-1 in each sample.

[13]SL was determined using helium-neon laser diffraction (Cross et al., 1981).

[a,b,c,d]Means within star probe group with different superscripts are significantly different ($P < 0.05$).

[x,y]Means within rows and day of aging with different superscripts are significantly different ($P < 0.05$).

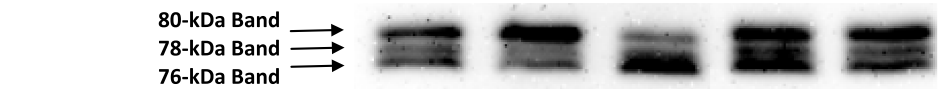
LM = *longissimus dorsi*; SL = sarcomere length.

demonstrate a moderate correlation ($r = -0.54$; $P < 0.01$) with sensory tenderness values (Huff-Lonergan et al., 2002) and a strong correlation ($r = 0.84$; $P < 0.01$) with Warner-Bratzler shear force values (Schulte et al., 2019) in pork loins. In a similar study of aged pork loins, Carlson et al. (2017b) determined a higher sensory tenderness value in the LSP group compared with the HSP group. These combined results demonstrate the expected tenderness difference in star probe values between categories.

The LSP group had less purge at 14- and 21-d aging ($P < 0.01$) and less cook loss at 8- and 21-d aging ($P < 0.05$) than the HSP group. This difference could be partially accounted for by significant differences in pH and desmin degradation. Postmortem proteolysis not only impacts meat tenderness but also impacts the ability of meat to retain water (Kristensen and Purslow, 2001; Huff-Lonergan and Lonergan, 2005). Bee et al. (2007) showed that drip loss at 1, 2, and 4 d of storage was positively correlated to intact desmin (0.41, 0.45, and 0.42, respectively) and talin (0.35, 0.60, and 0.51, respectively). In pork, early postmortem and ultimate pH is negatively correlated with drip and purge loss (Huff-Lonergan et al., 2002; Melody et al., 2004; Bee et al., 2007; Boler et al., 2010; Richardson et al., 2018; Watanabe et al., 2018).

High pH values will result in greater pork quality regardless of intramuscular fat content (Lonergan et al., 2007). However, intermediate pH ranges ($5.50 < \text{pH} > 5.95$) may be influenced by lipid content (Lonergan et al., 2007). When factors such as genetics, management techniques, and harvest day were restricted along with a set range of ultimate pH (5.48 to 5.79), marbling did not influence eating quality (Rincker et al., 2008). Additionally, some research has suggested that consumers' purchasing intent has shifted towards the desire to purchase pork with less intramuscular fat content (Brewer et al., 2001; Papanagiotou et al., 2013). This has influenced genetic decisions for leaner, more efficient growth in pigs and —adversely—has negatively impacted pork quality (Lonergan et al., 2001). A small yet significant difference in pH was observed between star probe groups. The LSP group demonstrated greater pH values at 1-, 14-, and 21-d aging ($P < 0.05$) compared with the HSP group. Additionally, the LSP group had greater marbling scores at all aging time points ($P < 0.05$) compared with the HSP group. The combination of pH and marbling score of these chops within these medium pH ranges may be influencing quality attributes and proteolysis; however, the differences were small. Chop visual color scores and Hunter L, a, and b values were not different between star probe groups at any aging time point ($P > 0.05$).

Calpain-1 plays a significant role in the degradation of myofibrillar, cytoskeletal, and intermediate filament proteins (Wheeler et al., 2000; Lametsch et al., 2004; Geesink et al., 2006; Koohmaraie and Geesink, 2006). The 76-kDa autolysis product of calpain-1 was negatively correlated to desmin (−0.57), vinculin (−0.18), and talin (−0.66), demonstrating the close association of calpain-1 degradation and autolysis (Bee et al., 2007). Calpain-1 activity and autolysis is impacted by pH decline (Melody et al., 2004; Bee et al., 2007), oxidative conditions (Maddock-Carlin et al., 2006), and nitric oxide (Li et al., 2014; Zhang et al., 2018; Liu et al., 2016; Liu et al., 2019); calpain-1 rate of activation and autolysis could impact the extent of proteolysis that occurs. Desmin, troponin-T, titin, tropomyosin, actin, and myosin light chain I are substrates of calpain-1 (Huff-Lonergan et al., 1996; Lametsch et al., 2004; Geesink et al., 2006; Anderson et al., 2012; Carlson et al., 2017a, 2017b). Although some autolysis occurred in every sample at 1 d post mortem, no significant difference between experimental groups was identified ($P > 0.05$) in the sarcoplasmic fraction (see Figure 3 for a representative western blot of calpain-1 autolysis and the corresponding star probe



Star probe value (kg)	7.78	11.32	8.83	11.51	6.08
Days Aged	1	1	1	1	1
Category	Low	High	High	High	Low

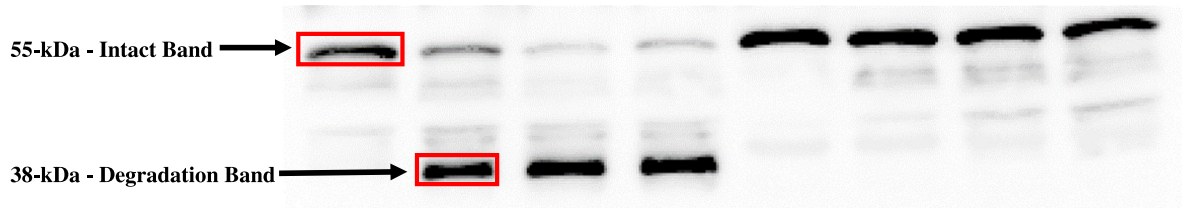
Figure 3. Representative western blot of sarcoplasmic calpain-1 autolysis in 1-d–aged *longissimus dorsi* (LM) muscle pork samples. Samples in low and high star probe groups were compared as a percentage of the fully autolyzed (76-kDa) band as a percentage of the total (all bands combined) bands. A 0-d/7-d mixed LM whole-muscle sample (Reference) was used to identify the presence of intact calpain-1 (80-kDa) and autolyzed (78-kDa and 76-kDa) protein bands. Star probe values (kg) are provided for the samples, and samples are labeled high or low depending on star probe force.

values for those specific samples). Melody et al. (2004) demonstrated that muscles with a faster rate of pH decline showed a quicker rate of calpain-1 autolysis in the sarcoplasmic protein fraction (Melody et al., 2004). The rate of pH decline during the early postmortem period was not collected with these samples. Differences in the rate of pH decline could explain differences in calpain-1 activity between samples. An extreme pH decline is not suspected because there were no significant differences in Hunter L or b values between star probe groups, but this was not measured and therefore cannot be concluded.

A representative western blot of desmin degradation analysis and the corresponding star probe values is shown in Figure 4. Postmortem proteolysis has a significant impact on the development of meat tenderness (Taylor et al., 1995; Melody et al., 2004; Carlson et al., 2017a, 2017b). Desmin functions to integrate the myofibril with surrounding organelles while also interlinking myofibrils at the Z-line region (Clark et al., 2002). Troponin-T is an integral part of the troponin complex that regulates the cross-bridging of myosin and actin (Clark et al., 2002). Degradation of desmin and troponin-T has consistently shown to be related to differences seen in instrumental tenderness values of pork muscles (Wheeler et al., 2000; Melody et al., 2004; Carlson et al., 2017b). Degradation of desmin can alter alignment of myofibrils, ultimately increasing meat tenderness. The results demonstrate that abundance of intact desmin decreased from 1- to 8-d aging (Table 1; $P < 0.01$) in both star probe groups. The LSP group had significantly less intact desmin at 14- and-21 d aging ($P < 0.01$) compared with the HSP group. Abundance of intact desmin in the LSP group decreased by 67% from 1- to 21-d aging, whereas the HSP group only decreased by 30% during the entire aging period. No measurable 30-kDa troponin-T degradation product was detected at 1-d aging in either star probe group. Troponin-T degradation product abundance increased from 8- to 14- and from 14- to 21-d aging (Table 1; $P < 0.01$) in the LSP group. Conversely, troponin-T degradation product increased from 14- to 21-d aging (Table 1; $P < 0.01$) but was not different between 8- and 14-d aging in the HSP group. Within 8-, 14-, and 21-d aging, the LSP group had significantly greater abundance of 30-kDa troponin-T degradation product compared with the HSP group ($P < 0.01$). While no differences were observed between LSP and HSP groups in calpain-1 at 1-d aging, the rate of pH decline and the activation of calpain-1 over day aging may explain the differences observed in intact desmin and degraded troponin-T after 1-d aging.

2D-DIGE analysis

Both 2D-DIGE corresponding pH ranges identified a variety of proteins in the sarcoplasmic fraction related to glycolytic metabolism, other forms of energy metabolism, stress response, and regulatory proteins that differed in abundance between star probe groups. In the corresponding 3–10 pH range, of 444 spots, there was significance or a tendency for classification group to affect abundance of 23 spots. Seven of the most prevalent spots were chosen for identification (Figure 1, Table 2). Spot 248 was also picked due to prevalence ($P = 0.16$). In the refined pH range (4–7), of 462 spots, abundance of 32 spots was different or tended to be different due to classification group. The 13 most prevalent were chose for identification (Figure 2, Table 2). Spots 272, 273, 371, and 409 were picked due to prevalence between experimental groups and previous identification in aged pork loins as potential biomarkers for star probe differences (Carlson et al., 2017a).



Star probe value (kg)	5.60	3.70	5.14	4.76	8.87	10.26	8.53	9.35
Days Aged	1	8	14	21	1	8	14	21
Category	Low	Low	Low	Low	High	High	High	High

Figure 4. Representative western blot of intact and degraded desmin in pork *longissimus dorsi* (LM) whole-muscle samples aged over time. Intact bands (55-kDa) and degradation bands (38-kDa) were compared to corresponding bands of a 0-d–aged pork LM sample (Reference). Star probe values (kg) are provided for the samples, and samples are labeled high or low depending on star probe force.

Table 2. Identified proteins of 2D-DIGE experiment 11-cm immobilized pH gradient strips, pH 3–10 and 4–7

Spot Number	Immobilized pH Gradient	Protein	Ratio[1]	*P* value
246	3–10	Mitochondrial aldehyde dehydrogenase	−2.14	0.14
248	3–10	Pyruvate kinase	−1.84	0.16
272	3–10	Creatine kinase M-type	−1.64	0.12
305	3–10	Glyceraldehyde 3-phosphate dehydrogenase	−2.37	0.11
314	3–10	Glyceraldehyde 3-phosphate dehydrogenase	−1.59	0.15
353	3–10	Triosephosphate isomerase	−1.54	0.13
355	3–10	Triosephosphate isomerase	−1.52	0.13
444	3–10	Adenylate kinase isoenzyme 1	−2.08	0.08
161	4–7	Heat shock cognate 71-kDa protein	1.14	0.09
214	4–7	Myc box-dependent-interacting protein 1	−1.23	0.07
244	4–7	Mitochondrial dihydrolipoyl dehydrogenase	−1.19	0.05
256	4–7	Protein disulfide-isomerase	−1.41	0.04
264	4–7	Mitochondrial aldehyde dehydrogenase	−1.32	0.02
271	4–7	Mitochondrial ATP synthase subunit beta	−1.71	<0.01
272	4–7	Annexin A7	−1.33	0.15
273	4–7	Hsc 70-interacting protein	1.14	0.20
310	4–7	Mitochondrial isocitrate dehydrogenase subunit alpha	−1.25	0.06
332	4–7	Phosphoglycerate kinase 1	−1.36	0.04
371	4–7	Annexin A5	−1.20	0.11
409	4–7	Peroxiredoxin-2 (fragment)	−1.25	0.20
462	4–7	Myosin regulatory light chain 2 isoform	−1.46	0.05

[1]Ratio indicates spot abundance differences between low and high star probe samples. Positive values represent more abundant in the low star probe group. Negative values represent less abundant in the low star probe group.

2D-DIGE = two-dimensional difference gel electrophoresis; ATP = adenosine triphosphate.

Glycolytic metabolism

Several proteins spots were identified to be involved with glycolytic metabolism. These proteins included pyruvate kinase (Spot 248), triosephosphate isomerase (Spots 353 and 355), glyceraldehyde 3-phosphate dehydrogenase (Spots 305 and 314), and phosphoglycerate kinase 1 (Spot 332). Pyruvate kinase was numerically (84%) more abundant in the HSP samples, but not trending or significant (Figure 1; $P = 0.16$). Two spots identified as triosephosphate isomerase (Spot 353 and 355) were trending to be more abundant (54% and 52%, respectively; Figure 1; $P = 0.13$ for both spots) in the HSP group. This enzyme catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Alber et al., 1981). Triosephosphate isomerase has been identified as a potential biomarker for meat quality attributes, including tenderness (Lametsch et al., 2003; Hwang et al., 2005; Carlson et al., 2017a) and drip loss in pork (Di Luca et al., 2013) as well as intramuscular fat deposition in beef (Kim et al., 2009). The results of the current experiments are consistent with Carlson et al. (2017a) demonstrating a greater abundance of triosephosphate isomerase in the less tender experimental group.

Two spots identified as glyceraldehyde 3-phosphate dehydrogenase (Spots 305 and 314) tended (Figure 1; $P = 0.11$ and 0.15, respectively) to be greater in abundance by 137% and 59% in the HSP group. Glyceraldehyde 3-phosphate dehydrogenase catalyzes the oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate (Seidler, 2013). The only protein identified in the 4–7 pH range directly related to glycolytic energy production was phosphoglycerate kinase 1 (Spot 332; Figure 2). Phosphoglycerate kinase 1 was 36% more abundant in the HSP group ($P = 0.04$). Phosphoglycerate kinase catalyzes the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate through the transfer of a phosphoryl group (Ohlendieck, 2010; Tymoczko et al., 2013). Combined, these proteins involved in glycolytic metabolism further identify the need to understand their role in postmortem metabolism, extent of impacting ultimate pH, and subsequent meat quality development. A greater understanding of the impact of different isoforms is also warranted for future research to enhance this understanding.

Other energy metabolism

Several other energy metabolism proteins were found to express differential abundance between

experimental groups: creatine kinase M-type (Spot 272; Figure 1) as well as mitochondrial adenosine triphosphate (ATP) synthase subunit beta (Spot 271; Figure 2) and mitochondrial isocitrate dehydrogenase subunit alpha (Spot 310; Figure 2). Creatine kinase M-type tended to be more abundant (64%; Figure 1; $P = 0.12$) in the HSP group. Creatine kinase functions to reversibly catalyze the transfer of a phosphoryl group to adenosine diphosphate, through the phosphagen system, from phosphocreatine to produce ATP and creatine during postmortem anaerobic metabolism (Westerblad et al., 2010; Tymoczko et al., 2013). Mitochondrial isocitrate dehydrogenase subunit alpha was 25% more abundant (Figure 2; $P = 0.06$) in the HSP samples. Isocitrate dehydrogenase catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate in the citric acid cycle (Tymoczko et al., 2013). This is the rate-limiting step of the tricarboxylic acid cycle (Tymoczko et al., 2013).

In the refined pH range, 71% greater abundance (Figure 2; $P < 0.01$) of mitochondrial ATP synthase subunit beta was observed in the HSP group. This protein is an enzyme functionally active in the production of ATP through the use of a proton gradient in the electron transport chain (Stock et al., 1999). Specifically, the beta subunit is the water-soluble, catalytic domain. As demonstrated by Matarneh et al. (2018), this subunit in in vitro conditions is shown to extend ultimate pH decline when an inhibitor of ATP hydrolysis is present. Thus, a greater abundance of mitochondrial ATP synthase subunit beta in the sarcoplasmic fraction could help to explain why the HSP samples may have had a lower ultimate pH. Combined, these proteins could identify different isoforms that could be impacting postmortem metabolism and the extent of postmortem glycolysis.

Other proteins

Two proteins were identified in the corresponding 3–10 pH range, mitochondrial aldehyde dehydrogenase (Spot 246) and adenylate kinase isoenzyme 1 (Spot 244). Proteins found in experiment 2 included myc box-dependent-interacting protein 1 (Spot 214), mitochondrial dihydrolipoyl dehydrogenase (Spot 244), mitochondrial aldehyde dehydrogenase (Spot 264), annexin A7 (Spot 272) and annexin A5 (Spot 371), protein disulfide-isomerase (Spot 256), and mitochondrial isocitrate dehydrogenase subunit alpha (Spot 310). Spots of mitochondrial aldehyde dehydrogenase in both pH ranges demonstrated 114% and 32% greater abundance (Figures 1–2; $P = 0.14$ and 0.02; respectively) in the HSP group. This enzyme functions to protect cells from oxidative stress by catalyzing the oxidation of acetaldehydes to acetate (Jelski and Szmitkowski, 2008). The greater abundance of both protein spots could indicate cellular stress in the HSP group that may impact tenderness development.

Adenylate kinase is a phosphotransferase used to maintain homeostasis in the cell through the reversible reaction of ATP and adenosine monophosphate to 2 molecules of adenosine diphosphate (Janssen et al., 2003; Dzeja and Terzic, 2009). It was reported that the abundance of adenylate kinase increased over 24 h post mortem in beef *longissimus thoracis* muscle (Jia et al., 2007). However, the abundance of adenylate kinase was lesser in beef aged 7 d compared with beef aged 0 d (Oliveira et al., 2019). Laville et al. (2007) reported a greater abundance of adenylate kinase in the sarcoplasmic fraction of tough pork chops compared with tender pork chops (Laville et al., 2007). Comparable results in our study demonstrated a 108% greater abundance ($P = 0.08$) of adenylate kinase (Spot 444) in the HSP group. This greater abundance of adenylate kinase could mean a greater need for ATP production in the HSP group. Many of these proteins originate in the mitochondria. Obtaining a greater understanding of what causes this location change and their impact on meat quality development is needed.

Stress response proteins

In the refined pH 4–7 range, 3 proteins were identified related to stress response: peroxiredoxin-2 (fragment; Spot 409; Figure 2), Hsc 70-interacting protein (Spot 273), and heat shock cognate 71-kDa protein (Spot 161). Peroxiredoxin-2 was numerically (25%) more abundant ($P = 0.20$) in the HSP group. To confirm the results from the 2D-DIGE experiment, peroxiredoxin-2 abundance was quantified using one-dimensional western blots on the sarcoplasmic extracts at each day of aging. A representative western blot of peroxiredoxin-2 analysis and the corresponding star probe values for those specific samples is shown in Figure 5. Peroxiredoxin is a family of peroxidases that protect against oxidative damage or are involved in signaling through regulation of hydrogen peroxide (Rhee et al., 2001). Specifically, peroxiredoxin-2 reduces the consequences of oxidative stress in cells by removing hydrogen peroxide produced from normal metabolism, influencing oxidative stress resistance (Rhee et al., 2001; Oláhová et al., 2008). Carlson et al. (2017a) reported a greater abundance of peroxiredoxin-2 in

Star probe value (kg)	6.54	4.13	5.25	5.77	11.32	8.15	7.32	7.39
Days Aged	1	8	14	21	1	8	14	21
Category	Low	Low	Low	Low	High	High	High	High

Figure 5. Representative western blot of peroxiredoxin-2 monomer in pork *longissimus dorsi* (LM) whole-muscle samples aged over time. The band (22 kDa) was compared to corresponding band of a 1-d aged pork LM sample (reference). Star probe values (kg) are provided for the samples, and samples are labeled high or low depending on star probe force.

HSP aged pork loins at 14-d aging compared with LSP pork loins, potentially impacted by oxidative stress early post mortem. In the current study, peroxiredoxin-2 abundance decreased between 1- and 8-d aging (Table 1; $P < 0.01$) in LSP and did not change with further days' aging. In HSP, peroxiredoxin-2 abundance decreased between 8- and 14-d aging ($P < 0.01$). No differences between classification groups at 1-d aging were observed. The HSP group maintained a greater abundance of peroxiredoxin-2 at 8- and 21-d aging compared with the LSP group ($P < 0.01$), potentially a response to oxidative stress. However, the role of peroxiredoxin-2 regarding meat tenderness remains unclear, warranting further investigation with oxidative stress and the impact on meat tenderness or proteolysis.

Heat shock cognate 71-kDa protein (Spot 161; $P = 0.09$) and Hsc 70-interacting protein (Spot 273; Figure 2; $P = 0.20$) were 14% more abundant in the sarcoplasmic fraction of the LSP group. The Hsc 70-interacting protein functions as a binder at the ATPase domains of at least 2 heat shock cognate 70 molecules for activation (Ohtsuka and Suzuki, 2000). Heat shock cognate 71 was more abundant in sarcoplasmic fraction of beef *longissimus lumborum* and *triceps brachii* after aging 7 d (Oliveira et al., 2019). Heat shock cognate 71 protein plays a crucial role in the initial folding of myosin as well as the assembly of myosin through its chaperoning function (Srikakulam and Winkelmann, 2004). Understanding the role of stress response proteins on postmortem meat is key to being able to predict tenderness variations.

Regulatory proteins

The only regulatory protein identified in the sarcoplasmic protein fraction was myosin regulatory light chain 2, which was 46% more abundant (Spot 462; Figure 2; $P = 0.05$) in the HSP group. Myosin light chains are important for regulation of muscle contraction (Weeds and Lowey, 1971; Clark et al., 2002). The light chains may be phosphorylated, impacting the rate and extent of force being produced (Perrie et al., 1973; Sweeney et al., 1993). In beef bulls, the sarcoplasmic fraction of the *longissimus* muscle was analyzed between tough and tender samples based on the 7-d shear force value (Bjarnadóttir et al., 2012). Three spots of myosin regulatory light chain 2 were found, two of which were more abundant in the tender group and the other of which was more abundant in the tough samples (Bjarnadóttir et al., 2012). A similar beef study analyzing the *longissimus* myofibrils of aged (36 h) samples identified three myosin light chain 2 proteins within bands as being negatively associated and three myosin light chain 2 proteins as being positively associated with Warner-Bratzler shear force values at 36 h (Zapata et al., 2009). Myosin light chain 2 fragment abundance was found to be correlated to 1- and 4-d Warner-Bratzler shear force values (0.59 and 0.49, respectively) in pork *longissimus* muscle (Lametsch et al., 2003). The current results, in light of the published data, demonstrate that dynamic changes in myosin light chain 2 may be associated with the development of tenderness early post mortem.

Conclusions

The current study identified differences in the sarcoplasmic proteome 1 d post mortem to predict aged loin tenderness. Variations in star probe values were attributed to differences in pH, marbling, water-holding capacity, proteolysis, and sarcoplasmic protein profile at 1-d aging. The HSP group had greater abundance of metabolic, regulatory, and mitochondrial-associated proteins, whereas the LSP group had greater abundance of stress response proteins. The sarcoplasmic proteome analysis results confirm a difference in glycolytic metabolism capabilities between star probe

groups, thus demonstrating the need to investigate more deeply the role of metabolic and regulatory proteins in the development of pork tenderness. Identification of many mitochondrial proteins in the sarcoplasmic proteome may suggest solubilization of by-products of the mitochondrial electron transport chain (Matarneh et al., 2018). This difference in proteins may be due to a greater number of mitochondria or rupture of mitochondrial membrane; however, this was not tested. Once the status of these proteins is defined, robust protein biomarkers can identify products of differing tenderness.

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