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Label-free quantitative proteomic analysis reveals muscle contraction and metabolism proteins linked to ultimate pH in bovine skeletal muscle

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ABSTRACT

The purpose of this research was to investigate the causes and consequences of pHu variations in beef cattle. A group of 176 Nellore beef cattle was evaluated and classified into two different pHu groups: High (≥6.0, N = 17) and Normal (< 5.8, N = 159). Plasma concentrations of cortisol and adrenocorticotropic hormone, lactate and glycogen muscular content, meat color, shear force and *Longissimus thoracis* muscle proteomic profile were evaluated and compared between pHu groups. Muscle glycogen content, meat color and shear force statistically differed between pHu groups. Label-free quantitative proteomic analysis revealed ten differentially abundant proteins between pHu groups, involved in metabolic processes and muscle contraction, which also were significantly correlated with pHu. Thirty-six and 31 proteins were exclusively present in Normal and High pHu group, respectively, which were related to TCA cycle, cortisol production, calcium regulation, and antioxidant function. The MYH7, UGP2, H2AFJ and VDAC3 were identified as potential indicators of pHu variations. CALM and NNT appeared to be interesting proteins to understand the metabolic pathways behind pHu. Data are available via ProteomeXchange with identifier PXD009320.

1. Introduction

The decrease of pH during the conversion of muscle into meat is a highly important factor for the meat industry. Notably, the abnormal pH after slaughter can lead to significant changes in meat quality resulting in severe economic losses. Dark Firm Dry (DFD) or Dark Cutting is the principal defect concerning beef quality (Adzitey & Nurul, 2011), which is determined when the ultimate pH is higher than 5.8 measured after 24 h *post-mortem* (Holdstock et al., 2014; Mahmood et al., 2017). DFD usually is discriminated against by retailers because of its reduced shelf life (Ponnampalam et al., 2017) and by consumers because of its unusual appearance.

DFD is a common problem for many countries. According to Miller (2007), the USDA Agricultural Marketing Service reported a loss of $ 165 million with dark cuttings in the year 2000. Whereas in Canada, 1.3% of DFD represented $ 1.4 million economic losses in the years of 2010/11 (Holdstock et al., 2014). In Brazil, close to 40% of all beef produced from Nellore cattle can be classified as DFD, and until now, there is no information about the economic impact of this occurrence to the industry (Rosa et al., 2017).

It is well-known that high pHu values of meat are frequently associated with the pre-slaughter stress of the animals (Ferguson & Warner, 2008). A stressful situation triggers an increased secretion of hormones (cortisol, adrenaline, noradrenaline, catecholamines, etc.) that exacerbate the muscular activity and promote the depletion of muscle glycogen stores, which are the metabolic substrates to maintain homeostasis and, therefore, a constant ATP level. Low glycogen content in muscles affects the glycolytic metabolism after the slaughter, resulting in dark-cutting meat (Wulf, Emnett, Leheska, & Moeller, 2002).

The variability in meat quality traits resulting from the pHu is dependent on specific changes in the cellular structure of the muscle as well as the variations in the energy metabolism (Huff-Lonergan &

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Lonergan, 2007). Thus, the muscle proteins and their interactions are keys during the *post-mortem* period. Proteomic is a tool to determine the protein profile of muscle and has been applied in meat science to promote understanding of molecular mechanisms taking place in muscle and affecting meat quality (Carvalho et al., 2014; Gagaoua et al., 2015; Picard et al., 2015; Zhang et al., 2014). Quantitative proteomics by high-resolution label-free mass spectrometry (MS^E) has the advantage of identifying the entire protein expression profile of a sample, regardless of whether or not the proteins are significantly differentially abundant between groups as a rapid, reproducible, and accurate quantification strategy (Delahunty & Yates 3rd., 2007).

Therefore, to enhance our knowledge of the molecular mechanisms involved in pHu variations in beef cattle, the objectives of this study were to investigate the causes and consequences of pHu variations in the beef. Plasma concentrations of cortisol and adrenocorticotropic hormone (ACTH), the muscular content of lactate and glycogen, meat color, shear force, and *Longissimus thoracis* muscle proteomic profile were evaluated and compared between two different pHu groups: High ($\geq$6.0) and Normal ($<$ 5.8).

2. Material and methods

2.1. Animals

One hundred seventy-six animals were selected for this study according to discriminatory meat quality parameters between Normal (pH $<$ 5.8, N = 159) and High (pH $\geq$ 6.0, N = 17) ultimate pH (pHu) groups. The animals came from an experiment which consisted of 241 male cattle of the Nellore breed reared on pasture until approximately 21 $\pm$ 1 month of age and finished in a feedlot. They were fed a diet composed of 74.8% of total digestible nutrients, 14.4% of crude protein, sugarcane bagasse (15%, dry basis), ground corn (50%), soybean meal (5.6%), corn gluten feed (28%), mineral premix (1%) and urea (0.4%). The experiment was approved by the Animal Care and Use Committee from the College of Animal Science and Food Engineering, University of São Paulo. The animals were slaughtered at the age of 24 $\pm$ 1.2 months and with a live weight of 508 $\pm$ 39 kg. The carcasses were stored in cold chambers (4 °C) for 24 h for further boning (Poleti et al., 2015).

2.2. Sample collection

The blood samples for quantification of the adrenocorticotropic (ACTH) and cortisol hormones were collected into a vacutainer tube containing sodium heparin at two-time points. The first blood samples were collected by jugular venipuncture at weighing, 28 d after animals entered the feedlot (*ante-mortem*). The second blood samples were collected *post-mortem* during the exsanguination at the time of slaughter (Tume & Shaw, 1992). The blood was centrifuged at 3000 $\times g$ for 15 min (5 °C) immediately after the collection to obtain the plasma, which was frozen at −20 °C until the quantification of hormones. Approximately 30 min and 24 h after slaughter, samples of the *Longissimus thoracis* (LT) muscle from the same area were excised from the right half of each carcass and snap-frozen in liquid nitrogen and stored at −80 °C in the freezer awaiting muscle glycogen and lactate levels determinations. After the carcasses chilled for 24 h, meat samples were excised and immediately frozen in liquid nitrogen for protein extraction. Also, three 2.5 cm thick beef were collected between the 11th and the 13th thoracic vertebrae from LT muscle from the left carcass to determine cooking loss, shear force and color. The steaks were vacuum-packaged (Selovac M160) in high barrier flexible film, Polyfilm® and aged for one, seven, and 14 days at 2 °C.

2.3. Meat quality analyses

Carcass pH was measured in the left half carcass at one and 24 h after slaughter using a portable digital pH-meter (pH 11 Economy Meter, Oakton instruments) equipped with a penetration probe. From the pH values at 24 h (pHu), two distinct groups were created: Normal (N = pH $<$ 5.8), and High (H = pH $\geq$ 6.0). These classes were used to conduct the statistical analysis. On the particular storage days, steaks were removed from the packages and were performed the analysis of color, shear force (SF) and cooking loss (CL). The meat color was measured using a portable colorimeter (Minolta CM-2500d spectrophotometer, Osaka, Japan) with a D_{65} light source, 10° observer, and 30-mm aperture. The L*, a*, and b* values were determined according to the CIE-L*a*b* evaluation system. Three different measurements were performed on the surface of the aged steaks after a 20-min bloom time. The steaks were cooked in a conventional electrical oven (Luxo Classic 2.4, Lary) pre-heated with a thermostat adjusted to 170 °C. The steaks internal temperatures were individually monitored. The steaks were turned over after reaching an internal temperature of 40 °C and removed from the oven when they arrived a final internal temperature of 71 °C. After cooking, the steaks were cooled at room temperature and stored overnight at 4 °C. On the next day, six cores with a 1.25 cm diameter were removed parallel to the direction of muscle fibers. At last, the cores were sheared using the Warner-Bratzler equipment, and the shear force values were expressed in kg. The cooking loss was determined by the difference between the steaks weights before and after roasting. The cooking losses (CL) values were presented in percentage (%).

2.4. Endocrine and metabolic parameters

Hormones and metabolites were measured to evaluate the stress status of animals at slaughter. The concentration of plasma cortisol and adrenocorticotropic hormone (ACTH) were measured using commercially available enzyme immunoassay kits (Cortisol Accu Bind, Monobind Inc., Lake Forest, CA, USA and ACTH ELISA Bioamerica Inc., Irvine, CA, USA). Inter- and intra-assay standards were used to validate each plate. Muscle samples (0.5 g) were homogenized in 2.5 mL of perchloric acid (0.6 M) using a Turratec TE-102 homogenizer. The homogenate was then centrifuged at 3000 $\times g$ (4 °C) for 10 min, and the supernatant was filtered through the Whatman No. 54 filter paper. The pH of the filtered supernatant was adjusted (7.0–7.5) to determine the concentrations of muscle lactate and glycogen spectrophotometrically, using the EnzyChrom™ Lactate Assay (Bioassay Systems, Hayward, USA) and EnzyChrom™ Glycogen Assay (Bioassay Systems, Hayward, USA) kits, respectively.

2.5. Muscle protein extraction

Samples of LT muscle from 6 animals chosen randomly from each pHu group were used to perform the proteomic analysis. Frozen muscle tissue (0.5 g) was homogenized in 2 mL of an ice-cold lysis buffer containing 8 M urea, 2 M thiourea, 1% DTT, 2% CHAPS and 1% protease inhibitor cocktail with a polytron. Crude extracts were vigorously shaken for 30 min at 4 °C, followed by centrifugation at 10,000 $\times g$ for 30 min at 4 °C (Bouley et al., 2005). The supernatant was harvested and protein concentration determined as described in the PlusOne 2-D Quant Kit (GE Healthcare). The BSA protein standard was used to get a calibration curve.

2.6. Sample preparation for mass spectrometry

Protein extracts previously resuspended in lysis buffer were desalinized using Amicon Ultra-0.5 mL 3 K-NMWL filter devices (Millipore, Ireland). The sample was washed out four times with approximately 400 µL of a solution containing 50 mM NH_4CO_3 (pH 8.0) and 2 M urea. The retained protein solution (100 µL) was harvested, and total protein concentration determined using a Bradford Protein Assay kit (Bio Rad). For trypsin digest, 50 µL of each sample (1 µg/µL) was denatured with