



Comparative proteomics to reveal muscle-specific beef color stability of Holstein cattle during post-mortem storage



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ABSTRACT

Label-free strategy was applied to elucidate muscle-specific beef (*M. longissimus lumborum* (LL) and *M. psoas major* (PM)) color stability of Holstein cattle during post-mortem storage at $4\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$. LL showed greater ($p < 0.05$) redness (a^*) value than PM at day 4 and 9 storage, while the proportion of metmyoglobin in PM exhibited a greater increase than in LL muscle. Furthermore, an overabundance of proteins with the functions of antioxidation, protection, and repair in LL were conducive to its color stability, whereas the overabundant proteins/subunits involved in tricarboxylic acid (TCA) cycle and mitochondrial electron transport chain (ETC) in PM indicated greater oxidative metabolism and degradation of ETC complexes, resulting in poor color stability. Bioinformatic analyses indicated that these proteins mainly participated in oxidation-reduction processes, TCA cycle, and ETC processes. All of these results provided a deeper understanding of muscle-specific beef color stability from the perspective of proteomics.

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1. Introduction

Protection of meat from discoloration is a formidable task for scientists and manufacturers, and there is much research concerning the complicated biological mechanisms involved in meat discoloration (Faustman, Sun, Mancini, & Suman, 2010; Suman & Joseph, 2013), and methods to improve meat color stability during post-mortem or retail display (Suman, Hunt, Nair, & Rentfrow, 2014).

Meat color is influenced by many extrinsic factors (temperature, O_2 availability, packaging, etc.) and intrinsic factors (breed, age of animal, muscle type, metabolism, the ultimate pH of meat, etc.) (Bekhit & Faustman, 2005). Different muscle types exhibit distinct color stability, which is attributed to their inherent muscle biochemical profile and metabolic function. Researchers have demonstrated that *longissimus lumborum* (LL) showed greater surface redness, color stability and metmyoglobin reducing activity during post-mortem storage than *psoas major* (PM), meanwhile, PM had higher degree of oxygen consumption rate, lipid oxidation and protein oxidation than LL muscle (McKenna et al., 2005; Seyfert et al., 2006). Therefore, LL and PM muscles can be used as models with opposing color stabilities (color-stable and

color-labile) to elucidate underlying mechanisms of discoloration during post-mortem storage.

However, meat is a complex matrix containing abundant proteins, lipid, carbohydrates and other compounds, and these compounds participate in complex biochemical and physiological changes during meat storage, which may alter meat quality attributes e.g. meat color. A promising omics strategy coupled with bioinformatics provides a new perspective to illustrate underlying mechanisms and discover potential biomarkers to improve meat quality. Proteomics is a pivotal tool to unravel the changes of muscle biochemistry taking place at the protein level during post-mortem. Two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry (MS) is a common approach used in meat quality research (Polati et al., 2012); it has been successfully used to investigate muscle-specific meat color stability (Joseph, Suman, Rentfrow, Li, & Beach, 2012; Wu et al., 2016), and interpret the mechanism of meat discoloration during storage (Canto et al., 2015; Wu et al., 2015). Label-free approach is known as a reliable, versatile, and cost-effective strategy (Neilson et al., 2011). It has gained much attention due to its notable superiority in identifying biomarkers (Dai et al., 2016; Sandin, Chawade, & Levander, 2015). Recently, it has been successfully used to evaluate changes in protein abundance in meat samples (Gallego, Mora, Aristoy, & Toldra, 2015; Hernández-Castellano et al., 2016).

However, application of label-free proteomics strategy in investigating meat color stability has not been reported yet, while the

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differential abundance of proteomes and their changes during post-mortem storage in color-stable and color-labile beef muscles remain unclear. Therefore, in the present study, label-free approach coupled with western blotting was firstly applied for comparative proteomics to reveal muscle-specific beef color stability at protein level. The objectives of the current study were to compare the color attributes and proteome changes of beef LL (color-stable) and PM (color-labile) muscles during post-mortem storage, and to elucidate underlying mechanisms of meat discoloration with the help of bioinformatics analyses.

2. Materials and methods

2.1. Chemicals

Tris-HCl was obtained from Solarbio (Beijing, China). Dithiothreitol was from Genview Scientific Inc. (El Monte, CA). Trypsin was obtained from Promega (Madison, WI). Protease Arrest acting as protease inhibitor was purchased from G-Biosciences (Saint Louis, MO). Urea, NH_4HCO_3 , formic acid, iodoacetamide and acetonitrile were purchased from Sigma Aldrich (Saint Louis, MO). Bovine serum albumin (BSA) was from Amresco (Solon, OH). Retinal dehydrogenase 1 (ALDH1A1) polyclonal antibody (ab9883), aconitase 2 (ACO2) polyclonal antibody (ab83528), NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5 (NDUFB5) polyclonal antibody (ab96228), heat shock protein beta-6 (Hsp20/HSPB6) polyclonal antibody (ab68977), and myosin light chain 3 (MYL3) polyclonal antibody (ab172073) were obtained from Abcam Trading (Shanghai) Company Ltd. (Pudong, Shanghai, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (YM3029) was from ImmunoWay Biotechnology Company (Plano, TX). Goat anti-rabbit IgG (H + L), bovine anti-goat IgG (H + L), and goat anti-mouse IgG (H + L) were from Beijing TDY Biotech Co., Ltd. (Beijing, China).

2.2. Samples

This study was carried out using beef of Holstein cattle from Fucheng meat plant, Dachang country, Hebei Province, China. Animals were fed the same diet and were slaughtered following industrial practice at the age of approximately 36 months with a live weight of 450 ± 5 kg. The samples of *M. longissimus lumborum* and *M. psoas major* were collected from the carcasses of three Holstein cattle after 36 h post mortem. Each sample was cut into 2.54-cm steaks, kept in styrofoam trays, and overwrapped with polyethylene (PE) film ($13,000 \pm 20\%$ $\text{cm}^3/\text{m}^2/24$ h oxygen transmission rate). All trays were stored in a refrigerator at 4 ± 1 °C, and samples were analyzed at 0, 4, and 9 days of storage, respectively. The proteome samples (2 g from each storage time) were frozen immediately in liquid nitrogen until analysis.

2.3. pH and meat color attributes

An FE-20 pH-meter (Mettler Toledo, Zurich, Switzerland) was used to measure pH of meat. Each sample (5 g) was homogenized with 50 mL deionized water and the homogenate was measured in triplicate.

The surface CIE lightness (L^*), redness (a^*), and yellowness (b^*) values of samples were measured by a CR-400 Minolta colorimeter (Konica Minolta Sensing Americas Inc., Ramsey, NJ) with illuminant D_{65} . Calibration was performed prior to color measurement using white plate ($Y = 87.0$, $x = 0.3180$, and $y = 0.3355$) provided by the manufacturer. Each sample was evaluated at six locations on the meat surface.

The proportions of myoglobin redox forms were determined according to the method described by Wu et al. (2016).

2.4. Analysis of proteomics

2.4.1. Proteome extraction and digestion

Frozen muscle tissue samples (2 g) were ground using liquid nitrogen in a mortar, and homogenized with cold extraction buffer (8.0 M urea, 100 mM Tris-HCl, 10 mM dithiothreitol (DTT), Protease Arrest (1X), pH 8.0). The homogenate was centrifuged at 4 °C, 10,000g, for 30 min, and then the supernatants were filtered and stored at -80 °C for further analysis. A BCA protein assay kit (Thermo Fisher Scientific Inc. Waltham, MA) was used to determine the protein concentration of proteome extract from each sample.

Whole muscle proteomes were digested according to the method of Shi et al. (2016) with some modifications. Accordingly, 40 mM DTT was used to reduce the extract solution of each sample (100 μg protein) at 60 °C for 1 h. Iodoacetamide (50 mM) was added to alkylate cysteines, and then samples were incubated for 1 h in a dark room at 20 °C. Trypsin (Promega Corp., Madison, WI) with a protein/trypsin ratio of 50 (w/w) was used to digest proteins at 37 °C for 16 h. Peptides were acidified with formic acid (FA) (10%, v/v) and desalted by reversed-phase extraction using C18 ZipTip pipette tips and re-suspended in 0.1% formic acid for high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis.

2.4.2. Detection of digested samples by HPLC-MS/MS

Ultimate 3000 nano HPLC system coupled with QExactive mass spectrometer (Thermo Scientific, Bremen, Germany) was utilized for label-free analysis. Digested peptides with 500 ng were loaded and pre-concentrated on a self-made C_{18} trap column (3 μm , 100 $\mu\text{m} \times 20$ mm). Then, the sample was analyzed by the self-made analytical column (1.9 μm , 120 mm $\times$ 150 μm , C_{18}) at a flow rate of 600 nL/min, in which the eluents were solvent A, which contained 0.1% (v/v) formic acid (FA) in H_2O , and solvent B, which contained 0.08% (v/v) FA in acetonitrile/ H_2O (80:20%, v/v). Chromatographic conditions of gradient elution went from 6% B up to 14% B in 24 min, from 14% B up to 40% B in 51 min, followed by an increase to 95% B in 3 min, and then held 7 min at 95% B. The gradient decreased sharply from 95% B to 6% B in 1 min, and then maintained at 6% B for 4 min. The total procedure time was 90 min. Q-Exactive HF MS/MS was applied to identify the separated peptide fragments. Full-scan MS spectra were acquired at a resolution of 120,000 at m/z 200 with an automatic gain control (AGC) target value of 3×10^6 . The mass range was set as m/z 300–1400. The 20 most abundant precursor ions in the MS scan per cycle were selected for tandem data-dependent analysis using higher energy collision-induced dissociation (HCD) fragmentation at a resolution of 15,000 at m/z 200 with an AGC target value of 5×10^4 and a maximum injection time of 45 ms. HCD spectra were acquired using a normalized collision energy of 30%. The isolation window was set to 1.6 amu, and the intensity threshold was 1.1×10^4 .

2.4.3. Protein identification and quantification

The generated raw data from label-free LC-MS/MS were further examined by Mascot 2.2 and Proteome Discoverer software 2.0. Database searches were performed against the forward Uniprot database for *Bos taurus* (uniprot_Bos_160304.fasta) with a peptide mass tolerance of ± 15 ppm and fragment mass tolerance of 20 mmu. The proteins were cleaved by trypsin, and the two missed cleavages were accepted. Oxidations of methionine and acetylation on protein N-term were designated as variable modifications, and carbamidomethylation of cysteine was appointed as a fixed modification. The peptide length was set to >6 , and the peptide false dis-

covery rate (FDR) was set to ≤ 0.01 . The peak intensities of the report ions of the only unique peptides in the MS/MS spectra were used to quantify the protein.

2.5. Western blot

The protein solution of samples was diluted and then heated at 95 °C for 5 min. Twenty microgram of proteins were loaded per lane and separated by 12% SDS-PAGE gel electrophoresis using a Bio-Rad Mini-Protein system at 160 V. Then, proteins were transferred to 0.45- μ m nitrocellulose membranes (Millipore, Billerica, MA) at 300 mA for 1 h. Following transfer, membranes were blocked with 5% skim milk in Tris-buffered saline (20 mM Tris-HCl and 500 mM NaCl, pH 7.5) with 0.1% Tween 20 (TBST) for 1 h, and incubated with primary antibodies (ALDH1A1 polyclonal antibody with dilution of 1:2000, ACO2 polyclonal antibody with dilution of 1:2000, NDUFB5 polyclonal antibody with dilution of 1:1000, HSPB6 polyclonal antibody with dilution of 1:2000 and MYL3 polyclonal antibody with dilution of 1:1000) overnight at 4 °C in TBST containing 3% BSA (w/v). GAPDH monoclonal antibody diluted 1:20,000 was used as a loading control. After primary antibodies incubation, membranes were washed 5 times with TBST, and incubated with horseradish peroxidase (HRP)-coupled secondary antibodies (goat anti-rabbit IgG with dilution of 1:10,000, bovine anti-goat IgG with dilution of 1:5000, and goat anti-mouse IgG with dilution of 1:10,000) for 2 h. After that, the membranes were washed 6 times (3 min each) with TBST and visualized by Pierce Enhanced Chemiluminescence (ECL) Plus Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA) and X-ray film. Gel-Pro Analyzer (Media Cybernetics, Rockville, MD) was applied for the resulting image analyses.

2.6. Statistical analysis

All data of meat color attributes of LL and PM muscles during post-mortem storage were analyzed by one-way analysis of variance using SPSS 20.0 software (SPSS Inc., Chicago, IL). The differences among means were detected by the Duncan's multiple range test at the 5% level. The resultant data matrices of proteomes were imported to MetaboAnalyst 3.0 for principal component analysis (PCA), and volcano plot analysis which combined fold-change analysis and *t*-tests. Prior to statistical analyses, all variables were normalized by the quantile, transformed by generalized log transformation, and scaled to range variance. Proteins with a minimum fold change of 2 (ratio > 2 or < 0.5, $p < 0.05$) were considered to be regulated differently in three comparison groups (LL vs. PM at Day 0, 4, and 9, respectively). Gene ontology (GO) enrichment, KEGG pathway enrichment analyses, and protein-protein interaction (PPI) were performed by String 10.0 to obtain more functional information regarding differentially abundant proteins. The results with false discovery rate <0.01 were reported.

3. Results and discussion

3.1. Meat color and biochemical attributes

L^* value, reflecting the water-holding capability of meat, exhibited decreasing trends during post-mortem storage in both LL and PM from 37.66 and 36.90 at Day 0 to 33.95 and 32.59 at Day 9, respectively (Table 1). This result was consistent with the research of Canto et al. (2015), who reported that L^* -value of beef muscle decreased significantly during refrigerated retail display. However, there was no significant difference ($p > 0.05$) between LL and PM in L^* value. The b^* value declined significantly ($p < 0.05$) in both LL and PM from 16.13 and 14.74 at Day 0 to 10.57 and 8.03 at Day

9, respectively. Previous research on Chinese Luxi yellow cattle also indicated a decreasing trend of b^* value in LL and PM muscles following extended storage (Wu et al., 2016). Moreover, LL showed greater ($p < 0.05$) b^* -value than PM throughout the extended storage, which was consistent with previous research (Seyfert et al., 2006). The a^* value, a vital parameter for fresh beef color, dropped significantly ($p < 0.05$) in both muscles during post mortem. LL had a greater a^* value ($p < 0.05$) than PM at Day 4 and 9 storage, which indicated a greater color stability of LL than PM (Joseph et al., 2012; Seyfert et al., 2006; Wu et al., 2016).

The pH of meat can affect meat color by influencing the metmyoglobin reducing activity (Bekhit & Faustman, 2005). In the present study, pH increased ($p < 0.05$) in both muscles following the extended storage. The increase of pH was mostly due to the deamination of meat proteins and accumulation of metabolites of bacteria during storage (Bekhit & Faustman, 2005). In addition, PM demonstrated a higher pH than LL throughout the extended storage.

Myoglobin is the principal protein responsible for meat color, which mainly exists in the forms of deoxymyoglobin (DeoMb), oxymyoglobin (OxyMb), and metmyoglobin (MetMb). OxyMb exerts a bright red color in meat, and its proportion declined continuously ($p < 0.05$) in both muscles with prolonged storage (Fig. 1). At the same time, the relative proportion of MetMb increased significantly ($p < 0.05$), resulting in increased brown color of the meat. The gradually disabled MetMb reductase and the depletion of the NADH pool facilitated the accumulation of MetMb during post-mortem storage; subsequently, the subsurface layer of MetMb thickens and gradually replaces the OxyMb layer as MetMb concentration increases (AMSA, 2012). As a consequence, the surface color changed from bright red to brown, which was reflected by the decrease of the a^* -value of the meat. Furthermore, the relative proportion of MetMb in PM exhibited a greater increase than in LL muscle, mainly due to the fact that PM possesses lesser metmyoglobin reducing activity and less capacity to reduce the oxidized form of myoglobin (Joseph et al., 2012; Wu et al., 2016). Thus, PM muscles showed lower color stability than LL during post-mortem storage.

3.2. Comparative proteomics in LL and PM muscles

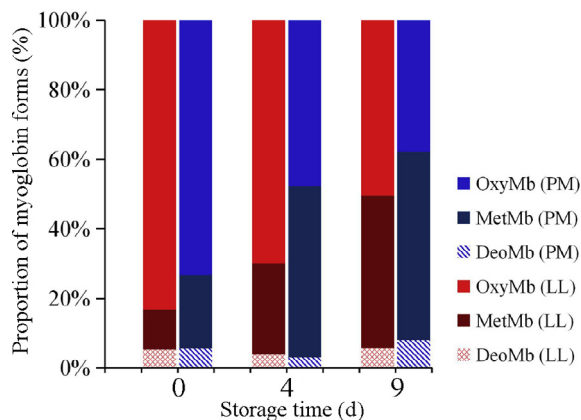
PCA was performed as an exploratory data analysis tool to visualize and differentiate the two (LL and PM) muscle groups based on mutual proteins of each comparison (LL vs. PM at Day 0, 4, and 9, respectively). Score plot of PCA analysis at Day 0 storage (Fig. 2A1) showed that 66.4% of the variability was explained by the first two principal components, which accounted for 40.0%, and 26.4% of the total variance, respectively. The PCA result indicated that the LL muscles could be separated from their PM counterparts, suggesting the existence of differentially abundant proteins between the two muscle groups. Accordingly, PCA results of muscles from Day 4, and Day 9 post-mortem storage (Figs. 2B1 and 2C1) showed similar results, which implied that differences in color stability of the two muscles (LL and PM) could be closely related to the abundance of proteomes.

Volcano plot is a versatile figure that can present fold-change analysis and *t*-test simultaneously. Specifically, there were 30 differentially abundant proteins in LL vs. PM at day 0 storage (Fig. 2A2) (19 overabundant in LL and 11 in PM). Forty-four differentially abundant proteins were identified in LL vs. PM at Day 4 (Fig. 2B2) (19 overabundant in LL and 25 in PM), while 31 were detected in LL vs. PM at Day 9 (Fig. 2C2) (13 overabundant in LL and 18 in PM). The detailed results of the differentially abundant proteins in LL and PM are summarized in Tables 2, S1 and S2, where critical information about the proteins was provided,

Table 1

Instrumental color and pH value of LL and PM muscles from Holstein cattle during post-mortem storage (0, 4, and 9 days) at 4 °C ± 1 °C.

Attributes		Post-mortem storage time (day)		
		0	4	9
L*	LL	37.66 ± 3.39 ^{ax}	35.46 ± 2.06 ^{abx}	33.95 ± 1.46 ^{bx}
	PM	36.90 ± 1.80 ^{ax}	35.40 ± 1.29 ^{bx}	32.59 ± 1.46 ^{cx}
b*	LL	16.13 ± 1.54 ^{ax}	13.82 ± 0.98 ^{bx}	10.57 ± 1.14 ^{cx}
	PM	14.74 ± 0.60 ^{ay}	12.59 ± 1.03 ^{by}	8.30 ± 1.43 ^{cy}
a*	LL	24.32 ± 1.75 ^{ax}	20.33 ± 0.49 ^{bx}	15.32 ± 1.31 ^{cx}
	PM	23.57 ± 1.07 ^{ax}	17.81 ± 0.75 ^{by}	10.45 ± 1.14 ^{cy}
pH	LL	5.54 ± 0.05 ^{bx}	5.52 ± 0.06 ^{bx}	5.65 ± 0.09 ^{ax}
	PM	5.64 ± 0.04 ^{by}	5.56 ± 0.07 ^{cx}	5.84 ± 0.03 ^{ay}

**Fig. 1.** Changes in the relative proportions of MetMb, OxyMb, and DeoMb in LL and PM of Holstein cattle during post-mortem storage at 4 ± 1 °C.

including accession numbers in UniProt, protein description, and fold-changes (LL/PM) (ratio > 2 or < 0.5, $p < 0.05$).

3.2.1. Metabolic enzymes

3.2.1.1. Overabundance of enzymes in LL. Carbonic anhydrase 3 (CA3) is responsible for proton homeostasis, through its role in facilitating CO₂ diffusion and diverse processes involving H⁺ and HCO₃⁻ transport. This protein was overabundant in LL at Day 0 and Day 4 with 2.37 and 2.10-fold compared with PM, respectively (Tables 2 and S1). Correlations between CA3 and beef tenderness have been demonstrated (D'Alessandro et al., 2012), although a clear mechanism remains unknown. Moreover, Damon et al. (2013) concluded that gene expression of CA3 was correlated negatively with ultimate pH and positively with drip loss and L*-value in pork *longissimus* muscle. Retinal dehydrogenase 1 (ALDH1A1) catalyzes the conversion of retinal, NAD⁺, and H₂O to retinoate and NADH, and this reaction is involved in the pathway of retinol metabolism (<http://www.uniprot.org/>). NADH contributes to meat color stability by reacting with reactive oxygen species (ROS) (Kirsch & Groot, 2001) and serves as a substrate of complex I, component of ETC-linked pathway for MetMb reduction (Tang, Faustman, Mancini, Seyfert, & Hunt, 2005). MetMb can also be reduced non-enzymatically by NADH or NADPH in the presence of EDTA or MnCl₂ (Koizumi & Brown, 1972). Therefore, the overabundance of ALDH1A1 in LL throughout post-mortem storage could offer more NADH for myoglobin protection. These results were in accordance with our previous research (Wu et al., 2016), in which ALDH1A1 showed a positive correlation ($r = +0.835$) with a*-value of beef *M. longissimus lumborum* from Chinese Luxi yellow cattle during post-mortem storage. Glutathione S-transferase Mu 1 (GSTM4) and GSTM1 protein (GSTM1) belong to the glutathione S-transferase (GST) superfamily with the molecular function of

glutathione transferase activity (<http://www.uniprot.org/>). GST plays an important role in regulating the formation and elimination of ROS (Hui et al., 2013). High levels of cell ROS could lead to a state of oxidative stress which can cause cellular damage through the peroxidation of cellular proteins, nucleic acids and lipids, deactivation of enzymes, and deregulation of redox-sensitive reactions and signaling pathways (Zhao et al., 2010). Thus, overabundance of GSTM4 and GSTM1 in LL (Table S1) could protect meat samples against oxidative damage, which may alleviate lipid oxidation-induced myoglobin oxidation (Faustman et al., 2010). Ubiquitin carboxyl-terminal hydrolase isozyme L3 (UCHL3) is one of the isozymes of ubiquitin C-terminal hydrolases with the de-ubiquitinating function. The ubiquitin system can regulate numerous cellular processes (such as cell cycle progression, signal transduction, protein quality control, transcriptional regulation, and growth control), and the role of ubiquitination in most of these processes is to promote the degradation of specific proteins (Amerik & Hochstrasser, 2004). Moreover, researchers have concluded that lack of UCH-L3 expression increases fatty acid oxidation in the skeletal muscles (Setsuie et al., 2009). Therefore, we speculated that the overabundance of UCHL3 in LL (Table S1) could reduce protein degradation and fatty acid oxidation, which in turn may play a pivotal role in maintaining color stability of meat. Protein-L-isoaspartate O-methyltransferase (PCMT1) catalyzes the methylation of L-isoaspartyl residues in peptides and proteins and facilitates their restoration to L-aspartyl residues. The spontaneous conversion from L-aspartyl residues to L-isoaspartyl residues is the major source of spontaneous protein damage, which can result in the defunctionalization of proteins and cells (Clarke, 2003). Methyltransferase can recognize and repair the damaged aspartyl residues (Clarke, 2003). The overabundant expression of protein-L-isoaspartate O-methyltransferase in LL (Table S2) may provide more repair processes for damaged proteins, which were probably associated with color stability of meat.

3.2.1.2. Overabundance of enzymes in PM. Several enzymes involved in the tricarboxylic acid cycle (CS, ACO2, IDH3A, IDH3G, and MDH2) were detected with higher abundances in PM than in LL (Tables 2, S1 and S2). It was noticed that aconitate hydratase (ACO2) also named as aconitase, is an essential enzyme located in the mitochondria that catalyzes the isomerization of citrate to isocitrate via *cis*-aconitate in the tricarboxylic acid (TCA) cycle. This enzyme contains a [4Fe-4S]²⁺ cluster in its active site, and it has been proposed that the [4Fe-4S]²⁺ aconitase can be oxidized by superoxide, generating the inactive [3Fe-4S]¹⁺ aconitase and hydrogen peroxide, which facilitate the formation of hydroxyl radical through the Fenton reaction in mitochondria (Vasquez-Vivar, Kalyanaraman, & Kennedy, 2000). In the present study, ACO2 was overabundant in PM throughout the post-mortem storage, which could enhance mitochondrial susceptibility in response to hydroxyl radicals attack, causing mitochondrial oxidative damage. Moreover, Joseph et al. (2012) demonstrated that the overabun-

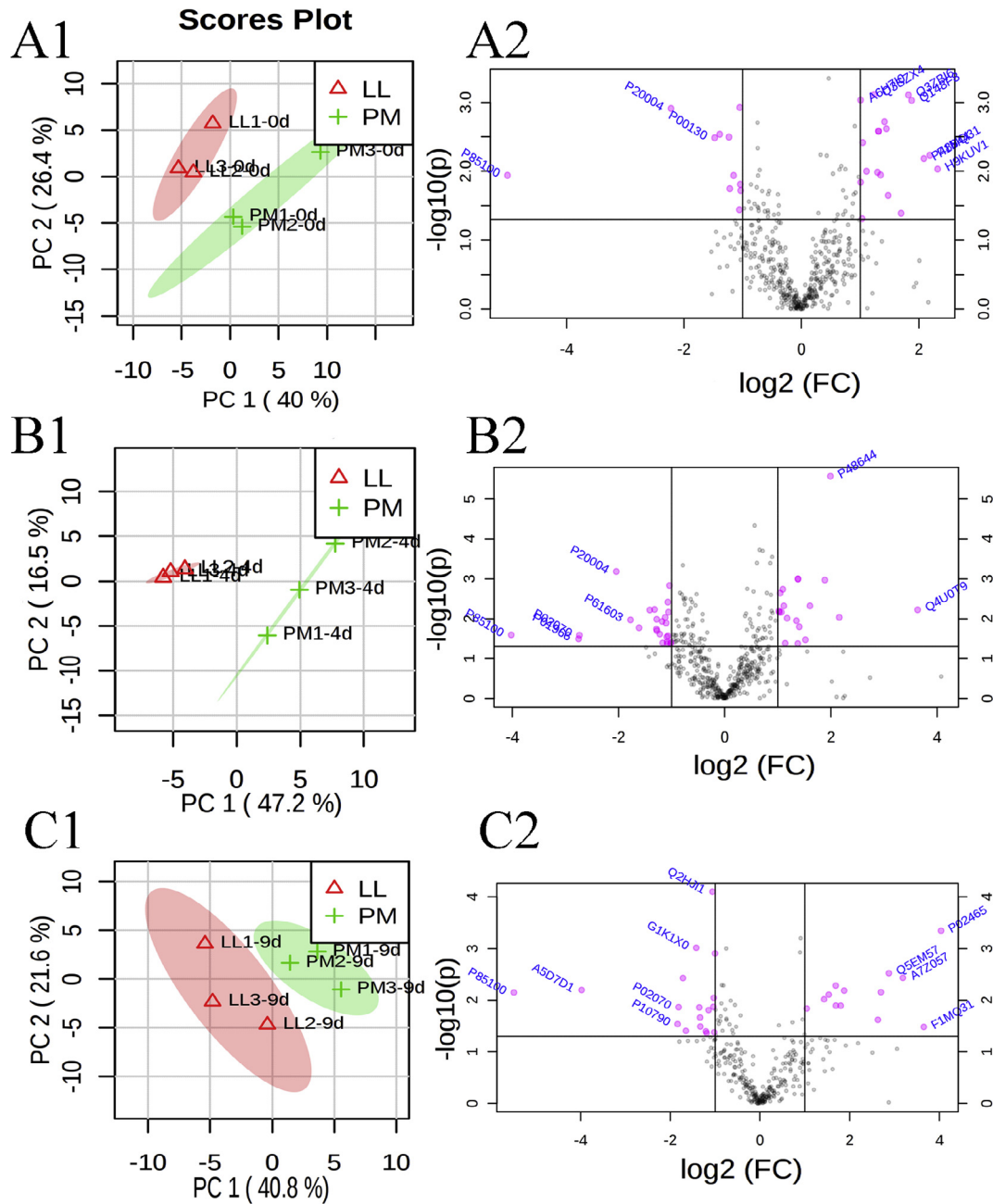


Fig. 2. The PCA score plots of mutual proteins in LL and PM during post-mortem storage at 4 °C ± 1 °C (A1: Day 0 storage, B1: Day 4 storage, C1: Day 9 storage). The volcano plots in the comparison of LL/PM at different post-mortem storage days (A2: Day 0 storage, B2: Day 4 storage, C2: Day 9 storage), in which the points with pink color located in the left of plots ($\log_2(\text{FC}) < -1$) represented the proteins which were overabundant in PM with a minimum fold-change of 2 ($p < 0.05$), while on the right of the plots ($\log_2(\text{FC}) > 1$) are represented the proteins which were overabundant in LL.

dant aconitase in PM could accelerate free radical-induced and iron-catalyzed lipid oxidation and subsequent pigment oxidation, giving rise to greater discoloration compared to LL. Malate dehydrogenase (MDH2), a key enzyme in TCA cycle, catalyzes the reversible conversion of malate and oxaloacetic acid. MDH2 is also involved in redox shuttling, which plays an important role in the export of reducing equivalents from mitochondria and the maintenance of mitochondrial NADH/NAD⁺ homeostasis (Yoshida & Hisabori, 2016). Previous research demonstrated that malate dehydrogenase was correlated negatively with *a**-value of LL muscle during post-mortem storage (Wu et al., 2016).

Overabundant protein subunits of complex I (NDUFB5, NDUFA2, NDUFS4, ND1, NDUFS7, NDUFV1, NDUFB9), complex II

(SDHC and SDHA), complex III (UQCRC10, UQCRCQ, UQCRC1, UQCRC2), and complex IV (COX6C and COX5A) of mitochondrial electron transport chain (ETC) were identified in PM (Tables 2, S1 and S2). An ETC-linked pathway for MetMb reduction was identified by Tang et al. (2005), and they proposed that the possible site(s) where electrons became available for MetMb reduction appears to be located between complexes III and IV. Additionally, Belskie, Van Buiten, Ramanathan, and Mancini (2015) concluded that NADH generated *via* reversed electron flow from complex II to complex I can be utilized for metmyoglobin reduction through both electron transport-mediated and enzymatic pathways. A greater amount of subunits of complexes in PM could imply a higher degree of complex degradation, and destruction of mito-

Table 2

Differentially abundant proteins in Holstein beef LL and PM muscles at day 0 post-mortem storage at 4 °C ± 1 °C.

Accession	Description	FC	p-value
<i>Overabundant in LL</i>			
<i>Enzymes</i>			
Q3SZX4	Carbonic anhydrase 3 (CA3)	2.3658	0.0008
P48644	Retinal dehydrogenase 1 (ALDH1A1)	4.2435	0.0065
Q2HJ33	Obg-like ATPase 1 (OLA1)	2.5459	0.0113
Q3SYR3	Probable C→U-editing enzyme APOBEC-2 (APOBEC2)	2.4541	0.0104
<i>Heat shock protein family</i>			
Q148F8	Heat shock protein beta-6 (HSPB6)	3.6737	0.0009
<i>Binding proteins</i>			
A7Z057	14-3-3 protein gamma (YWHAG)	2.0623	0.0038
F1MQ31	Brevican core protein (BCAN)	4.5544	0.0058
Q3ZBI6	Four and a half LIM domains protein 3 (FHL3)	3.5360	0.0008
P62958	Histidine triad nucleotide-binding protein 1 (HINT1)	2.7840	0.0225
Q5EM57	LIM protein (Fragment)	2.6655	0.0019
Q3SYZ8	PDZ and LIM domain protein 3 (PDLIM3)	2.1570	0.0100
H9KUV1	Protein S100 (Fragment) (S100A1)	4.9920	0.0093
<i>Structural proteins</i>			
F1MRC2	Myosin-2 (MYH2) (GN = MYH2 PE = 4 SV = 1)	2.4851	0.0026
Q9BE41	Myosin-2 (MYH2) (GN = MYH2 PE = 2 SV = 1)	2.4851	0.0026
<i>Others</i>			
A6H7I0	KBTD5 protein (KBTD5)	2.0108	0.0009
A6QPR1	PCYOX1 protein (PCYOX1)	2.0119	0.0145
P00974	Pancreatic trypsin inhibitor	2.0488	0.0489
A5PK61	Histone H3.3C (H3F3C)	2.7273	0.0024
B6VAP7	CDC42 protein (CDC42)	3.2432	0.0408
<i>Overabundant in PM</i>			
<i>Enzymes</i>			
P20004	Aconitate hydratase, mitochondrial (ACO2)	0.2145	0.0012
F1MN74	Isocitrate dehydrogenase [NAD] subunit, mitochondrial (IDH3A)	0.4294	0.0179
Q02380	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial (NDUFB5)	0.4253	0.0032
Q02370	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2 (NDUFA2)	0.4882	0.0190
Q02375	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial (NDUFS4)	0.4492	0.0115
AOA0B5H0C3	NADH-ubiquinone oxidoreductase chain 1 (ND1)	0.4810	0.0362
P00130	Cytochrome b-c1 complex subunit 9 (UQCRI0)	0.3595	0.0033
P05631	ATP synthase subunit gamma, mitochondrial (ATP5C1)	0.4861	0.0155
<i>Structural proteins</i>			
P85100	Myosin light chain 3 (MYL3)	0.0311	0.0115
<i>Others</i>			
Q3ZBU2	CDGSH iron-sulfur domain-containing protein 1(CISD1)	0.3813	0.0029
Q3T0S7	Chromosome 14 open reading frame 166 ortholog (C10H14ORF166)	0.4831	0.0012

chondrial ETC, which in turn results in poor meat color stability. Two subunits of Complex V (ATP5C1 and ATP5I) were found with higher abundance in PM compared with LL. Mitochondrial membrane ATP synthase (Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane (<http://www.uniprot.org/>). More subunits of Complex V suggested a higher level of its degradation, which could affect the homeostasis of ATP and ADP, subsequently influencing meat color stability, but the underlying relationship between Complex V and meat color remains unknown.

It is noteworthy that two important enzymes (CPT1B and ACADVL) involved in fatty acid β -oxidation were overabundant in PM at Day 4 post mortem (Table S1). Gureev, Shmatkova, Bashmakov, Starkov, and Popov (2016) demonstrated that enhancement of oxidative processes caused by increasing the hepatic gene expression responsible for β -oxidation of fatty acids in peroxisomes and mitochondria resulted in an increase in the rate of ROS production. High level of ROS is detrimental to meat color stability.

3.2.2. Heat shock proteins

Heat shock protein beta-6 (HSPB6) and heat shock 27 kDa protein 2 (HSPB2) belong to small heat shock protein (sHSP) family, and these two proteins were more abundant in LL compared to PM (Tables 2 and S1). The role of sHSP in maintaining muscle struc-

tural integrity against intracellular stresses, e.g., changing pH, has been proved to be important in meat quality (Pulford et al., 2009). Small heat shock proteins also have been shown to protect different types of cells against oxidative stress (Garrido, Paul, Seigneuric, & Kampinga, 2012). Therefore, the overabundance of small heat shock proteins in LL, and its anti-apoptotic and chaperone functions could protect proteins against damage and contribute to meat color stability. Similarly, Joseph et al. (2012) identified two heat shock proteins (HSP-70 kDa and HSP-27 kDa) that were overabundant in beef *Longissimus lumborum* (color-stable) muscles. Meanwhile, higher abundance of 10 kDa heat shock protein (HSPE1) and 60 kDa heat shock protein (HSPD1) located in mitochondria were found in PM (Tables S1 and S2). These two proteins are mitochondrial matrix proteins induced by stress and play an important role in maintaining normal mitochondrial function (Lau, Patnaik, Sayen, & Mestril, 1997).

3.2.3. Binding proteins

14-3-3 protein gamma (YWHAG), overabundant in LL in this study (Tables 2 and S2), belongs to the 14-3-3 proteins family that is involved in cellular signaling, trafficking, apoptosis, cell cycle, and stress response (Jin et al., 2006). This protein was found to be up-regulated in tender meat (D'Alessandro et al., 2012). However, the clear mechanisms of these proteins involved in meat color are still indefinable. Protein S100 (Fragment) (S100A1) belongs to a

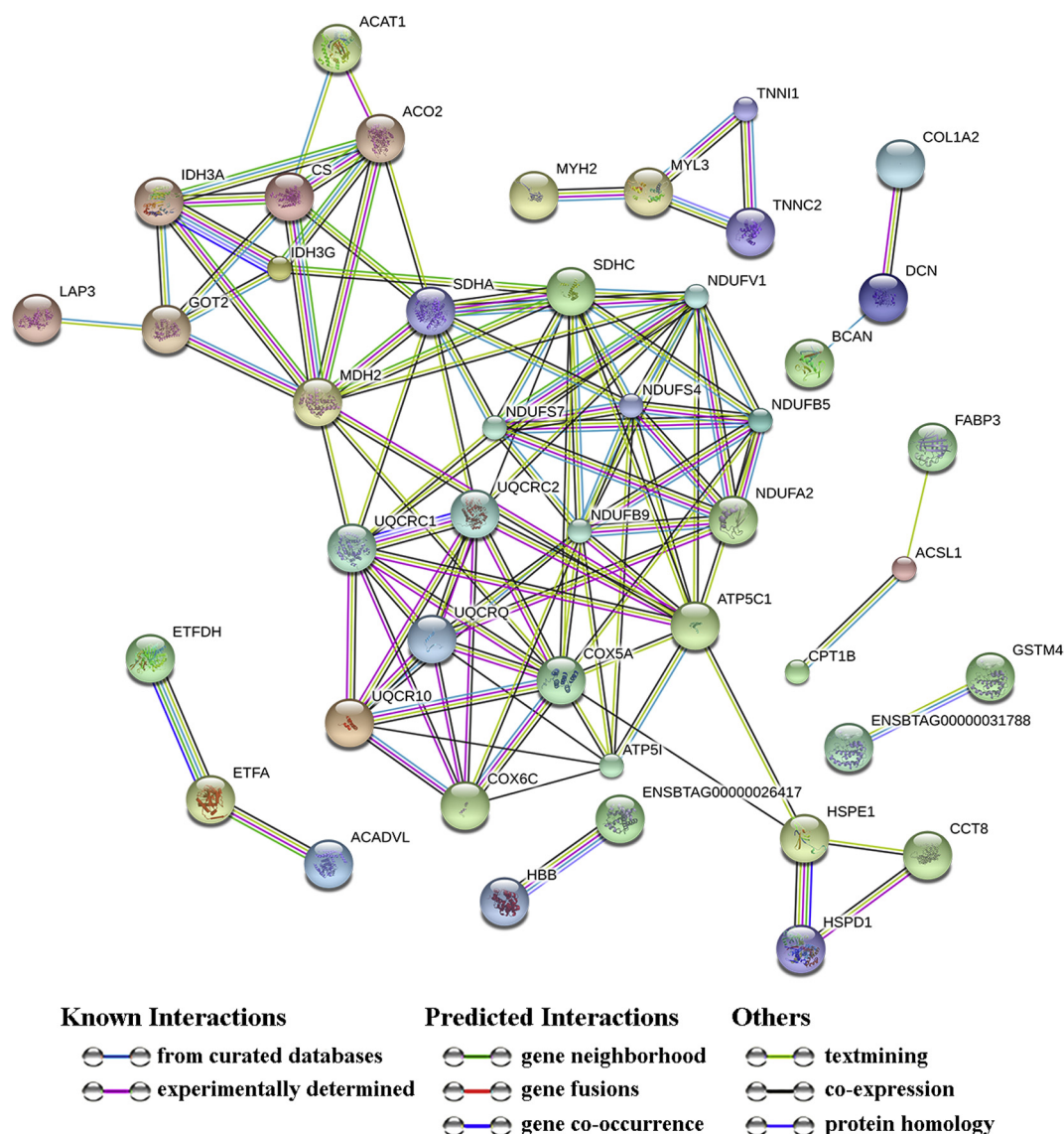


Fig. 3. Protein-protein interaction (PPI) network of differentially expressed proteins. The network nodes represent proteins, and edges represent protein-protein associations.

family of small EF-hand calcium-binding proteins, and it can regulate many physiological processes such as cell growth and differentiation, phosphorylation, apoptosis, transcription, and inflammation (Yamaguchi et al., 2012). S100A1 was overabundant in LL at Day 0 and Day 9 post mortem with 4.99 and 6.48-fold compared with PM, although the underlying relationship between this protein and color stability is still unknown.

3.2.4. Structural proteins

Structural proteins and their degradation play a critical role in the development of meat tenderness during post-mortem aging (Lametsch et al., 2003; Polati et al., 2012). Myosins contain a family of ATP-dependent motor proteins that are involved in muscle contraction and various intracellular functions (cell migration and adhesion, intracellular transport and localization of organelles and macromolecules, and signal transduction; Krendel & Mooseker, 2005). In the present study, overabundant myosin-2 (MYH2) was identified in LL, whereas overabundant myosin light chain 3 (MYL3) was identified in PM. Troponin comprises three subunits: C subunit binds calcium, I subunit inhibits the acto-

myosin ATPase activity, and T subunit binds tropomyosin (Polati et al., 2012). The higher levels of troponin C type 2 (Fast) (TNNC2) were found in LL with 2.55 and 3.23-fold compared with PM at Day 4 and Day 9 post mortem (Tables S1 and S2), respectively. However, limited research is available about the specific role of structural proteins in meat discoloration.

3.2.5. Others

BAG3 protein (BAG3) can regulate Hsp70/Hsc70 chaperone activity via their conserved C-terminal domains, and it plays novel roles in stabilizing myofibril structure and inhibiting myofibrillar degeneration in response to mechanical stress by interacting with Hsc70 protein and actin capping protein CapZ (Hishiya, Kitazawa, & Takayama, 2010). Thus it was speculated that overabundant BAG3 in LL (Table S1) could provide protection against structural proteins degeneration. In addition, the overabundance of hemoglobin subunit alpha (HBA) and hemoglobin subunit beta (HBB) in PM could suggest a higher degree of degradation of hemoglobin compared with LL (Tables S1 and S2).

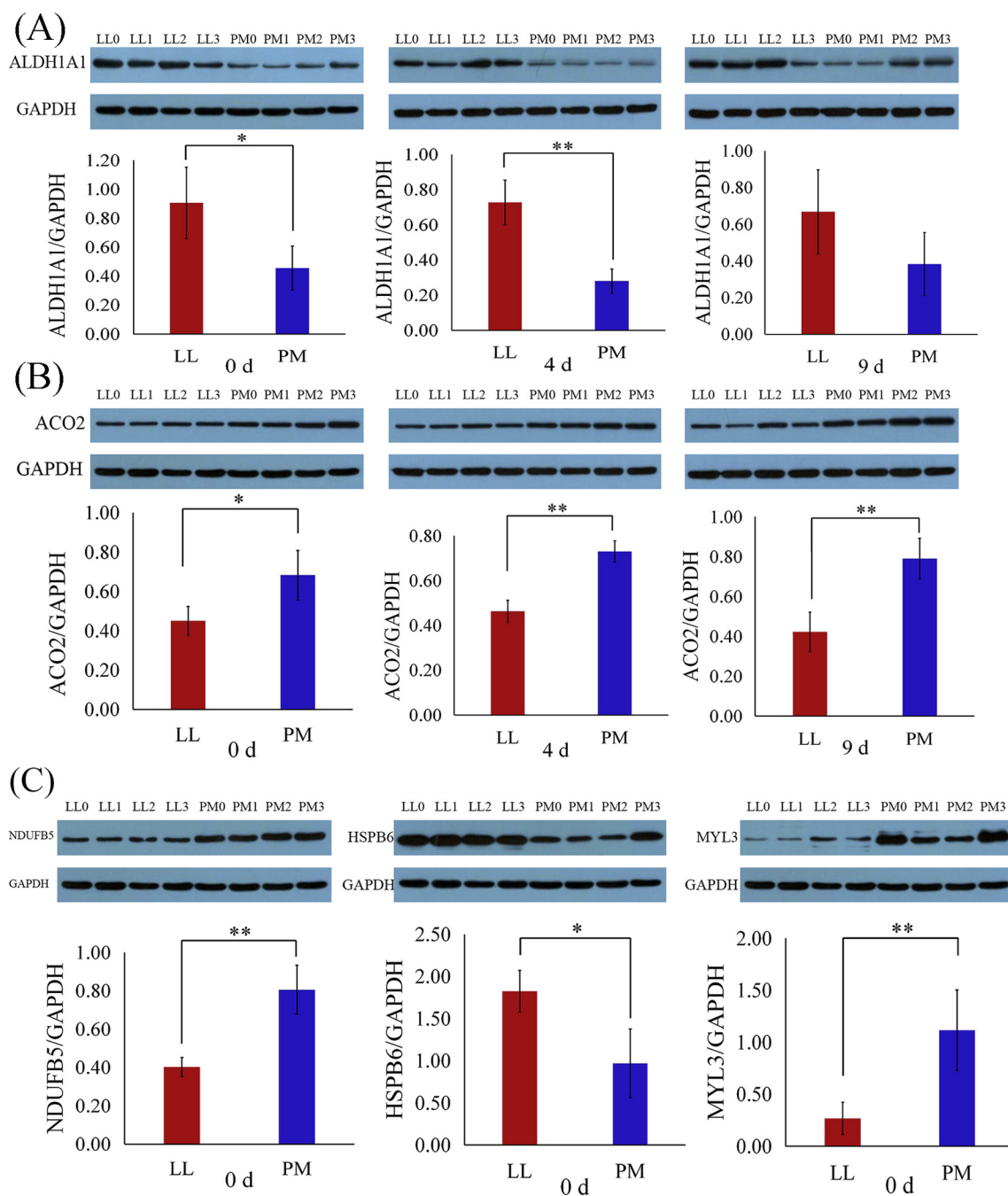


Fig. 4. Western blot profiles of ALDH1A1, ACO2, NDUFB5, HSPB6 and MYL3 in LL and PM at different post-mortem storage days. Relative intensity of protein is expressed as the mean $\pm$ standard deviation ($n = 4$). LL0 represents the mixture sample of three biological replicates from LL, whereas PM0 represents the mixture sample of three biological replicates from PM. LL1, LL2 and LL3 refer to three biological samples from LL. Analogously PM1, PM2 and PM3 refer to three biological samples from PM. Asterisks represent levels of significance (t-test: * $p < 0.05$, ** $p < 0.01$).

3.3. Bioinformatics analyses

Bioinformatics analyses of all the differentially abundant proteins (presented in Tables 2, S1 and S2) were performed using String 10.0. The results of PPI with minimum required interaction score of 0.700 are presented in Fig. 3, and only connected nodes (proteins) in the network are displayed. Protein subunits of complexes from ETC were connected closely with each other, suggesting the critical role of ETC-linked pathway for MetMb reduction and meat color stability. Enzymes involved in TCA cycle (CS, ACO2, IDH3A, IDH3G, and MDH2) interacted strongly with each other and the more abundant these enzymes, the more oxidative

metabolism and poorer color stability in PM (color-labile) muscles. In addition, four structural proteins (MYH2, MYL3, TNNC2, and TNN1) interacted closely as well.

The results of GO enrichment of all differentially abundant proteins for biological processes, molecular function, and cellular components are presented in Table S3. Some proteins involved in important biological processes were enriched significantly, such as oxidation-reduction process (GO.0055114), metabolic process (GO.0008152), cellular respiration (GO.0045333), tricarboxylic acid cycle (GO.0006099), electron transport chain (GO.0022900), and fatty acid beta-oxidation (GO.0006635). As for molecular function, proteins related to binding (GO.0005488), catalytic activity

(GO.0003824), metal ion binding (GO.0046872), and oxidoreductase activity (GO.0016491) were enriched significantly. For cellular components, the major classes of these proteins were cytoplasm (GO.0005737) and mitochondrial part (GO.0044429). String 10.0 provides pathway enrichment analysis (Table S3), in the present study; proteins participating in oxidative phosphorylation (00190), metabolic pathways (01100), citrate cycle (TCA cycle) (00020), carbon metabolism (01200), and fatty acid degradation (00071) were markedly enriched. Thus, the bioinformatics analyses provide more valuable information to comprehend the function of proteins and give a hint on the importance of the abovementioned pathways in regulating meat color stability.

3.4. Protein verifications by western blot

ALDH1A1, ACO2, NDUFB5, HSPB6 and MYL3 were selected for western blot analyses to confirm MS observations. The average band intensity of protein (normalized with GAPDH) was obtained for the statistical analysis (Fig. 4). Label-free results indicated that ALDH1A1 was overabundant in LL throughout post-mortem storage. Similarly, its band intensities were higher in LL than in PM, although the repeatability in samples at Day 9 storage was poor. ACO2 was overabundant in PM throughout post-mortem storage, and the western blot intensity of ACO2 was significant higher ($p < 0.05$) in PM than in LL, regardless of storage time. Moreover, protein verifications of NDUFB5, HSPB6 and MYL3 at Day 0 storage were performed, and the western blot intensities of these three proteins were consistent with their respective MS expression. The band intensities of NDUFB5 and MYL3 were significant higher ($p < 0.01$) in PM than in LL at Day 0, whereas HSPB6 showed significantly higher ($p < 0.05$) band intensity in LL at Day 0.

4. Conclusion

Comparative proteomics strategy was applied to elucidate the muscle-specific beef color stability of LL and PM by label-free mass spectrometry. LL exhibited better color stability than PM during post-mortem storage. The overabundance of proteins with the functions of antioxidation (GSTM4 and GSTM1), protection (UCHL3, HSPB6, HSPB2, and BAG3) and repair (PCMT1) in LL may contribute to its better color stability, whereas overabundance of proteins involved in TCA cycle (CS, ACO2, IDH3A, IDH3G, and MDH2) in PM indicated more oxidative metabolism and poorer color stability. In addition, more degradation of mitochondrial ETC complexes in PM affected MetMb reducing capacity and color stability. All of these current findings combined with bioinformatic analyses gave an insightful understanding in muscle-specific color stability of beef and pave the way for further metabolomics research, although more work is needed for protein verification.

Conflicts of interest

The authors declare no competing interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2017.03.004>.

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