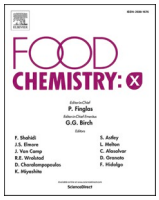
Food Chemistry: X 17 (2023) 100608

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Investigation of changes in proteomes of beef exudate and meat quality attributes during wet-aging

Qianqian Yu [a], Shimeng Li [a], Bei Cheng [a], Yuan H. Brad Kim [b], Chengfeng Sun [a,*]

[a] College of Life Science, Yantai University, No. 30 Qingquan Road, Laishan District, Yantai 264005, Shandong, China
[b] Meat Science and Muscle Biology Laboratory, Department of Animal Science, Purdue University, West Lafayette, IN 47906, United States

ARTICLE INFO

Keywords:
Meat quality
Beef exudates
Wet-aging
Label-free proteomics

ABSTRACT

This study was performed to evaluate the effects of wet-aging (3, 7, 14, 21, and 28 d at 2 °C) on beef (*longissimus lumborum* muscles) exudate proteome and meat quality changes. The pH, purge loss, and tenderness of beef increased with aging ($P < 0.05$), while color and lipid oxidative stabilities decreased, especially when long-term (14 and 21 d) aged meat were repackaged and displayed under retail condition ($P < 0.05$). Nineteen proteins changed significantly with aging (FDR < 0.05), in which most of them progressively accumulated in exudates over aging periods. Combined with partial least squares discriminant analysis, 16 proteins (including 9 structural proteins, 3 metabolic enzymes, 1 heat shock protein, 2 binding proteins, and KBTBD10 protein) were screened as characteristic proteins that could be used for potential meat quality indication. These findings offered novel insight into the utilization of exudates for meat quality assessment.

1. Introduction

Postmortem aging is a common post-harvest technique to improve the edibility characteristic of meat such as tenderness, juiciness, and/or flavor. Different aging methods with their unique characteristics are well-documented and practiced, including wet-aging, dry-aging, and novel stepwise-aging (dry-then-wet aging) (Kim et al., 2018b). Wet-aging is the most commonly used method due to its flexibility, affordability, and ease of storage (McMillin, 2017). A process of wet-aging is storing fresh meat in vacuum-packaged bags at refrigerated temperatures for a certain period of time (Kim et al., 2018b). Significant improvements in meat tenderness occur during postmortem aging through a breakdown of the structural proteins into smaller constituents comprised of polypeptides or amino acids by the various endogenous proteases (Lana & Zolla, 2016; Matarneh, England, Scheffler, & Gerrard, 2017). Meanwhile, with the release of flavor-related compounds, such as polypeptides, nucleotides, reducing sugars, amino acids, free fatty acids, and lipid oxidation-related compounds, meat flavor can be also enhanced after a period of aging (Kim et al., 2017; Kim et al., 2018b).

However, not all the effects of postmortem aging are positive. Greater aging time can result in reduced oxidative stability, mitochondria degeneration, and depletion of reducing substrates (Ke et al., 2017; Ramanathan et al., 2020). Consequently, longer aging time may induce adverse impacts on meat shelf-life upon being repackaged into a retail display packaging format (e.g. oxygen permeable overwrap film), resulting in a rapid decline in color stability and severe lipid oxidation during display storage (Mitacek et al., 2019; Ramanathan et al., 2020; Yu, Cooper, Sobreira, & Kim, 2021). In this regard, Kim et al. (2018b) proposed a smart-aging strategy, by which the positive impacts of postmortem aging on meat palatability can be maximized, while minimizing the oxidation-related quality defects through developing a tailored post-harvest processing system.

Recently, the utilization of meat exudate for meat quality prediction has been proposed as a potential post-harvest processing system for the optimized aging practice (Castejón, García-Segura, Escudero, Herrera, & Cambero, 2015; Kim et al., 2015; Setyabrata, Ma, Cooper, Sobreira, & Kim, 2018; Yu et al., 2021). Meat exudate is a natural aqueous solution released from meat during wet-aging or storage, which contains sarcoplasmic proteins, peptides, amino acids, nucleotides, and soluble enzymes (Castejón et al., 2015). In fact, several studies utilizing metabolomics profiling of meat exudate (using ^{1}H nuclear magnetic resonance spectroscopy or liquid chromatography coupled with tandem mass spectrometry), demonstrated the value of analyzing meat exudate for its relevance to meat quality attributes of beef (Castejón et al., 2015; Setyabrata et al., 2018), pork (García-García, Herrera, Fernández-Valle, Cambero, & Castejón, 2019; Yu et al., 2021) and chicken (Xing et al.,

* Corresponding author.
E-mail address: cfsunytu@126.com (C. Sun).

https://doi.org/10.1016/j.fochx.2023.100608
Received 8 December 2022; Received in revised form 6 February 2023; Accepted 20 February 2023
Available online 23 February 2023

2020).

High-throughput proteomics platforms have been extensively adopted in numerous studies for the meat quality biomarkers discovery (Fuente-García, Sentandreu, Aldai, Oliván, & Sentandreu, 2021; Gagaoua et al., 2021; Huang et al., 2020). However, nearly all of those proteomics studies focus on analyzing muscle tissue, very few researches have been performed to elaborate the exudate proteome profiling and its relationship with meat quality.

Thus, there is a clear knowledge gap of understanding the effect of postmortem aging on changes in proteome profile in meat exudate. Taken together, we hypothesized that meat exudates could provide useful background biochemical information to indirectly reflect meat quality attribute changes during aging. The aim of this study was to identify proteomes of beef exudate during wet-aging (3, 7, 14, 21, and 28 d) by label-free proteomics strategy. The findings of the present study could provide practical insights into a development of meat quality prediction systems using the exudate of fresh meat during aging.

2. Materials and methods

2.1. Sampling

Beef loin (*longissimus lumborum*) muscles (B grade according to Chinese National Standard GB/T 29392–2012) from one side of nine beef carcasses (Chinese *Luxi* yellow cattle, steers, approximately 22 months old, fed the same diet) at 2 d postmortem was obtained from a commercial abattoir (Yangxin Huasheng Meat CO., LTD, Shandong, China.). Muscles were vacuum packaged, placed in cryogenic storage containers with ice bags, then transported to the meat laboratory at Yantai university. Each loin was divided into five sections, vacuum packaged (polyethylene terephthalate material, 0.08 mm thickness), and then randomly assigned into five aging treatments (3, 7, 14, 21, and 28 days of additional aging) in the dark of a 2 °C cooler. After completion of each aging period, about 1 mL of exudate from each section was collected in the freezing tube (Corning® 2 mL external threaded polypropylene cryogenic vial, Corning Incorporated, New York, USA), and stored at −80 °C for proteomics analysis. The initial weight of the beef sections before aging and the final weight after each aging treatment were recorded for purge loss calculation. Three 2.54 cm thick steaks were cut from each section for cooking loss, shear force value, display color, and lipid oxidation evaluation. The remaining samples were used for pH, centrifugal loss, and myofibril fragmentation index (MFI) analyses immediately.

2.2. Meat quality attributes

2.2.1. pH of meat

A total of 3 g meat samples in duplicates were homogenized with 30 mL of deionized water. The pH of homogenate was recorded by a pH meter (FE-20, Mettler Toledo, Zurich, Switzerland) coupled with a 3-in-1 plastic pH electrode (LE438, Mettler Toledo, Zurich, Switzerland). Prior to measurement, the pH electrode was calibrated with standardized buffers (pH 4.0 and 7.0).

2.2.2. Purge loss

Purge loss of beef section was calculated by the following formula:

$$Purge\ loss\ (\%) = 100 \times \left(1 - \frac{final\ weight\ after\ aging}{initial\ weight\ prior\ to\ aging}\right)$$

2.2.3. Cooking loss of meat

Cooking loss was performed as described by Yu et al. (2021). In brief, beef steaks (2.54 cm thick) were cooked at 135 °C on a flat-top electric griddle (LR-X2901, Liven, Beijing Liven Technology Co., Ltd, Beijing, China). Each steak was flipped when the core temperature was 41 °C and then cooked until 71 °C. Cooking loss was calculated by the following formula:

$$Cooking\ loss\ (\%) = 100 \times \left(1 - \frac{weight\ of\ cooked\ sample}{weight\ of\ raw\ sample}\right)$$

2.2.4. Centrifugation loss

Duplicates of raw meat sample (around 10 g, 1 cm × 1 cm × 6 cm of each) were wrapped with filter paper and centrifuged at 1500 *g* for 5 min at 4 °C. Centrifugation loss was calculated according to the following formula:

$$\text{Centrifugation loss}\ (\%) = 100 \times (1 - \frac{weight\ after\ \text{centrifugation}}{weight\ before\ \text{centrifugation}})$$

2.2.5. Warner-Bratzler shear force and MFI of meat

Aforementioned cooked beef steaks were chilled at 4 °C for 24 h prior to using for shear force assessment. Meat cores were collected parallel to the fiber orientation by a hand-held coring device (1.27 cm diameter) and used for shear force measurement. Peak shear force values of six replicates were recorded using a TMS-Pro Texture Analyzer (Food Technology Corporation, Virginia, USA) coupled with a Warner-Bratzler V-shaped blade attachment with 60 mm/min speed. The average value expressed in Newtons was used for statistical analysis.

MFI was performed in triplicates as described by Culler, Smith, and Cross (1978). The absorbance of diluent myofibrillar suspension (with a protein concentration of 0.5 mg/mL) at 540 nm was recorded using a UV spectrophotometer (T6 Beijing Purkinje General Instrument Co., Ltd, Beijing, China). The absorbance reading was multiplied by 200 to give values for MFI.

2.2.6. Color and lipid oxidative stability during display

For display color and lipid oxidative stability evaluation, two steaks from each assigned aging section were placed individually on plastic trays with water absorbent pads and overwrapped with polyvinyl chloride film (20,000 ± 20 % cm^3/m^2/24 h oxygen transmission rate), and then stored at 2 °C vertical display cabinets (equipped with glazed doors and four-layer shelves, Amoi Technology Co., LTD, Xiamen, China) for 6 days display. No extra light treatment was performed, but the room with the cabinets has natural light. One of the steaks was used for display color evaluation. Instrumental color attributes including lightness (L^*), redness (a^*), and yellowness (b^*) were recorded randomly in three locations daily using a colorimeter (CS-800, Hangzhou Color Spectrum Technology Co., Ltd.) with an illuminant of D_{65}, 11 mm diameter aperture, and 10°standard observer. The chroma and hue angle were calculated according to the procedures from AMSA guidelines (King et al., In Press).

Around 20 g of meat was collected daily from another steak, stored at −20 °C, and used for lipid oxidation measurement. The 2-thiobarbituric reactive substances (TBARS) assay was conducted to evaluate lipid oxidation using the protocol described by Setyabrata and Kim (2019). The TBARS value was expressed as the value of absorbance multiplied by 5.54.

2.3. Proteomics analysis of beef exudate

2.3.1. Protein extraction and digestion

A total of 25 exudate samples (5 randomly selected biological replicates × 5 aging treatments) were used for label-free proteomics analysis. SDT lysis buffer (4% sodium dodecyl sulfate, 100 mM dithiothreitol, 100 mM, Tris-HCl pH 8.0) was used for protein extraction. After boiling for 3 min, the samples were ultrasonicated for 2 min and then centrifuged at 16,000 *g* for 20 min. A BCA Protein Assay Kit (Bio-Rad, USA) was used to measure the protein concentration of supernatant. Protein (300 μg of each sample) was digested using the filter-aided sample preparation (FASP) method according to the procedures from Wiśniewski, Zougman, Nagaraj, and Mann (2009). Briefly, an

appropriate amount of 1 M dithiothreitol was added to each sample to keep the final concentration of 100 mM, samples were boiled for 5 min, and cooled to room temperature. UA buffer of 200 μL (8 M Urea, 150 mM Tris-HCl, pH 8.0) was added, and the mixture was transferred into a 10 KDa ultrafiltration centrifuge tube, centrifuged at 12,000 *g* for 15 min, discarded the filtrate, and repeated once. Iodoacetamide (100 μL, 50 mM iodoacetamide in UA buffer) was added and mixed at 600 rpm for 1 min, kept in a dark place, and then centrifuged at 12,000 *g* for 10 min. Then, the sample was washed twice with 100 μL UA buffer and 100 μL NH_4HCO_3 buffer, respectively. The protein suspension was digested with trypsin (6 μg Trypsin in 40 μL NH_4HCO_3 buffer) with a ratio of 50:1 overnight at 37 °C. The peptides were collected by centrifugation at 12,000 *g* for 10 min and desalted with a C_{18} cartridge, and re-suspended in 0.1% trifluoroacetic acid for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

2.3.2. LC-MS/MS analysis

A QE HF-X mass spectrometer coupled with Easy-nLC 1200 (Thermo Scientific, Bremen, Germany) was used for peptide detection. The peptide was loaded to a trap column (100 μm × 20 mm, 5 μm, C_{18}, Dr. Maisch GmbH, Ammerbuch, Germany) in buffer A (0.1% formic acid in water), and then separated by reverse-phase high-performance liquid chromatography using a C_{18} column (75 μm × 150 mm; 3 μm, Dr. Maisch GmbH, Ammerbuch, Germany). The mobile phase A was 0.1% formic acid in water, and B was 0.1% formic acid in acetonitrile/water (80%:20%, *v/v*). The flow rate was 300 nL/min. Peptides were eluted over 120 min with a linear gradient of buffer B: from 5% B to 8% in 2 min, from 8% to 23% in 88 min, and increased to 40% in 10 min, followed by an increase up to 100% within 8 min, and then kept 100% for 12 min. MS data were acquired using a data-dependent top 20 method which dynamically chose the most abundant precursor ions from the survey scan (300–1800 *m/z*) for HCD fragmentation. The MS/MS full scan was acquired at a resolution of 15,000 at *m/z* 200. The maximum injection time was set to 50 ms for MS/MS. The normalized collision energy was 28 and the isolation window was set to 1.6 Th.

2.3.3. Data process and analysis

The MS data were processed using Proteome Discoverer software version 2.4 (Thermo Scientific, Bremen, Germany), and were searched against the database with Uniprot-*Bos taurus* (Bovine) [9913]-47017–20210903.fasta. Trypsin was selected as a digestion enzyme. The maximal two missed cleavage sites and the mass tolerance of 10 ppm for precursor ions and 0.02 Da for fragment ions were defined for database search. Carbamidomethylation of cysteine was appointed as a fixed modification, while oxidation (M), deamidation (N, Q), and acetyl (Protein *N*-term) were set as variable modifications for database searching. The database search results were filtered with false discovery rate (FDR) < 0.01 both at the peptide-spectral matching level and protein level. The razor and unique peptides were used for protein quantification. Five biological replicates were performed, and only those proteins that were detected in at least 3 out of 5 biological replicates for each treatment were retained (Poleti et al., 2018) for further analyses. The remaining missing values were imputed using K-Nearest Neighbours (KNN). Prior to statistical analyses, all proteins were normalized by a constant sum, transformed by generalized log transformation, and scaled to range variance. One-way ANOVA and partial least squares discriminant analysis (PLS-DA) statistical analysis were performed by MetaboAnalyst 5.0. Proteins with FDR < 0.05 by ANOVA were considered significant changes among aging periods.

2.3.4. Bioinformatics analyses

Bioinformatics analyses were conducted by STRING 11.5 (https://cn.string-db.org/). Enriched GO and KEGG pathways were statistically significant at the FDR < 0.01 level.

2.4. Statistical analysis

In this study, the experimental design of this study was a completely randomized design, where each loin served as an experimental unit and each carcass served as a block. Specifically, a repeated measure design was used to measure instrumental color at the retail display. The collected data were processed using the SPSS software (SPAA Inc., Chicago, IL, USA) for ANOVA to assess the significance of aging period treatments at the level of $P < 0.05$. Pearson's correlation analysis was performed using SPSS and $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Effects of wet-aging on meat quality attributes

3.1.1. pH, purge loss, cooking loss, and centrifugation loss

Beef pH increased with aging ($P < 0.05$) from 5.49 in 3 d to 5.57 and 5.61 in 21 and 28 d, respectively (Table 1). This would be likely by the changes in charges caused by proteolytic enzymes during aging, moreover vacuum packaging excluded oxygen transfer which might also prevent the increase in acidity of meat (Boakye & Mittal, 1993).

Purge loss increased from 1.44% in 3 d to 3.08% in 28 d aging treatments ($P < 0.05$), but meanwhile no significant changes were found in centrifugal loss and cooking loss among aging groups (Table 1). Similar trends in purge loss and cooking loss were found in our previous study of wet-aged pork meat (Yu et al., 2021). The increased purge loss during aging processing might be attributed to myofibrillar and cytoskeletal protein degradation. These proteins are degraded to various degrees during postmortem aging and resulted in the loss of the linkage between myofibrils and the cell membrane, thus promoting some of the water migration from the muscle cell structure (Straadt, Rasmussen, Andersen, & Bertram, 2007; Warner, 2017). Usually, cooking loss and centrifugal loss are related to how much water is available and how easily it can leave the muscle structure network. Purge loss increased following aging which may indicate a lesser volume of water available in muscle to be easily run off. However, about 85% of the total water, termed "immobilized" or "entrapped" water, is held by steric effects or by attraction to the bound water (Huff-Lonergan & Lonergan, 2005; Pearce, Rosenvold, Andersen, & Hopkins, 2011), which does not easily leave the structure but can be removed by drying and be lost during the rigor process and through protein degradation (Warner, 2017). Thus, it can be postulated that this group of water may be lost much more easily after a longer aging time, especially given some degree of external forces. Therefore, it was reasonable that even increased amount of water lost as a purge during the aging period, no significantly changed in cooking loss and centrifugal loss were found with aging.

3.1.2. MFI and shear force

Meat aged for 3 days showed the highest shear force value (40.06 N),

Table 1
The pH, purge loss, cooking loss, and centrifugation loss changes of beef loins* during postmortem aging.

	Aging times (d)				
	3	7	14	21	28
pH	5.49 ± 0.01 a	5.53 ± 0.01 ab	5.55 ± 0.02 ab	5.57 ± 0.03 bc	5.61 ± 0.03 c
Purge loss (%)	1.44 ± 0.14 a	1.80 ± 0.22 a	2.19 ± 0.26 ab	2.80 ± 0.20 bc	3.08 ± 0.45 c
Centrifugal loss (%)	17.30 ± 1.34	18.61 ± 1.34	16.73 ± 0.67	17.31 ± 1.09	16.68 ± 1.06
Cooking loss (%)	29.66 ± 1.27	33.02 ± 1.65	30.64 ± 0.86	32.39 ± 0.95	31.66 ± 1.45

*Beef loins obtained at 2 days postmortem.
Results are expressed as the mean ± standard error. The means with different letters (a-c) in a row are different ($P < 0.05$).

and the value decreased ($P < 0.05$) to 29.75 and 28.26 N when aged for 21 and 28 d, respectively (Fig. 1). No difference in shear force values was found between 21d and 28d ($P > 0.05$). Meanwhile, aging had a significant impact on MFI, which gradually increased ($P < 0.05$) from 43.67 in the 3 d sample to 73.44 in the 28 d sample. The improved tenderness of postmortem aged meat is generally caused by the destruction of the myofibrillar structure and degradation of myofibrillar and cytoskeletal proteins by endogenous proteolytic enzymes, including calpain system, cathepsins, apoptosis-mediated caspase system, and/or proteasomes (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010; Lana & Zolla, 2016).

3.1.3. Oxidative stabilities of meat color and lipid

Significant interactions between aging periods and display were found at L^*, a^*, b^*, hue angle, and chroma of beef loins. For each aging treatment, a^* was affected by display duration and showed decreases ($P < 0.01$) from 0 d to 6 d display (Fig. 2b). Beef steaks aged for 3 d had the highest a^* value at the beginning and the ending of display ($P < 0.01$). Steaks aged for 14 d and 21 d had different a^* values at 0 d display with 18.69 *vs*. 17.74 ($P < 0.01$), but their a^* value were similar ($P > 0.05$) from the 2 d display to the 5 days display. In contrast, steaks from the longest aging time group (28 d) exhibited the lowest a^* value during 1 to 6 days of display ($P < 0.01$), especially in the later stages of display (4 to 6 days). Hue angle is an indication of discoloration over time, higher values indicate the less red and greater formation of metmyoglobin (King et al., In Press). Beef steaks aged for 28 d had the highest hue angle values ($P < 0.05$) among all aging groups during retail display, while steaks aged for 3 d had the lowest values through the whole display period (Fig. 2d). Chroma represents the color intensity of the meat, which was also affected by aging time ($P < 0.01$), steaks aged for 28 d had the lowest chroma values ($P < 0.01$) during retail display (Fig. 2e). Meanwhile, for each aging treatment, chroma values dramatically decreased during retail display periods ($P < 0.01$) (Fig. 2e). These results indicated that extended aging duration was detrimental to beef color stability during subsequent retail display. This observation was consistent with the findings of Mitacek et al. (2019), where beef sections aged for 28 days had a 30.4% decrease in a* value than that for 3 days. Similar results were also observed by Ma et al. (2017), where an increase in discoloration was found in beef muscle with wet-aging. The declined color stability in aged beef could be due to the increasingly severe mitochondrial damage, depletion of metabolites related to myoglobin redox stability, and/or increased oxidative stress of meat (Ke et al., 2017; Mitacek et al., 2019; Ramanathan et al., 2020).

No interaction between aging and display was found at TBARS values of beef loins ($P = 0.590$). Beef aged for longer time (21d and 28d) had higher TBARS values ($P < 0.05$) than that aged for 3, 7, and 14 d through the whole retail display period (Fig. 2f). This result indicated that a longer aging time could induce more lipid oxidation at the end of aging. In support, Ma et al. (2017) also reported a similar finding, indicating an increase in TBARS value with extended aging time. In addition, TBARS values were greatly affected by retail display regardless of aging time, where its value increased along with retail display duration ($P < 0.05$). Similarly, Mitacek et al. (2019) reported an increase in TBARS value with display, and greater lipid oxidations in long-term (14 d and 21 d) aged meat at 6 d display than that in short-term (3 d and 7 d) ones. The depletion of endogenous antioxidants and loss of structural integrity of cells with aging may cause muscles to be more susceptible to oxidation when it was exposed to aerobic conditions (Kim, Kim, Seo, Setyabrata, & Kim, 2018a; Yu et al., 2021).

3.2. Proteomic analysis of beef exudate

There were 1023 proteins identified in beef exudates from different aging duration, only those proteins that were detected at least 3 biological replicates in each treatment were retained. Thus, 776 proteins were considered for further analysis, 79% of them with molecular weight below 70 KDa (28% of proteins with molecular weight of 40–70 KDa, and 51% with below 40 KDa, Fig. S1a). KEGG analysis was carried out to extract the significantly enriched biological pathways related to the exudate proteins. The top 15 significant pathways (FDR < 0.01) were revealed and presented in Fig. S1b. The significantly enriched pathways included glycolysis/gluconeogenesis, proteasome, biosynthesis of amino acids, pentose phosphate pathway, glutathione metabolism, and pyruvate metabolism.

Enrichments of meat exudate proteins in the top 10 items (FDR < 0.01) of three different categories based on GO analysis were presented in Table S1. As for biological processes, the small molecule metabolic process (GO:0044281), protein folding (GO:0006457), catabolic process (GO:0009056), and oxidation–reduction process (GO:0055114) were enriched significantly. The cellular components analysis indicated that exudate proteins were significantly enriched in proteasome complex (GO:0000502), myofibril (GO:0030016), and sarcomere (GO:0030017) components. In the molecular functions, proteins related to cytoskeletal protein binding (GO:0008092), actin binding (GO:0003779), protein binding (GO:0005515), and catalytic activity (GO:0003824) were enriched significantly.

A complex series of energetic, enzymatic, biochemical, and physical changes occur in the postmortem muscles, and ultimately determine the edible quality of the meat. As expected, proteins related to these complex changes were detected as well in beef exudates. After slaughter,

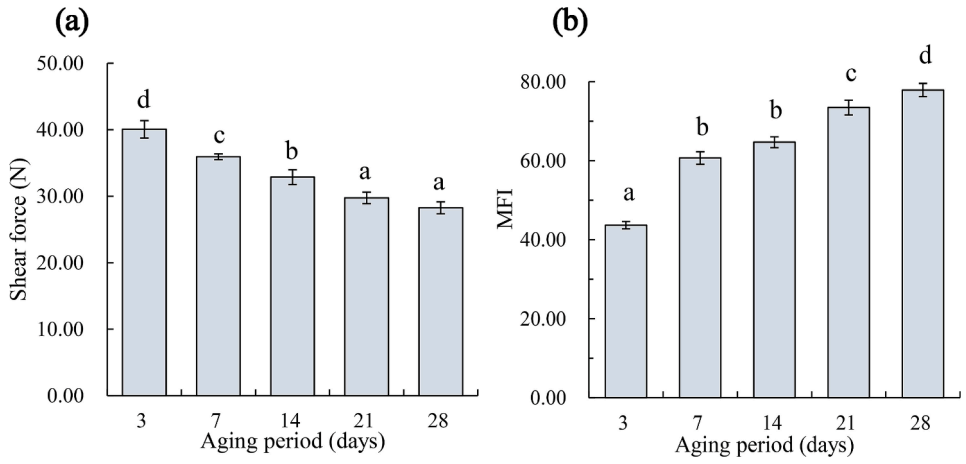


Fig. 1. Effect of wet-aging on shear force (a) and myofibril fragmentation index (MFI, b) of beef meat. Means with different letters (a-d) are different ($P < 0.05$). Error bars indicate the standard error of the mean.

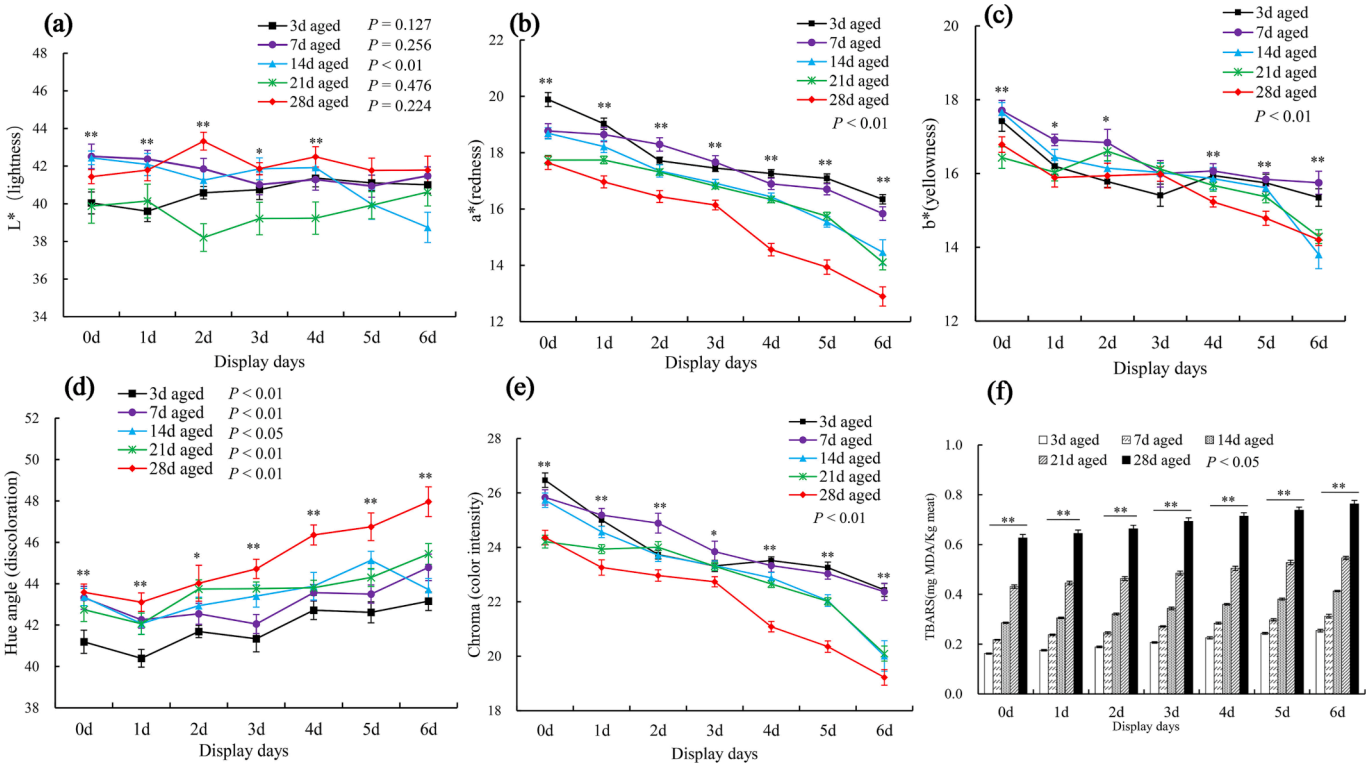


Fig. 2. Effect of wet-aging on lightness (a), redness (b), yellowness (c), hue angle (d), chroma (e), and TBARS (f) changes during further 6 days of display. The significant differences among aging treatments at the same display time were marked with asterisks (* means $P < 0.05$; ** means $P < 0.01$). Error bars indicate the standard error of the mean.

oxidative phosphorylation in muscles breaks down quickly, and aerobic metabolism is gradually switched to anaerobic metabolism during the conversion of muscle to meat (Matarneh et al., 2017). This process may continue in wet-aging until muscle glycogen is depleted. In the present study, glycolysis/gluconeogenesis was one of the significantly enriched pathways, where 19 relevant proteins were detected in beef exudates (Fig. S1b).

The proteasome is a large protein complex consisting of a proteolytic core and cofactors with various regulatory functions (https://www.kegg.jp). It has the capacity to degrade both sarcoplasmic and myofibrillar proteins into their amino acid components (Matarneh et al., 2017), and this process requires metabolic energy (Tanaka, 2009). With postmortem aging, myofibrillar proteins could be released into the sarcoplasmic fraction due to proteolysis (Bowker, Eastridge, & Solomon, 2014; Laville et al., 2009), and be detected in meat exudates. Thus, some exudate proteins enriched in proteasome complex, myofibril, sarcomere, and contractile fiber items were revealed by GO analyses. In addition, some proteins enriched in activities related to threonine-type peptidase/endopeptidase were also identified via GO analyses. This observation is in accordance with our previous work on the metabolomic profile of pork exudate (Yu et al., 2021), where over 20 overabundant dipeptides/tripeptides were identified in pork purge aged for 9 d and 16 d (longer aging samples). These results indirectly indicated that the proteolysis process has taken place in postmortem muscle tissue.

3.3. Characteristic proteins discovery

Nineteen exudate proteins changed significantly with wet-aging by Tukey's HSD ANOVA analysis (FDR < 0.05, presented in Table S2). PLS-DA is a statistical method of supervised discriminant analysis. It can be used to establish the relationship model between variables' expression level and sample category to achieve the discrimination of sample category. Variable importance in projection (VIP, usually with VIP value > 1.0 as the screening criteria) is usually used to measure the influence magnitude and explanatory ability of each variable's expression pattern on the classification and discrimination of each group of samples. Thus, PLS-DA was performed to find differentially characteristic proteins that contribute most to the aging time classification and may be used as a source of potential biomarkers for predicting aging times and meat quality changes. In this study, the variables with the top 20 proteins (VIP > 1) were selected as potentially important proteins (Fig. 3). Combined with ANOVA and VIP value, 16 proteins (FDR < 0.05 and top 20 VIP ranking) were selected as characteristic proteins of beef exudates during wet-aging periods (Table 2 and Fig. 4), in which most of them were gradually accumulated in beef exudate with extended aging. Meanwhile, Pearson's correlation analysis indicated significant correlations between most of the 16 characteristic proteins and meat quality traits (Table 2). These proteins can be preliminarily categorized into five groups according to their functions as metabolic, structural, heat shock, binding, or other proteins.

3.3.1. Metabolic enzymes

Gp_dh_N domain-containing protein (A0A3Q1LPH5, belongs to glyceraldehyde 3-phosphate dehydrogenase family) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, P10096) decreased with aging (Table 2 and Fig. 4). Those proteins were positively correlated with shear force (r = 0.529** and r = 0.529**, respectively), but negatively correlated with MFI (r = -0.705** and r = -0.601**, respectively) and TBARS (r = -0.795** and r = -0.748**, respectively). The down-regulated expression of GAPDH in exudate during wet-aging was consistent with research results from Bowker et al. (2014), who found that the relative abundance of GAPDH in exudate decreased with aging and was positively correlated with overall shear force of beef (r = 0.62**). Meanwhile, GAPDH as a biomarker of beef tenderness has been well demonstrated (Gagaoua et al., 2021), thus it stands to reason to suggest that GAPDH could be a potential biomarker in meat exudate for

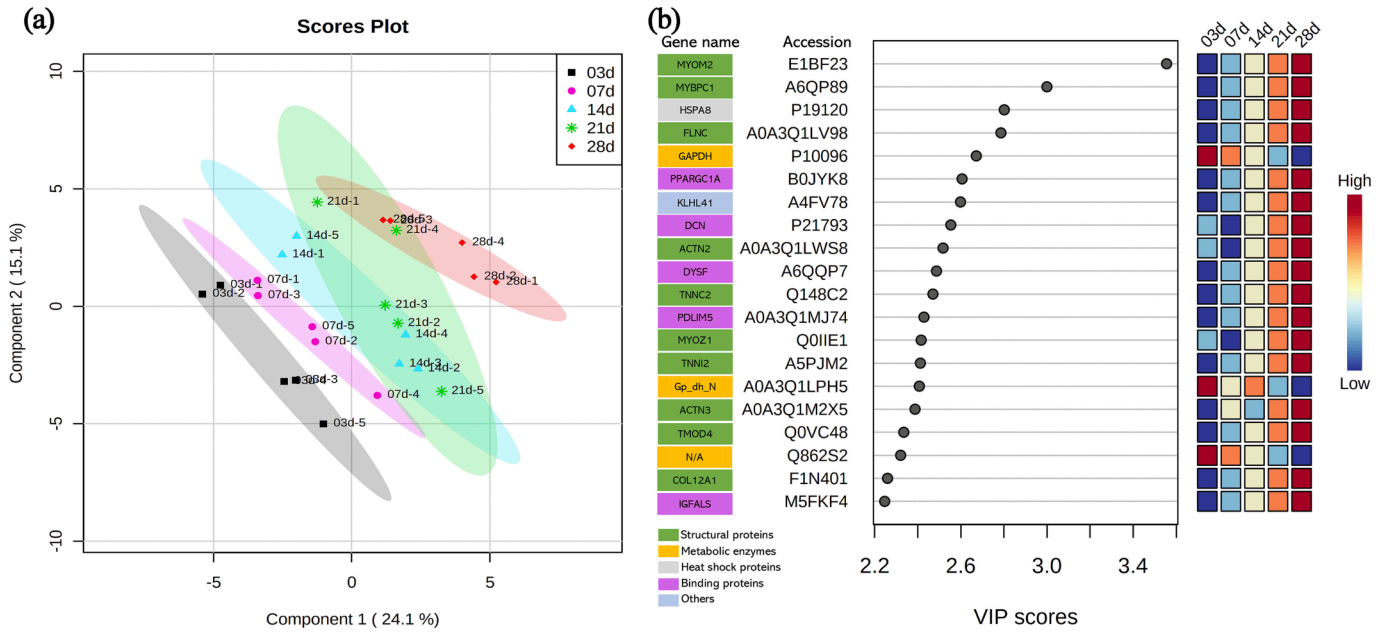


Fig. 3. The partial least squares discriminant analysis (PLS-DA) score plots (a) and the top 20 exudate proteins with VIP > 1 (b), the colored boxes on the right indicate the relative concentrations of the corresponding protein in each group under study.

Table 2
Sixteen characteristic proteins that were screened by ANOVA and PLS-DA, and correlations with meat quality attributes.

Accession	Protein Name	Gene Name	ANOVA	PLS-DA VIP	Correlation coefficients with meat quality[1]				
			FDR	Comp. 1	Purge loss	Shear force	MFI	a*	TBARS
Metabolic enzymes									
A0A3Q1LPH5	Gp_dh_N domain-containing protein	Gp_dh_N domain-containing protein	9.32E-04	2.41		0.529**	−0.705**		−0.795**
B0JYK8	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	PPARGC1A	1.10E-02	2.61		−0.669**	0.725**		0.805**
P10096	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	2.47E-03	2.67	−0.421*	0.529**	−0.601**		−0.748**
Structural proteins									
A0A3Q1LV98	Filamin C	FLNC	9.60E-05	2.79	0.406*	−0.612**	0.773**	−0.653**	0.897**
A0A3Q1M2X5	Alpha-actinin-3	ACTN3	1.60E-03	2.39		−0.653**	0.641**		0.846**
A5PJM2	TNNI2 protein (Fragment)	TNNI2	1.54E-02	2.41	0.422*	−0.556**	0.724**	−0.457*	0.825**
E1BF23	Myomesin 2	MYOM2	1.48E-04	3.56		−0.675**	0.687**		0.847**
Q0IIE1	MYOZ1 protein	MYOZ1	1.10E-02	2.42	0.470*	−0.506**	0.626**	−0.527**	0.854**
Q0VC48	Tropomodulin-4	TMOD4	3.75E-02	2.33		−0.556**	0.653**	−0.457*	0.736**
Q148C2	Troponin C type 2 (Fast)	TNNC2	1.60E-03	2.47		−0.667**	0.657**		0.768**
P21793	Decorin	DCN	3.48E-04	2.55		−0.421*	0.523**	−0.401*	0.826**
A6QP89	MYBPC1 protein	MYBPC1	1.48E-04	3.00		−0.667**	0.752**	−0.508**	0.900**
Heat shock proteins									
P19120	Heat shock cognate 71 kDa protein	HSPA8	2.47E-03	2.80	0.562**	−0.703**	0.797**	−0.475*	0.790**
Binding proteins									
A0A3Q1MJ74	PDZ and LIM domain 5	PDLIM5	4.67E-02	2.43	0.437*	−0.484*	0.631**		0.725**
A6QQP7	Dysferlin	DYSF	2.59E-02	2.49	0.430*	−0.664**	0.688**		0.780**
Others									
A4FV78	KBTBD10 protein	KLHL41	1.10E-02	2.60		−0.720**	0.723**		0.687**

MFI: myofibril fragmentation index; a*: redness; TBARS: The 2-thiobarbituric reactive substances.
[1] Pearson's correlation between characteristic proteins and meat quality attributes was performed using data from 5 biological replicates.

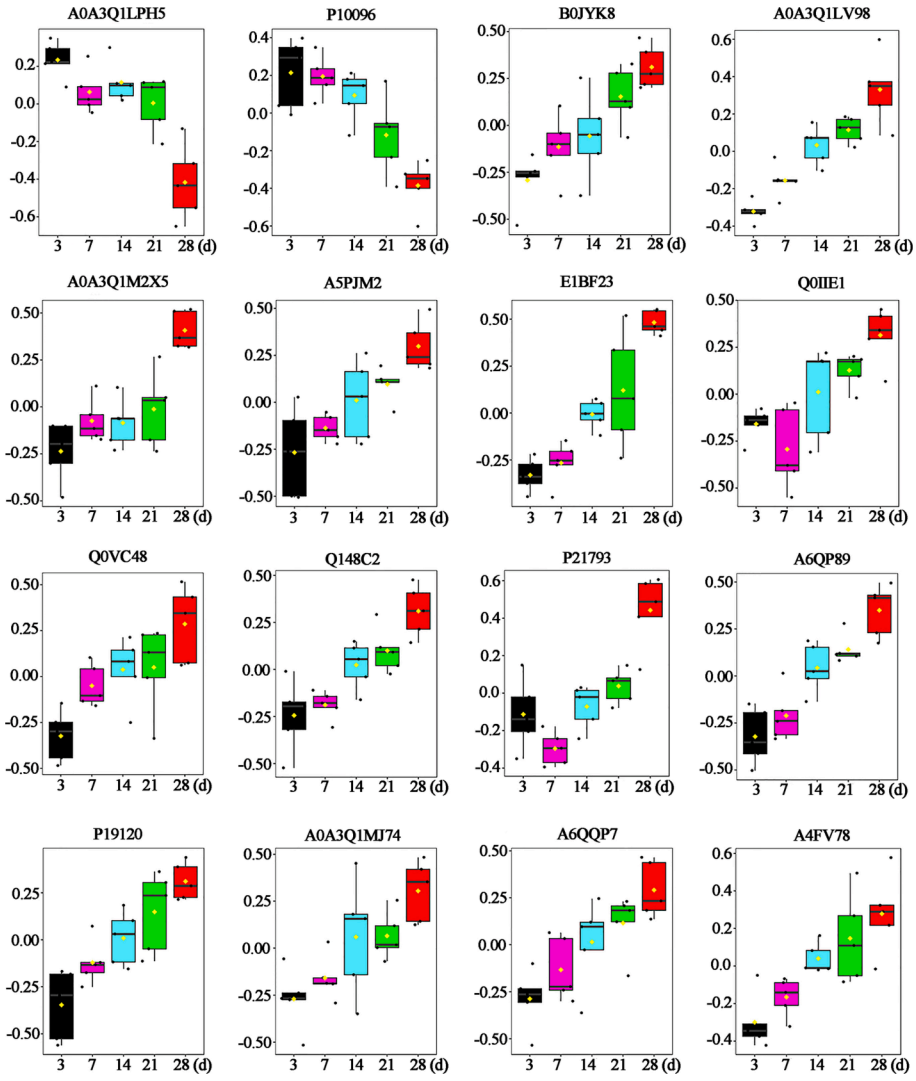


Fig. 4. The 16 characteristic proteins in beef exudates changed with aging.

meat tenderness changes during wet-aging. Further research should be warranted to validate this postulation.

3.3.2. *Structural proteins*

It has been reported that intact myofibrillar proteins could be released into the sarcoplasmic fraction due to proteolysis (Anderson, Lonergan, & Huff-Lonergan, 2012), and the sarcoplasmic fraction of muscles might also contain degradation products of myofibrillar proteins (Bowker et al., 2014; Laville et al., 2009). Therefore, it was reasonable to state that some of the structural proteins or their fragments were released from meat into exudates with purge loss, and progressively increased over the aging period (Table 2 and Fig. 4). This was supported by studies from Kim et al. (2015) and Di Luca, Elia, Mullen, and Hamill (2013), in which structural protein fragments generated from myofibrillar proteins proteolysis were identified and accumulated in the exudate during postmortem aging as well. Changes occur in muscles structure during postmortem aging, in which sarcomeres continuously be degraded, length of sarcomeres increases due to the enlargement of I-bands accompanied with Z-disk degradation (Kołczak, Pospiech, Palka, & Łącki, 2003). These breakdowns of muscle myofibrillar and structural proteins are responsible for the improved meat tenderness that was reflected by increased MFI and decreased shear force (Fig. 1). Proteins from thick and thin filaments, Z- disk components, such as myosin, actin, troponin, and desmin, have been well-demonstrated as potential biomarkers for meat tenderness (Gagaoua et al., 2021; Picard & Gagaoua, 2020). In the present study, nine structural proteins were accumulated significantly in beef exudates with aging (Table 2 and Fig. 4), and showed high VIP values from PLS-DA analysis (Fig. 3b). Specifically, filamin C (FLNC), alpha-actinin-3 (ACTN3), and MYOZ1 protein (MYOZ1) are located in Z-disks (Van Der Ven et al., 2000), meanwhile, myomesin 2 (MYOM2) also known as M-protein that located in the M-band of muscle, and interacted with myosin and titin. MYBPC1 protein (MYBPC1) known as myosin-binding protein C slow type associates with myosin and titin and plays an important role in muscle contraction (https://www.uniprot.org). Troponin C type 2 (Fast) (TNNC2) is a Ca^{2+}-binding subunit of troponin, which forms a complex with troponin I and troponin T (Ohtsuki & Morimoto, 2013). These proteins were all correlated negatively with shear force, while positively with MFI and TBARS values (Table 2). Although the underlying mechanism by which these structural proteins were accumulated in beef exudates still needs further investigation, these proteins could indirectly reflect the changes of meat tenderness during wet-aging process.

3.3.3. *Heat shock proteins (HSPs)*

Heat cognate 71 kDa protein (HSPA8) belongs to the heat shock protein 70 family, known as a molecular chaperone in various cellular processes, including folding and transport of newly synthesized

polypeptides, protection of the proteome from stress, activation of proteolysis of misfolded proteins, and the formation and dissociation of protein complexes (https://www.uniprot.org). The 70 kDa HSPs expression is often induced in response to ischemic and hypoxic conditions and as a response to increased cellular stress caused by ROS, and those proteins have been proven as good biomarkers of tenderness (Gagaoua et al., 2021). In the present study, an increase in the detection of (up-regulated) HSPA8 in exudates with aging (FDR < 0.05) was found. The more detection of HSPA8 in exudates could indicate the more release of HSPA8 from muscle cells, possibly suggesting the loss of anti-apoptotic function of muscles with aging (Ma & Kim, 2020). It has been suggested that a decrease in HSPs activity may result in a more apoptosis-mediated tenderization process via possible coupling interactions with multiple-apoptotic cascade (i.e. less interference with cytochrome *c*, apoptosome, and/or caspase-3) processes (Kim et al., 2018b). Given its relatively high VIP value (Fig. 3b) from PLS-DA analysis and significant correlations with shear force (r = -0.703**), MFI (r = 0.797**), TBARS (r = 0.790**), and a* values (r = -0.475*), the HSPA8 could be one of the potential characteristic proteins for aging time and subsequent meat quality prediction (Table 2). A further study should be warranted to validate this postulation.

3.3.4. Binding proteins

PDZ and LIM domain 5 (PDLIM5, A0A3Q1MJ74) and dysferlin (DYSF) were up-regulated (FDR < 0.05) in exudate during wet-aging (Fig. 4), and have relatively high VIP value (Fig. 3b) from PLS-DA analysis. DYSF is a critical calcium ion sensor involved in the Ca^{2+}-triggered synaptic vesicle-plasma membrane fusion and plays a role in the sarcolemma repair mechanism of both skeletal muscle and cardiomyocytes (https://www.uniprot.org/). It locates predominantly on the subsarcolemmal surface of the muscle membrane. The up-regulation of DYSF was negatively associated with shear force (r = − 0.664**), while positively correlated with MFI (r = 0.688**) with aging, which may reflect the collapse of muscle membrane structure to some extent.

4. Conclusions

In summary, the extended aging improved instrumental tenderness and myofibrillar protein fragmentation of beef loins as expected. However, adverse impacts of long-term aging on color and oxidative stability of beef loins were also confirmed. The proteome profiling of beef exudates revealed a total of 19 exudate proteins changed significantly with wet-aging, in which most of them progressively accumulated in exudates with aging. Combined with VIP values from PLS-DA analysis results, 16 proteins (Gp_dh_N domain-containing protein, PPARGC1A, FLNC, ACTN3, TNNI2, MYOM2, MYOZ1, TMOD4, TNNC2, DCN, MYBPC1, HSPA8, PDLIM5, DYSF, and KLHL41) were identified as potential characteristic proteins that could be used as meat quality biomarkers. These novel findings could suggest that beef exudate can be used as an analytical medium to understand biochemical and concomitant changes in beef muscles during aging. Future studies determining the efficacy of using candidate protein biomarkers in beef exudate for meat quality prediction would be beneficial for the practical implication for the meat industry.

CRediT authorship contribution statement

Qianqian Yu: Conceptualization, Resources, Writing – original draft, Funding acquisition. **Shimeng Li:** Data curation. **Bei Cheng:** Data curation. **Yuan H. Brad Kim:** Conceptualization, Writing – review & editing. **Chengfeng Sun:** Investigation, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This research was supported by National Natural Science Foundation of China (Grant number 32102031). The authors would like to acknowledge Shanghai Bioprofile Biotechnology Co., Ltd for technological assistance in the proteomics experiment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100608.

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