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Intramuscular Variations in Color and Sarcoplasmic Proteome of Beef *Semimembranosus* during Postmortem Aging

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Abstract: Beef *semimembranosus* exhibits intramuscular difference in color stability, and the inside region (ISM) of the muscle is color-labile, whereas the outside region (OSM) is color-stable. Variations in sarcoplasmic proteins are known to contribute to this intramuscular color difference. Sarcoplasmic proteome and beef color are affected by postmortem aging. The objective of the present study was to examine the effect of aging on intramuscular color variations and the sarcoplasmic proteome of beef *semimembranosus*. *Semimembranosus* muscles obtained from 8 beef carcasses (n = 8) were subjected to aging at 2°C for 0, 7, 14, and 21 d. On each aging day, the muscles were fabricated into ISM and OSM steaks and allotted to refrigerated storage (2°C) under aerobic packaging. Instrumental color and metmyoglobin reducing activity were evaluated on d 0, 3, and 6 of storage. Samples frozen on d 0 and d 21 of aging were utilized for sarcoplasmic proteome analysis. Color attributes of both ISM and OSM steaks were influenced by aging, with steaks aged for 21 d having the lowest (P < 0.05) color stability. The ISM steaks had greater (P < 0.05) lightness than OSM counterparts, and the difference in lightness was not negated by aging. The ISM and OSM had similar (P > 0.05) redness on d 0 of storage, whereas ISM had lower (P < 0.05) redness compared to OSM on d 3 and d 6 of storage. Several proteins associated with glycolysis and energy metabolism were of greater abundance (P < 0.05) in OSM than in ISM after 21-d aging. Furthermore, the influence of 21-d aging on sarcoplasmic proteome was observed at a greater extent in OSM than in ISM, indicating that the effect of aging on sarcoplasmic proteome of beef *semimembranosus* was influenced by the location within the muscle.

Keywords: beef color, color stability, muscle-specificity, proteomics, sarcoplasmic proteome
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Introduction

Surface color and color stability are critical determinants of consumer acceptance of fresh meat, and have a significant economic impact on the US beef industry (Smith et al., 2000). Beef color stability is a muscle-specific trait, and various muscles in a beef carcass discolor at different rates (Hunt and Hedrick, 1977a; O'Keeffe and Hood, 1982; McKenna et al., 2005). From this standpoint, the biochemical bases of intermuscular variations in beef color have been studied extensively (McKenna et al., 2005; Seyfert et al., 2006; Joseph et al., 2012; Wu et al., 2016; Nair et al., 2018).

Semimembranosus is a large muscle in the beef hindquarter and exhibits intramuscular variation in

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color (2-toned color). Based on the color and location within the carcass, beef *semimembranosus* muscle can be separated into outside (OSM) and inside (ISM) regions (Hunt and Hedrick, 1977a). The OSM is darker in appearance and color-stable during retail display compared to ISM, which is lighter and color-labile (Sammel et al., 2002a). The ISM chills at a slower rate than OSM during postmortem storage due to its internal location within the carcass, leading to variations in temperature and pH within the same muscle (Sammel et al., 2002a). Tarrant (1977) observed pH values below 6.0 in ISM even when carcass temperature was above 30°C.

Proteomic approaches have characterized the fundamental basis of color stability variations in fresh beef, such as muscle-specificity (Joseph et al., 2012; Wu et al., 2016; Nair et al., 2018) and animal effect (Canto et al., 2015). Previous investigations (Nair et al., 2016) on beef ISM and OSM steaks (48 h postmortem) reported an increased abundance of glycolytic enzymes in sarcoplasmic proteome of ISM, which could possibly lead to a faster pH drop in ISM during the postmortem period while the carcass temperature is still high. This high temperature-low pH combination is known to denature muscle proteins leading to pale, soft, and exudative-like conditions in beef (Hunt and Hedrick, 1977a, 1977b; Fischer and Hamm, 1980; Aalhus et al., 1998; Warner et al., 2014; Kim et al., 2014). Furthermore, such conditions promote myoglobin oxidation and denaturation, thereby compromising meat color stability (Suman and Joseph, 2013; Faustman and Suman, 2017; Neethling et al., 2017).

Postmortem aging is a common industry practice to improve beef quality attributes such as flavor and tenderness. However, aging can also influence the initial color intensity (bloom) and the color stability of meat during subsequent retail display (Lee et al., 2008; Mancini and Ramanathan, 2014; English et al., 2016). Marino et al. (2014) reported that aging can affect color, pH, and water holding capacity of meat by altering the sarcoplasmic protein patterns. Moreover, recent research (Nair et al., 2018) reported that aging affects intermuscular variations in color and the sarcoplasmic proteome profile. However, the effect of aging on intramuscular color variations and the sarcoplasmic proteome profile of beef *semimembranosus* has not been examined yet. Therefore, the objective of the current study was to examine the effect of aging for 0, 7, 14, or 21 d on color attributes of beef ISM and OSM steaks. Further, the variations in the sarcoplasmic proteome profile of ISM and OSM after 0- and 21-d aging were examined.

Materials and Methods

Beef fabrication

Beef carcasses (n = 8; USDA Choice; A maturity; black-hided crossbred cattle) were obtained from the USDA-inspected meat laboratory at the University of Kentucky. At 24 h postmortem, *semimembranosus* muscles were separated from both sides of the carcasses and were divided into 2 equal-length sections, resulting in 4 sections per carcass. Each muscle section was randomly assigned to aging at 2°C for 0, 7, 14, or 21 d after vacuum packaging (99% vacuum; Sipromac Model 600A, Drummondville, Quebec, Canada) in Prime Source vacuum pouches (3 mil, Bunzl Koch Supplies Inc., Kansas City, MO). At the end of each aging period, the muscles were fabricated into 1.92-cm thick ISM and OSM steaks based on their location and visual color. During fabrication, samples for proteome analysis (approximately 50 g) were collected from 0-d aged and 21-d aged ISM and OSM, and were frozen in vacuum packaging at –80°C until protein extraction. Six steaks were placed individually in Styrofoam trays, were overwrapped with oxygen-permeable polyvinyl chloride film (15,500 to 16,275 cm^3/m^2 per 24 h oxygen transmission rate at 23°C), and were allocated for refrigerated storage (2°C) in darkness (Mancini et al., 2009). Two steaks were utilized on each storage day for evaluating instrumental color and metmyoglobin reducing activity.

Instrumental color

The instrumental color attributes (CIE L^*, a^*, and b^* values) were evaluated using a HunterLab LabScan XE colorimeter (Hunter Associates Laboratory, Reston, VA) with a 2.54 cm diameter aperture, illuminant A, and 10° standard observer. The steaks were allowed to bloom for 2 h (at 2°C) after fabrication on d 0 of storage (and on each aging day) before color measurement. The color readings were obtained from 3 random locations and averaged. The colorimeter was calibrated with standard black and white plates. Ratio of reflectance at 630 nm and 580 nm (R630/580) obtained from the colorimeter was used to estimate the surface color stability (American Meat Science Association, 2012).

Metmyoglobin reducing activity (MRA)

Metmyoglobin reducing activity (MRA) was evaluated using methodology by Sammel et al. (2002b). Cubes of meat (2.5 × 2.5 × 2.5 cm) with minimal fat

and connective tissue were obtained from the surface of the steaks and immersed in 0.3% sodium nitrite solution (Sigma-Aldrich, St. Louis, MO) for 20 min. This facilitated conversion of the surface myoglobin to metmyoglobin. After 20 min, samples were removed from the solution, blotted dry, and vacuum packaged. The surface reflectance spectra from 700 to 400 nm were obtained immediately after vacuum packaging using HunterLab LabScan XE colorimeter and were used to calculate the surface metmyoglobin percentage (pre-incubation). The samples were incubated at 30°C for 2 h to facilitate metmyoglobin reduction. After incubation, reflectance data were obtained again to calculate the post-incubation metmyoglobin percentages (American Meat Science Association, 2012). The MRA was calculated using the equation: MRA = 100 × [(% pre-incubation surface metmyoglobin – % post-incubation surface metmyoglobin) / % pre-incubation surface metmyoglobin].

Isolation of sarcoplasmic proteome

The ISM and OSM samples aged for 0-d (ISM0 and OSM0) and 21-d (ISM21 and OSM21) were used for sarcoplasmic proteome profiling. Samples (n = 8) frozen at –80°C during fabrication were used for proteome isolation as described previously (Joseph et al., 2012). Five-gram frozen muscle tissue devoid of any visible fat and connective tissue was homogenized in 25 mL ice-cold extraction buffer (40 mM Tris, 5 mM EDTA, pH 8.0) using a blender (Waring Commercial, Torrington, CT). The homogenate was further centrifuged at 10,000 × g for 15 min. After centrifugation, the supernatant was filtered using Whatman No. 1 filter paper (GE Healthcare, Little Chalfont, UK), and the sarcoplasmic proteome extract obtained was utilized for subsequent analysis.

Two-dimensional electrophoresis (2-DE)

Bradford assay using the Bio-Rad Protein Assay kit (Bio-Rad Lab., Hercules, CA) was utilized to determine the protein concentration of the sarcoplasmic proteome extract from each sample (Bradford, 1976). The sarcoplasmic proteome (900 µg) was mixed with rehydration buffer (7 M urea, 2 M thiourea, 20 mM DTT, 4% CHAPS, 0.5% Bio-Lyte 5/8 ampholyte, and 0.001% Bromophenol blue) and was loaded on to immobilized pH gradient (IPG) strips (pH 5 to 8, 17 cm; Bio-Rad Lab.). The strips were subjected to passive rehydration for 16 h. Isoelectric focusing (first dimension separation) allows separation of proteins based on isoelectric point and was done using a Protean IEF Cell system (Bio-Rad Lab.). A low voltage (50 V) was applied during the initial active rehydration (4 h), followed by a linear increase in voltage and a final rapid voltage ramping to attain a total of 60 kVh. The IPG strips were then equilibrated with equilibration buffer I [6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT; Bio-Rad Lab.] followed by equilibration buffer II [6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide; Bio-Rad lab.] for 15 min each. The second dimension was resolved based on molecular weight on 13.5% SDS polyacrylamide gel (38.5:1 ratio of acrylamide to bis-acrylamide) using a Protean II Multicell electrophoresis system. The equilibrated strips were loaded on to the gel using an agarose overlay, and electrophoresis was run using running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS). A constant voltage of 100 V was applied for approximately 16 h for separation of proteins in the second dimension. Each sample (ISM or OSM; n = 8) on each aging day (0 or 21) was run in duplicate. The gels were washed in deionized water for 15 min and stained for 48 h using Colloidal Coomassie Blue to visualize the protein spots. Gels were then destained in deionized water for 48 h or until sufficient background clearing was achieved.

Gel image analysis

VersaDoc imager (Bio-Rad Lab.) was used to obtain the gel images, and PDQUEST software (Version 7.3.1; Bio-Rad Lab.) was used for subsequent image analysis. Three comparisons were made during image analysis: (1) ISM and OSM after 21-d aging (ISM21 vs. OSM21); (2) OSM before and after 21-d aging (OSM0 vs. OSM21); and (3) ISM before and after 21-d aging (ISM0 vs. ISM21). The sarcoplasmic proteome analysis of ISM and OSM before aging (ISM0 vs. OSM0) has been reported previously (Nair et al., 2016), and therefore was not repeated in the current study. All gel images were processed and analyzed under similar parameters. Images were first subjected to automatic spot detection and matching optimized by the aid of landmark protein spots, and the matched spots were normalized as a percentage of the total volume of all spots detected on the gel. A spot was considered differentially abundant when it demonstrated 1.5–fold intensity difference between the treatments and was associated with $P < 0.05$ in a pairwise Student's t test.

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI–MS/MS) analysis

Duplicate spots from the corresponding gels were subjected to mass spectrometric analysis for confirma-

tion of protein identity. The protein spots differentially abundant between the treatments were excised from the gel and subjected to dithiothreitol reduction, iodoacetamide alkylation, and in-gel trypsin digestion. The resulting peptides were extracted, concentrated, and injected for nano-LC–MS/MS analysis using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with an Eksigent Nanoflex cHiPLC system (Eksigent, Dublin, CA) through a nano electrospray ionization source. The separation of peptides was achieved using a reverse phase cHiPLC column (75 µm × 150 mm) operated at a flow rate of 300 nL/min. Mobile phase A consisted of water with 0.1% (v/v) formic acid, and mobile phase B contained acetonitrile with 0.1% (v/v) formic acid. A 50 min gradient was applied: initial 3% mobile phase B was increased linearly to 50% in 24 min and further to 85% and 95% for 5 min each before it was decreased to 3%, and the column was re-equilibrated. The mass analysis method consisted of 8 scan events per segment. The first scan event was an Orbitrap MS scan (100 to 1,600 m/z) with 60,000 resolutions for parent ions which was followed by data dependent MS/MS for fragmentation of the 7 most intense ions through collision induced dissociation.

MS/MS protein identification

For MS/MS protein identification, the LC–MS/MS data were submitted to a local Mascot server via Proteome Discoverer (version 1.3, Thermo Fisher Scientific) against a *Bos taurus* database from National Center for Biotechnology Information. The MASCOT MS/MS ion search was done using the following parameters: trypsin digest with a maximum of 2 miscleavages, cysteine carbamidomethylation, methionine oxidation, a maximum of 0.001% MS error tolerance, and a maximum of 0.8 Da MS/MS error tolerance. A decoy database was built and searched. Filter settings that determine false discovery rates (FDR) were used to distribute the confidence indicators for the peptide matches. Peptide matches that passed the filter associated with the strict FDR (target setting of 0.01) were assigned as high confidence. For the MS/MS ion search, proteins with 2 or higher confidence peptides were considered unambiguous identifications without manual inspection. Proteins identified with one high confidence peptide were manually inspected and confirmed.

Statistical analysis

The ISM and OSM from 8 beef carcasses (n = 8) were utilized in the experiment. The experimental design was a split-split plot with muscle source (ISM and OSM) as whole plot and aging time (0, 7, 14, and 21 d) as subplot. For color characteristics, storage day (0, 3, and 6) was set as sub-sub plot. Carcass was considered as random effect. The data were analyzed using PROC MIXED procedure of SAS (Version 9.4; SAS Inst. Inc., Cary, NC), and the differences among the means were detected using the least significant difference at 5% level.

Results and Discussion

Instrumental color and biochemical attributes

There was no 3-way interaction (aging × muscle × storage) for lightness (L^* value) of the steaks (P = 0.6092; Table 1). Muscle specificity (P = 0.0002) in L^* value was observed, with ISM having greater lightness than OSM throughout the storage. The difference in lightness between the 2 regions of *semimembranosus* muscle is one of the most distinguishable characteristics, with ISM looking paler in color compared to darker OSM. Aging for 21-d was unable to negate the difference in lightness between ISM and OSM. The L^* value of the muscles increased with aging (P < 0.0001), with the 7-d aged steaks having greater (P = 0.0315) lightness than non-aged (0-d aged) steaks. However, there was no significant increase in lightness with further aging; i.e., the 7-d aged samples had similar lightness as the 14-d and 21-d aged samples. The L^* value decreased during storage for 6 d (P < 0.0001), and these results are in agreement with our previous findings (Nair et al., 2016) and those of Sammel et al. (2002a) on ISM and OSM.

Table 1. Effect of aging on surface lightness (L^* value) of beef inside (ISM) and outside *semimembranosus* (OSM) steaks (n = 8) during refrigerated storage (2°C) for 6 d under aerobic packaging (SEM = 1.0384)

	Muscle	Storage d (S)				
Aging (A)	(M)	0	3	6	*P*-values	
0 d	ISM	47.65	47.25	47.28	A	0.0037
	OSM	39.10	41.80	41.02	M	0.0002
					S	< 0.0001
7 d	ISM	50.55	48.76	48.15	A × M	0.9695
	OSM	42.08	41.67	41.58	A × S	< 0.0001
					M × S	0.0005
14 d	ISM	51.54	49.28	47.19	A × M × S	0.6092
	OSM	42.99	43.07	40.65		
21 d	ISM	52.00	49.59	48.76		
	OSM	44.06	43.90	40.78		

The surface redness (*a** value) of ISM and OSM after aging for 0, 7, 14, or 21 d is presented in Table 2. There was no 3-way interaction of aging, muscle, and storage ($P = 0.5259$). However, aging × muscle ($P = 0.0076$), aging × storage ($P = 0.0005$), and muscle × storage ($P < 0.0001$) interactions were observed for surface redness. Although ISM and OSM had similar redness before aging ($P = 0.6689$), OSM had greater redness ($P < 0.05$) than ISM after aging for 7, 14, and 21 d. Mancini and Ramanathan (2014) reported that the initial color intensity (bloom) of beef *longissimus lumborum* increased with longer aging time because of decreased mitochondrial oxygen consumption. However, these authors reported that color stability was compromised during aging due to the negative effects of storage time on mitochondria-mediated metmyoglobin reduction. Lee et al. (2008) reported that postmortem aging beyond 14 d adversely affected the redness of beef *gluteus medius*.

The ISM and OSM exhibited similar ($P = 0.4666$) redness on d 0 of storage after all aging times, whereas ISM had lower surface redness than OSM on d 3 ($P = 0.0023$) and d 6 ($P = 0.0027$) of storage. This observation is consistent with classification of OSM steaks as color-stable and ISM steaks as color-labile. Although not statistically different, the 0-d aged ISM had a 3-unit greater *a** value than 0-d aged OSM (32.75 in ISM vs. 29.13 in OSM) on d 0 of storage, suggesting the possibility of difference in their blooming capacity. However, the difference in blooming was not observed after aging for 7-d. These results indicate that postmortem aging of *semimembranosus* muscle for at least 7-d could possibly aid in minimizing the difference in the initial redness between ISM and OSM.

In agreement with our results, previous research has also reported a greater redness in ISM on the first day of display (no aging), whereas OSM had greater redness during later display days (Sammel et al., 2002a; Nair et al., 2016). Sammel et al. (2002a) attributed this difference to the greater oxygen consumption rate (OCR) of OSM compared to ISM. The greater OCR in OSM limits the oxygen available for myoglobin oxygenation resulting in lower blooming than in ISM. However, OCR measurements were not performed in the current study.

Yellowness (*b** value) of the steaks was affected by storage time ($P < 0.0001$; Table 3). The ISM had greater yellowness ($P = 0.0004$) on d 0 of storage compared to OSM. However, ISM and OSM demonstrated similar ($P > 0.05$) yellowness on d 3 and 6 of storage. Moreover, ISM exhibited greater yellowness than OSM after 0-d aging, whereas both ISM and OSM had similar ($P > 0.05$) yellowness on rest of the aging days. Our observations are in agreement with previous research which also reported greater *b** values in ISM than OSM on d 0 of display (Nair et al., 2016; Sammel et al., 2002a).

The color stability (R630/580) of ISM and OSM during storage after aging for 0, 7, 14, and 21 d is presented in Table 4. The ISM steaks had lower ($P = 0.0123$) color stability compared to OSM steaks, in agreement with the classification of ISM as color-labile and OSM as color-stable. The 21-d aged steaks had lower color stability ($P < 0.05$) compared to their 0-, 7-, and 14-d aged counterparts.

Metmyoglobin reducing activity was also muscle-specific, with OSM having greater MRA values than ISM (Table 5; $P = 0.0001$). The MRA values de-

Table 2. Effect of aging on surface redness (*a** value) of beef inside (ISM) and outside *semimembranosus* (OSM) steaks ($n = 8$) during refrigerated storage (2°C) for 6 d under aerobic packaging (SEM = 1.003)

	Muscle	Storage d (S)				
Aging (A)	(M)	0	3	6	*P*-values	
0 d	ISM	32.75	26.46	23.19	A	0.0762
	OSM	29.13	26.75	25.17	M	0.0838
					S	< 0.0001
7 d	ISM	32.46	25.74	20.62	A × M	0.0076
	OSM	32.31	30.31	24.50	A × S	0.0005
					M × S	< 0.0001
14 d	ISM	32.68	25.39	21.22	A × M × S	0.5259
	OSM	32.94	28.53	24.52		
21 d	ISM	31.34	25.08	19.50		
	OSM	32.08	28.85	21.93		

Table 3. Effect of aging on surface yellowness (*b** value) of beef inside (ISM) and outside *semimembranosus* (OSM) steaks ($n = 8$) during refrigerated storage (2°C) for 6 d under aerobic packaging (SEM = 0.6699)

	Muscle	Storage d (S)				
Aging (A)	(M)	0	3	6	*P*-values	
0 d	ISM	25.92	22.14	20.35	A	0.0852
	OSM	21.21	20.62	19.63	M	0.1474
					S	< 0.0001
7 d	ISM	25.71	22.20	19.82	A × M	0.0131
	OSM	24.43	23.46	19.73	A × S	0.0092
					M × S	0.0006
14 d	ISM	25.78	21.80	19.95	A × M × S	0.1153
	OSM	25.20	21.73	19.93		
21 d	ISM	25.10	21.45	19.05		
	OSM	24.51	22.47	18.66		

creased with storage in OSM ($P < 0.0001$). However, in ISM, there was an increase ($P < 0.05$) in the observed MRA values on d 6 of storage compared to d 3 when the steaks were aged for 7, 14, or 21 d. Such an increase was not observed in the 0-d aged ISM steaks. A similar trend in MRA values was observed during the later stages of storage in the color-labile *psoas major* muscle after aging (Nair et al., 2018). One possible explanation for this unexpected observation is that the combination of aging and long-term storage of an already color-labile ISM leads to extensive mitochondrial damage and the release of mitochondrial reducing enzymes, which in turn could lead to the observed increase in MRA values. Nonetheless, mitochondrial degradation and functionality were not examined to confirm this possibility.

Table 4. Effect of aging on color stability (R630/580) of beef inside (ISM) and outside *semimembranosus* (OSM) steaks ($n = 8$) during refrigerated storage (2°C) for 6 d under aerobic packaging (SEM = 0.3053)

	Muscle	Storage d (S)				
Aging (A)	(M)	0	3	6	*P*-values	
0 d	ISM	5.43	3.38	2.65	A	0.050
	OSM	5.71	4.00	3.47	M	0.0123
					S	< 0.0001
7 d	ISM	5.39	3.20	2.27	A × M	0.2569
	OSM	5.91	5.03	3.35	A × S	0.2067
					M × S	0.0548
14 d	ISM	4.98	3.27	2.73	A × M × S	0.0580
	OSM	6.16	4.40	3.22		
21 d	ISM	4.44	3.06	2.11		
	OSM	5.73	4.40	2.88		

Table 5. Effect of aging on metmyoglobin reducing activity (MRA; %) of beef inside (ISM) and outside *semimembranosus* (OSM) steaks ($n = 8$) during refrigerated storage (2°C) for 6 d under aerobic packaging (SEM = 6.1920)

	Muscle	Storage d (S)				
Aging (A)	(M)	0	3	6	*P*-values	
0 d	ISM	39.76	20.22	19.25	A	0.2630
	OSM	68.23	46.03	43.99	M	0.0001
					S	< 0.0001
7 d	ISM	28.13	9.57	20.53	A × M	0.9486
	OSM	59.12	50.75	30.34	A × S	0.2081
					M × S	0.0021
14 d	ISM	23.75	10.51	33.55	A × M × S	0.5521
	OSM	60.16	38.15	41.51		
21 d	ISM	22.10	11.63	25.97		
	OSM	59.84	43.30	40.32		

Sarcoplasmic proteome variations between ISM and OSM aged for 21 d

The sarcoplasmic proteome profile of ISM and OSM after aging for 21 d (ISM21 vs. OSM21) was compared for determining the effect of muscle source. Image analysis identified 6 protein spots differentially abundant between 21-d aged ISM and OSM steaks, and 3 proteins were identified by mass spectrometric analysis in these spots (Table 6). The representative gel images of sarcoplasmic proteome of ISM and OSM during aging are presented in Fig. 1. Multiple protein spots with similar molecular weight, but different isoelectric point (pI), were identified as the same protein, which could be attributed to the occurrence of protein isoforms or post-translational modifications such as phosphorylation (Canto et al., 2015; Anderson et al., 2014). However, protein phosphorylation was not analyzed in the present study to confirm this possibility.

The differentially abundant proteins were triosephosphate isomerase, β-enolase, and creatine kinase M-type. Triosephosphate isomerase is a glycolytic enzyme, which catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Albery and Knowles, 1976). The 2 spots identified as triosephosphate isomerase were more abundant in OSM21 than in ISM21 (Table 6). Creatine kinase reversibly catalyzes the transfer of phosphate between ATP and creatine phosphate, and plays a critical role in energy metabolism in postmortem muscles. Creatine phosphate is utilized for the production of ATP with concomitant production of creatine in postmortem skeletal muscles. Previous research indicated that creatine has antioxidant properties (Sestili et al., 2011; Lawler et al., 2002), which could be beneficial to minimize myoglobin oxidation in fresh meat. In the present study, 2 spots identified as creatine kinase were more abundant in color-stable OSM21, which indicated the antioxidant effect of this protein in fresh beef. Beta-enolase is also a glycolytic enzyme catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate, the ninth step of glycolysis (Hoorn et al., 1974), and was more abundant in OSM21.

Our results are in agreement with previous research (Nair et al., 2016) on the sarcoplasmic profile variations in ISM and OSM (48 h postmortem, no aging) that reported a greater abundance of triosephosphate isomerase and creatine kinase M-type in OSM than ISM. The trend in the abundance of these

Table 6. Differentially abundant sarcoplasmic proteins in beef inside (ISM) and outside *semimembranosus* (OSM) steaks (n = 8) after aging for 21 d

Protein	Accession number	Score/matched peptides	Sequence coverage, %	Over-abundant	Spot ratio (OSM21/ISM21)	Function
Triosephosphate isomerase	Q5E956	5679.71/30	93.98	OSM21	1.51	Glycolytic enzyme
Triosephosphate isomerase	Q5E956	5937.13/30	93.98	OSM21	1.56	Glycolytic enzyme
Beta-enolase	Q3ZC09	5035.90/60	77.88	OSM21	1.71	Glycolytic enzyme
Creatine kinase M-type	Q9XSC6	5929.44/57	84.25	OSM21	1.73	Energy metabolism
Creatine kinase M-type	Q9XSC6	8923.87/60	83.73	OSM21	2.20	Energy metabolism
Beta-enolase	Q3ZC09	3250.62/52	70.74	OSM21	1.65	Glycolytic enzyme

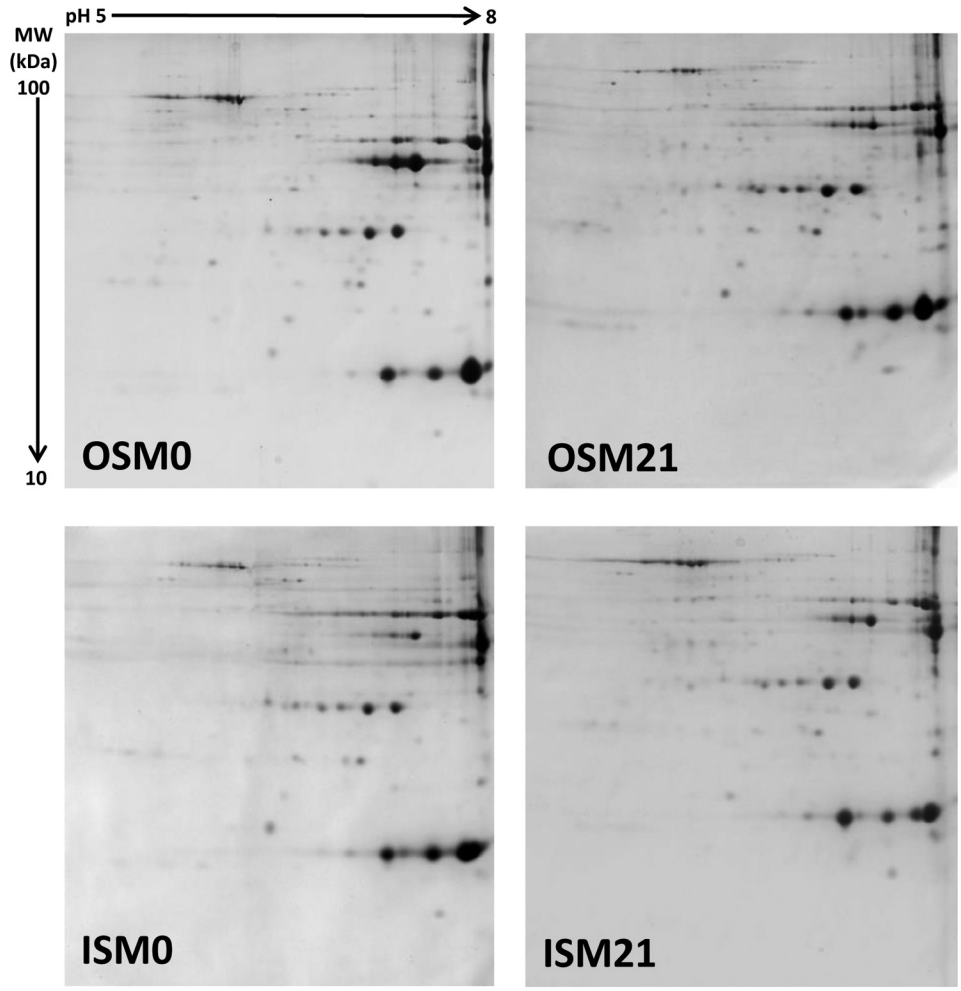

Figure 1. Representative 2-dimensional gel electrophoresis maps of sarcoplasmic proteome extracted from beef inside (ISM) and outside *semimembranosus* (OSM) after 0 (OSM0 and ISM0) and 21 (OSM21 and ISM21) d of postmortem aging separated using an immobilized pH gradient (IPG) 5 to 8 strip in the first dimension and 13.5% SDS gel in the second dimension.

enzymes was not influenced by aging for 21 d in the present study. However, Nair et al. (2016) reported a greater abundance of β-enolase in ISM compared to OSM. In the present study, this trend reversed, and OSM had greater abundance of β-enolase after 21 d of aging. Further, Nair et al. (2016) reported greater abundance of other glycolytic enzymes such as fructose bisphosphate aldolase A and phosphoglycerate mutase 2 in ISM compared to OSM. However, these enzymes were not detected as differentially abundant after 21-d aging.

In the present study, OSM21 had greater redness (P = 0.0314) and greater abundance of the triosephosphate isomerase, β-enolase, and creatine kinase than ISM21.

Joseph et al. (2012) also reported a greater abundance of β-enolase and creatine kinase in color-stable beef *longissimus lumborum* steaks than in color-labile *psoas major* steaks. Moreover, recently, Nair et al. (2018) reported that the color-stable *longissimus lumborum* and *semitendinosus* steaks had greater abundance of glycolytic enzymes and creatine kinase compared to *psoas major* steaks during postmortem aging.

Effect of 21-d aging on sarcoplasmic proteome of OSM

Comparison of the proteome profile of OSM before and after 21 d aging (OSM0 vs. OSM21) identified 10 protein spots as differentially abundant ($P < 0.05$; Table 7). The proteins identified were protein deglycase DJ-1, triosephosphate isomerase, myoglobin, creatine kinase M-type, and polyubiquitin-C. In general, these proteins, except for creatine kinase, were more abundant in aged OSM (OSM21) compared to non-aged OSM (OSM0). Multiple protein spots were identified as the same protein, and 2 protein spots identified as creatine kinase were more abundant in OSM0. However, triosephosphate isomerase, a glycolytic enzyme, was more abundant in OSM21 compared to OSM0.

Creatine kinase and triosephosphate isomerase were the only proteins associated with energy metabolism to be differentially abundant between OSM0 and OSM21. Overall, a differential abundance of proteins related to energy metabolism has been previously reported (Joseph et al., 2012; Canto et al., 2015) to be associated with differences in beef color stability, with color-stable steaks demonstrating greater abundance of these proteins than their color-labile counterparts. However, in the present study, both OSM0 and OSM21 steaks had similar redness after 21-d aging ($P = 0.3837$). In this perspective, the differential abundance of creatine kinase and triosephosphate isomerase observed in OSM0 and OSM21 is not readily explained.

Protein deglycase DJ-1 is a molecular chaperone that protects cells against oxidative stress and cell death by acting as a redox-sensitive chaperone and protease. This protein was differentially abundant in the sarcoplasmic proteome in relation to beef color, with a greater abundance in color-labile *psoas major* steaks compared to color-stable *longissimus lumborum* ones (Joseph et al., 2012). Chaperones play an important role in preventing protein aggregation and protein denaturation occurring during muscle-to-meat conversion (Sayd et al., 2006). Polyubiqutin-C was more abundant in OSM21 (Table 7). Although, ubiquitin contributes to regulation of many cellular events, its major function is to serve as a tag in the selective proteolysis of abnormal proteins by the 26S proteasome (Ciechanover et al., 1980; Jennissen, 1995). Both polyubiqutin-C and protein deglycase DJ-1 have been previously identified to be more abundant in 7-d aged *semitendinosus* compared to non-aged *semitendinosus* (Nair et al., 2018).

Three different protein spots identified as myoglobin were of greater abundance in OSM21, which indicated that the abundance of myoglobin isoforms within the same muscle varied during aging. However, biochemical analyses indicated no differences ($P > 0.05$) in myoglobin concentration between OSM0 (5.37 mg/g) and OSM21 (4.74 mg/g). The myoglobin spots of interest in the gels had similar molecular mass, but with different isoelectric point (pI), which is indicative of post-translational modifications such as phosphorylation (Anderson et al., 2014). Previous research also reported differential abundance of myo-

Table 7. Differentially abundant sarcoplasmic proteins in beef outside *semimembranosus* (OSM) steaks ($n = 8$) during aging (0 and 21) days

Protein	Accession number	Score/matched peptides	Sequence coverage, %	Over-abundant	Spot ratio (OSM0/OSM21)	Function
Protein deglycase DJ-1	Q5E946	245.59/21	72.49	OSM21	0.54	Chaperone
Triosephosphate isomerase	Q5E956	2366.47/25	93.98	OSM21	0.61	Glycolytic enzyme
Triosephosphate isomerase	Q5E956	3346.37/24	81.93	OSM21	0.55	Glycolytic enzyme
Myoglobin	P02192	122.70/12	63.64	OSM21	0.43	Oxygen transport
Protein deglycase DJ-1	Q5E946	567.35/33	97.35	OSM21	0.55	Chaperone
Creatine kinase M-type	Q9XSC6	2370.29/51	66.40	OSM0	1.90	Energy metabolism
Myoglobin	P02192	875.10/19	78.57	OSM21	0.66	Oxygen transport
Creatine kinase M-type	Q9XSC6	8567.76/70	87.14	OSM0	2.03	Energy metabolism
Myoglobin	P02192	2941.16/26	99.35	OSM21	0.50	Oxygen transport
Polyubiquitin-C	P0CH28	266.54/14	93.91	OSM21	0.39	Ubiquitylation

Table 8. Differentially abundant sarcoplasmic proteins in beef inside *semimembranosus* (ISM) steaks (n = 8) during aging (0 and 21) days

Protein	Accession number	Score/matched peptides	Sequence coverage, %	Over-abundant	Spot ratio (ISM0/ISM21)	Function
Beta-enolase	Q3ZC09	6855.75/61	85.94	ISM0	3.47	Glycolytic enzyme
Myoglobin	P02192	6636.18/30	99.35	ISM0	1.58	Oxygen transport

globin isoforms between color-stable and color-labile beef *longissimus lumborum* (Canto et al., 2015). These authors reported a greater abundance of a myoglobin isoform in color-labile steaks compared to color-stable steaks, suggesting that myoglobin post-translational modifications may influence meat color. In spite of the difference in the sarcoplasmic proteome profile, there were limited differences in the color of OSM before and after 21-d aging.

Effect of 21-d aging on sarcoplasmic proteome of ISM

Sarcoplasmic proteome profiles of ISM0 vs. ISM21 were compared to examine the effect of 21-d aging. There were only limited changes in the sarcoplasmic proteome of ISM with 21-d aging. The 2 protein spots identified as differentially abundant were β-enolase and myoglobin (Table 8), and were of greater abundance in ISM0. Marino et al. (2014) reported that the abundance of β-enolase decreased with aging in *longissimus dorsi* of Italian beef cattle breeds. However, β enolase was not differentially abundant in OSM during aging (Table 7). Myoglobin was more abundant in non-aged ISM (ISM0) compared to aged ISM (ISM21). However, biochemical analyses indicated no differences ($P > 0.05$) in myoglobin concentration between ISM0 and ISM21 (data not presented). In contrast, myoglobin isoforms were of greater abundance in OSM21 (compared to OSM0; Table 7). Further, the ISM0 steaks demonstrated greater redness ($P = 0.0031$) compared to ISM21, whereas the OSM0 and OSM21 steaks had similar redness. This reverse trend in ISM compared to OSM suggested that post-translational modifications of myoglobin could possibly be muscle-specific. The exact role of the myoglobin post-translational modifications in meat color is yet to be understood and needs further investigation.

Conclusions

The color attributes and sarcoplasmic proteome of beef ISM and OSM were affected by postmortem aging. Overall, the steaks aged for 21-d had lower color stability compared to other aging days. Nonetheless, the difference in lightness of ISM and OSM steaks was not negated by aging for 21 d. The influence of 21-d aging on sarcoplasmic proteome was observed at a greater extent in OSM than in ISM. Furthermore, after 21-d of aging, OSM had greater abundance of proteins and enzymes associated with glycolysis and energy metabolism compared to ISM, indicating that glycolytic and metabolic enzymes play a critical role in intramuscular variation in beef color attributes.

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