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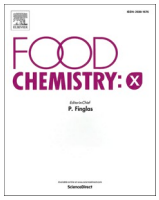

Meat exudate metabolomics reveals the impact of freeze-thaw cycles on meat quality in pork loins

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ABSTRACT

The aim of this study was to explore the effects of freeze-thaw (FT) cycles on meat quality, myofibrillar protein gelation and emulsification properties, and exudate metabolome changes in pork loins. Meat tenderness improved ($P < 0.05$), whereas water-holding capacity (WHC), meat color attributes declined ($P < 0.05$) with FT cycles. Multiple FT accelerated meat lipid and protein oxidations. Decreases in strength and WHC of myofibrillar protein gels with FT cycles were confirmed. Myofibrillar protein emulsions with more FT cycles showed a decrease in the emulsifying activity index ($P < 0.001$) and larger oil droplets, resulting in poorer storage stability. A total of 501 metabolites were tentatively identified in pork exudates, with 21 metabolites significantly correlated ($P < 0.05$ and $r > 0.6$) with meat quality attributes. These results demonstrated the potential of using the metabolomic information from exudates to elaborate on or even predict the FT cycles, or meat quality.

1. Introduction

Fresh meat is highly susceptible to bacterial decomposition and quality degradation due to its high water content and water activity (a_w) during prolonged storage. Freezing is a widely adopted method for preserving the quality, freshness, and shelf-life of meat and meat products (Coombs, Holman, Friend, & Hopkins, 2017). This preservation technique is particularly crucial in the meat export industry, as it ensures the safety and quality of meat distributed globally (Leygonie, Britz, & Hoffman, 2012). With adequate packing, the regularly used meat export storage temperature of −18 °C effectively prevents microbiological deterioration, maintaining acceptable quality for at least one year (Xiong, 2023).

Meat shelf-life is primarily governed by microbial activity, flavor, color, texture, and nutritional value (Singh & Singh, 2005). At the temperature of −20 °C, approximately 90 % of the water in meat is frozen (Calvelo, 1981), resulting in a low a_w level. This, combined with low temperatures, inhibits microbial growth and chemical or enzymatic spoilage reactions (Gracious Rinwi, Sun, Ma, & Wang, 2022). However, the freeze-thaw process negatively impacts the amount and distribution of moisture, adversely affecting the meat's overall physicochemical and textural properties (Jeong, Kim, Yang, & Joo, 2011). Researches indicate that freeze-thaw cycling accelerates protein denaturation, lipid/protein oxidation, discoloration, and reduces the water-holding capacity (WHC) of meat (Jeong et al., 2011; Leygonie et al., 2012). Additionally, freeze-thaw degradation typically exacerbates lipid oxidation and color deterioration in post-thaw meat during subsequent retail display (Jeong et al., 2011; Xiong, 2023). Consequently, this often results in a significantly shorter chilled shelf-life compared to meat that has not been previously frozen (Jeong et al., 2011; Xiong, 2023).

During the thawing process, significant moisture loss is inevitable, resulting in the loss of water-soluble nutrients and the release of exudate from the meat matrix. Various sarcoplasmic proteins, soluble enzymes, peptides, amino acids, and nucleotides are present in the meat exudate matrix (Savage, Warriss, & Jolley, 1990). The composition and volume of the exudate are typically influenced by the freeze-thaw rate, ice crystal location, and the duration of frozen storage (Pham & Mawson, 1997). Most research focuses on meat quality attributes rather than on meat exudates. Despite its valuable content, exudate is often discarded, even though it can provide insights that indirectly reflect meat quality to some extent (Setyabrata et al., 2023; Yu, Cooper, Sobreira, & Kim, 2021; Yu, Gu, Liu, Wen, & Sun, 2024). Using proteomics techniques, researchers identified unique protein expressions in exudates from fresh and freeze-thawed pork, with 22 proteins selected as discriminative markers for different types of pork (Kim et al., 2015). However, studies characterizing the metabolite changes in freeze-thawed meat exudates are few and far between.

We hypothesized that meat exudates from the thawing process could

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provide ponderable information for interpreting meat quality deterioration to a certain extent. The goal of this study was to assess the effects of freeze-thaw cycles on meat quality attributes (water-holding capacity, color traits, tenderness, lipid and protein oxidations), myofibrillar protein structures, gelation and emulsification properties, and the changes in meat exudate metabolomics for the purpose of finding characteristic metabolites and to contribute to a better understanding of the causes of meat quality deterioration.

2. Materials and methods

2.1. Chemicals and reagents

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) kit, 5 × loading buffer, and protein marker (molecular weight ranges from 10 to 170 KDa) were obtained from Beyotime Biotech Inc. (Shanghai, China). Bovine Serum Albumin (purity >98 %) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents including thiobarbituric acid (TBA), trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), sodium chloride (NaCl), magnesium chloride ($MgCl_2$), ethylenediaminetetraacetic acid (EDTA), tris base, glycine, urea, 5,5′-dithiobis (2-nitrobenzoic acid) (Ellman's reagent), and potassium bromide (KBr) were of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HPLC grade methanol, acetonitrile, and formic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Pork collection and freeze-thaw processing

Pork loins (*M. longissimus thoracis et lumborum* muscles) from the right side of six pig carcasses (*Landrace* × *Large White* female pigs, 48 h postmortem) were collected from an abattoir (Yantai Fuzu Livestock Breeding Co. LTD, Yantai, Shandong province, China). A total of 15 pork chops (2 cm thick) were obtained from each loin and randomly assigned to five freeze-thaw (FT) treatments (one-time freeze-thaw group: FT-1, two times freeze-thaw group: FT-2, three times freeze-thaw group: FT-3, four times freeze-thaw group: FT-4, and five times freeze-thaw group: FT-5). Each treatment consists of 18 individual pork chops (3 chops × 6 biological replicates). All these pork chops were individually vacuum-packaged and used for the freeze-thaw treatment. A freeze-thaw cycle was considered when pork chops were frozen in a freezer at −20 °C for 48 h and then thawed at 4 °C for 12 h. After completion of each treatment, freeze-thaw loss, water content, drip loss, and centrifugal loss changes were measured immediately. Meat exudates were collected in the freezing tube and stored at −80 °C for metabolomic analysis. One of the pork chops was utilized for cooking loss and shear force measurements, and the remaining samples were stored at −40 °C for further analysis.

2.3. Frozen-thawed loss and water content changes

The weights of the pork chops were documented before they were frozen and after thawed. Freeze-thaw loss was calculated as follow:

$$\text{Freeze} - \text{thaw loss}\ (\%) = 100 \times \left(1 - \frac{\textit{weight after}\ \text{thawing}}{\textit{initial weight prior to freezing}}\right)$$

The total water content of meat sample was determined using a method described by Naqvi et al. (2021). Drip loss was determined as described by Kim, Meyers, Kim, Liceaga, and Lemenager (2017). Centrifugation loss and cooking loss were measured according to those described by Yu, Li, Cheng, Brad Kim, and Sun (2023).

2.4. Meat color

The color attributes of lightness (L^*), redness (a^*), and yellowness (b^*) values in five random surface locations of each pork chop were recorded by a colorimeter (CS-800, Hangzhou Color Spectrum Technology Co., Ltd) with an illuminant of D_{65}, 11 mm diameter aperture, and 10° standard observer.

2.5. Meat tenderness

To assess the tenderness of pork meat, Warner-Bratzler shear force (WBSF), myofibril fragmentation index (MFI), and SDS-PAGE of myofibril proteins were measured. Cooked pork chops that were used for cooking loss were chilled at 4 °C overnight and used for WBSF evaluation. Meat cores were collected by a hand-held coring device (with a diameter of approximately 1.27 cm) parallel to the muscle fibers. Five cores of each pork chop were collected and used for WBSF measurement by a texture analyzer equipped with Warner-Bratzler V-shaped blade (TMS-Pilot, Food Technology Corporation, Virginia, USA). The testing speed was 120 mm/min. The results were expressed in Newtons.

MFI was assessed following the protocol by Yu et al. (2024). Briefly, 20 mL of pre-chilled MFI buffer was used to homogenize 2 g of ground meat. The mixture was subsequently centrifuged at 1000 *g* for 15 min at 4 °C. The precipitate was then resuspended in 20 mL cooled buffer, and the above procedure was repeated. After that, the pellet was again suspended in 5 mL of buffer and passed through a polyethylene filter (18 mesh). Additionally, 5 mL of buffer was added to facilitate the passage of myofibrils. The biuret method was used to measure the protein concentrations of the collected suspension. The absorbance of the suspensions (0.5 mg/mL protein concentration) in duplicates was measured at 540 nm. The absorbance readings were then multiplied by 200 to obtain the MFI values.

Myofibrillar protein extraction was performed as described above, and 1 mL protein solution (5 mg/mL) and 0.25 mL of 5 × loading buffer were mixed and heated at 100 °C for 5 min, followed by rapid cooling with ice water. The 5 % concentrating gel and 12 % separation gel were prepared by the SDS-PAGE gel electrophoresis kit. The protein solution and marker were loaded in separate lanes and electrophoresed at 80 V for the concentrating gel and 120 V for the separating gel. Bands were visualized by staining overnight and destained to visualize the bands.

2.6. Lipid oxidation

The 2-thiobarbituric reactive substances (TBARS) assay was performed in accordance with the method of Setyabrata and Kim (2019) for the evaluation of lipid oxidation in pork meat. Briefly, meat sample (5.00 g) was homogenized with 15 mL deionized water and 100 μL BHT for 30 s. Then, 1 mL of homogenate was mixed thoroughly with 2 mL of TCA/TBA reagent, and heated for 15 min at 80 °C. After cooling for 10 min in ice water, the samples were centrifuged at 2000 *g* for 10 min. The absorbance of the supernatant at 531 nm was measured. TBARS levels were expressed as mg malondialdehyde/kg meat.

2.7. Carbonyl and sulfhydryl contents of meat

Briefly, 2 g of minced meat was homogenized with 20 mL of 20 mM phosphate buffer (containing 0.6 M NaCl). The protein concentration of the homogenate was determined by biuret method. Carbonyl content was measured using a protocol modified from Oliver, Ahn, Moerman, Goldstein, and Stadtman (1987). The content of carbonyls was expressed as nmol carbonyl per mg protein.

The method of Ellman (1959) was used to determine the sulfhydryl content. Briefly, 8 mL of tris-glycine-urea solution was added to 1 mL of homogenate (2 mg/mL protein) and homogenized. Then, 4.5 mL of the homogenate was collected and mixed with 0.5 mL of Ellman's reagent for a 30 min reaction. After that, the mixture was centrifuged at 10,000 rpm for 15 min, and the absorbance of the supernatant was measured at 412 nm. The sulfhydryl content was expressed as nmol sulfhydryl per mg protein.

2.8. The tertiary and secondary structures of myofibrillar proteins

Myofibrillar proteins were collected according to the protocol from Zheng, Han, Ge, Zhao, and Sun (2019) with slight modifications. The chopped meat was dissolved in 4 vol of 10 mM phosphate buffer (pH 7.0, containing 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM EDTA) (4:1, *w/v*), then homogenized three times in an ice bath at 10,000 r/min for 40 s, and then centrifuged at 4 °C with 6000 *g* for 10 min. The precipitate was washed three times under the same centrifugation conditions using the phosphate buffer solution described above. Pellets were then rinsed with 0.1 M NaCl (1:4, w/v), filtered through four layers of gauze, and centrifuged at 6000 *g* for 10 min to obtain myofibrillar proteins.

The intrinsic fluorescence, surface hydrophobicity, and dityrosine content were measured to evaluate tertiary structure of myofibrillar protein. The myofibrillar protein pellets were dispersed in phosphate buffer (0.02 M, pH 7.0) to the final protein concentration of 0.5 mg/mL. The intrinsic fluorescence of myofibrillar protein dispersion was scanned by a fluorescence spectrophotometer (RF-6000, Shimadzu Corp., Japan) at wavelength range from 310 nm to 410 nm with excitation wavelength of 295 nm.

Surface hydrophobicity of myofibrillar protein was measured according the method described by Zhang et al. (2020). The myofibrillar protein dispersions were diluted to 5 mg/mL using 0.02 M phosphate buffer. After that, 1 mL of myofibrillar protein dilution was combined with 0.2 mL of 1 mg/mL bromophenol blue (BPB) solution, and 1 mL of phosphate buffer was set as control group. The mixtures were then shaken at 25 °C for 10 min, followed by centrifugation at 5000 *g* for 15 min at 4 °C. The absorbance of 10-fold diluted supernatant was measured at 595 nm, and used for the surface hydrophobicity calculation:

$$\text{Bound BPB (ug)} = \frac{200ug \times (A_{Control} - A_{Sample})}{A_{Control}}$$

The dityrosine content in myofibrillar protein was determined following the method described by Zhang, Li, Diao, Kong, and Xia (2017). The extracted myofibrillar protein was diluted to 1 mg/mL with phosphate buffer and then centrifuged at 5000 *g* for 5 min. The supernatant was collected, and fluorescence intensity was measured using a fluorescence spectrophotometer (RF-6000, Shimadzu Corp., Japan). Emission and excitation wavelengths were set at 420 nm and 325 nm, respectively. Dityrosine content was expressed in arbitrary units (A.U.), calculated by normalizing the measured fluorescence intensity to the protein concentration.

The secondary structure of the myofibrillar proteins were determined by fourier transform infrared (FTIR) spectroscopy according the protocol from Yu et al. (2022). Lyophilized myofibrillar proteins was mixed with KBr with 1:100 (*w/w*), and ground into a fine powder. The mixture was pressed to a pellet. The spectra of pellets were obtained using a Fourier-transform infrared spectrometer (IRAffinity-1, Shimadzu Corp., Japan) in the range of 4000–400 cm^{-1} for 32 scans. Peakfit software (version 4.12, SPSS Inc., Chicago, USA) was used to fit the second derivative of amide I band absorption spectrum (1700–1600 cm^{-1}) to obtain the secondary structure information of myofibrillar proteins.

2.9. Gelation properties of myofibrillar proteins

2.9.1. Heat-induced gel preparation

Myofibrillar protein was dissolved in phosphate buffer to make 80 mg/mL of protein and filled into plastic tubes (50 mL). Gel samples were obtained by heating in a water bath at 45 °C for 5 min and then at 75 °C for 30 min. The gels were quickly cooled and stored at 4 °C.

2.9.2. The WHC of gel

During heat-induced gel preparation, gel weights before and after heating were recorded and used to calculate cooking loss.

Centrifugal loss of gel was performed as described by Zhang et al. (2022). The gel (around 3 *g*) was centrifuged at 4000 *g* and 4 °C for 15 min. The weights of the gels were recorded for centrifugal loss calculation according to the following formula:

$$\text{Centrifugal loss (\%)} = 100 \times \left(1 - \frac{\textit{weight after centrifugation}}{\textit{initial weight prior to centrifugation}}\right)$$

2.9.3. Determination of gel strength

The cooled gels (cylinders with a 2.7 cm diameter and 1.5 cm height) were kept at 25 °C for 2 h prior to gel strength measurement. The measurements were performed with a TMS Pilot Texture Analyzer equipped with a cylindrical probe with a diameter of 3.81 cm. The test speed was 120 mm/min with a compression distance of 8 mm. The maximum compression force in Newtons was defined as gel strength.

2.9.4. Whiteness of gel

The color attributes of L^*, a^*, and b^* values in three random locations of gels were measured with an illuminant of D_{65}, 11 mm diameter aperture, and 10° standard observer. The whiteness was calculated as follow:

$$\text{Whiteness} = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

2.10. Emulsifying characteristic of myofibrillar proteins

The emulsifying activity index (EAI) and emulsifying stability index (ESI) of myofibrillar proteins were performed according to the method from Pan et al. (2022). The EAI and ESI were calculated as follows:

$$\text{EAI } (m^2/g) = \frac{2.303 \times 2 \times A_0 \times N}{C \times \Phi \times 10^4}$$

$$\text{ESI}(\%) = 100 \times \left(\frac{A_{10}}{A_0}\right)$$

where A_0 and A_{10} are the absorbance values of the diluted emulsion measured at 0 min and 10 min, respectively. *C* is the protein content (g/mL), and Φ is soybean oil accounting for the volume fraction of the emulsion.

2.11. Microstructure and storage stability of emulsion

Approximately 10 μL of the emulsion was taken and dropped on the microscope slide. The microscopic structure of the emulsion was observed under the microscope (DMI8, Leica microscope, Germany) to obtain the image of the emulsion.

The myofibrillar protein-soybean emulsions were divided into glass tubes and stored for 0 h, 6 h, 12 h, 24 h and 48 h at 4 °C, respectively. The creaming or oil phenomena were photographed during the storage periods.

2.12. Metabolomics of meat exudates

Metabolite extraction and detection were conducted according to the method from Yu et al. (2024). Following thawing at 4 °C, 100 μL of the pork exudate samples from FT-1, FT-3, and FT-5 were thoroughly mixed with 800 μL of pre-cooled methanol and 100 μL of water. After sonicating in an ice bath for 30 min, samples were stored at −20 °C for 2 h and then centrifuged at 16,000 *g* and 4 °C for 30 min. The supernatant was extracted and vacuum concentrated by a centrifuge concentrator. The sample was redissolved in 100 μL of methanol-water (1:1, *v*/v) solution and then centrifuged at 20,000 *g* for 20 min at 4 °C, the supernatant was obtained for mass spectrometric measurement. Ultra-high performance liquid chromatography (UHPLC; Shimadzu Nexera X2 LC-

30 CE, Shimadzu, Japan) in conjunction with a Q-Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany) was used to characterize the metabolome. The detailed parameters and processes of UHPLC-MS/MS were same with descriptions of our previous study (Yu et al., 2024).

Raw MS data were processed to align peaks, correct retention times, and extract peak areas using MS-DIAL software. Features were retained if both of the following conditions were met: (i) relative standard deviation (RSD) < 30 % in the QC samples ($n = 4$); (ii) the variables with more than 50 % of non-zero measured values in at least one group. Half of the minimum positive value was used to estimate the remaining missing values. Metabolites were identified by accurate mass (mass tolerance <0.01 Da) and MS/MS data (mass tolerance <0.02 Da), which were compared with Human Metabolome Database (HMDB), Massbank, other public databases, and the self-generated standard library of Shanghai Bioprofile Biotechnology Co., Ltd. The data were mean-centered using Pareto scaling. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was conducted with Fisher's exact-test and false discovery rates (FDR) correction for multiple testing.

2.13. Statistical analysis

The data were analyzed via SPSS software (SPAA Inc., Chicago, IL, USA) for one-way analysis of variance (ANOVA) to evaluate the significance of freeze-thaw cycles, and the differences among means were determined using Duncan's multiple range test at a threshold of $P < 0.05$. The discriminating metabolites between two treatment groups (FT-5 vs. FT-1, FT-3 vs. FT-1, and FT-5 vs. FT-3) were obtained using a two-tailed Student's t-test (P value <0.05) and fold-changes (FC > 1.5). MetaboAnalyst 5.0 online software was used for partial least squares discrimination analysis (PLS-DA). Before multivariate analyses, variables were normalized by constant sum, Log_{10} transformed, and auto-scaled. Pearson's correlation analysis was performed using SPSS, with significance defined as $P < 0.05$ and $r > 0.6$.

3. Results and discussion

3.1. Impacts of freeze-thaw cycles on multiple water loss of pork meat

Significant influences of freeze-thaw cycles were found on freeze-thaw loss and water content of pork meat ($P < 0.001$) (Table 1). Freeze-thaw loss significantly increased with cycles, with FT-5 showing the highest weight loss at 11.60 %. Concurrently, the water content of pork meat decreased significantly with freeze-thaw cycles, with pork meat after five cycles showing the lowest value of 68.40 %. The higher freeze-thaw loss and lower water content during repetitive freezing and thawing indicated more water leakage from the meat, resulting in less water retained. Thus, drip loss, centrifugal loss, and cooking loss should be reduced after repeated freeze-thaw cycles, however, no significant effects on these losses were found ($P > 0.05$, Table 1). This may indicate that meat with more freeze-thaw cycles has less WHC. Approximately 85 % of the total water, referred to as "immobilized" or "entrapped" water, is held by steric effects or by attraction to the bound water (Warner, 2023). Although this part of the water is difficult to release from the muscle structure, it may be removed by drying, loss during the rigor process, or degradation of proteins (Warner, 2023). Multiple freeze-thaws contribute to the repeated formation and melting of ice crystals, which may accelerate the disruption of the endomysium and the disintegration of myofibrils (Qi et al., 2012). Therefore, it was hypothesized that this group of water might be lost much more easily following multiple freeze-thaws when an extra force is applied. It is generally agreed that freeze-thawing reduces meat WHC, associated with muscle fiber structure disruption and protein modification and/or denaturation (Leygonie et al., 2012).

Table 1
The effects of repeated freeze-thaw treatments on water related indicators of pork meat.

Water related indicators (%)	FT-1	FT-2	FT-3	FT-4	FT-5	P value [1]
Freeze-thaw loss	3.07 ± 0.32 ^{c}	7.45 ± 0.67 ^{b}	8.54 ± 0.29 ^{b}	9.10 ± 0.70 ^{b}	11.60 ± 0.68 ^{a}	< 0.001
Water content	73.09 ± 0.60 ^{a}	72.06 ± 0.79 ^{ab}	71.57 ± 0.32 ^{ab}	70.35 ± 0.42 ^{b}	68.40 ± 0.80 ^{c}	< 0.001
Drip loss	4.15 ± 0.39	3.94 ± 0.32	3.74 ± 0.23	3.61 ± 0.31	3.38 ± 0.22	0.446
Centrifugal loss	22.26 ± 0.81	21.33 ± 0.64	20.54 ± 1.14	20.31 ± 1.07	20.16 ± 0.56	0.427
Cooking loss	21.46 ± 0.95	22.35 ± 1.19	19.64 ± 0.95	18.86 ± 0.91	21.33 ± 0.92	0.111

Results are expressed as the mean ± standard error.
1 The P values were conducted by one-way ANOVA to evaluate the significance of freeze-thaw cycles on water related indicators of pork meat.
Means with different letters (a–c) in a row are different by Duncan's multiple range test at a threshold of $P < 0.05$.

3.2. Influences of freeze-thaw on tenderness of pork meat

Shear force and MFI of pork meat were significantly affected by repeated freezing and thawing ($P < 0.01$, Fig. 1). The shear force value declined ($P < 0.01$, Fig. 1a), while MFI increased ($P < 0.01$, Fig. 1b) with freeze-thaw cycles. Consequently, the pork became softer after repeated freeze-thaws. SDS-PAGE of myofibrillar proteins was presented in Fig. 1c, with similar band profiles indicating that myofibrillar protein subunits like myosin heavy chain (MHC), desmin, and actin were not dramatically damaged by repeated freeze-thaw cycles. However, some electrophoretic bands (labeled 1, 2, and 3 in Fig. 1c) showed increased intensity in the FT-4 and FT-5 groups. There was a consensus among the studies that tenderness increased with freezing and thawing (Guimarães et al., 2024; Qi et al., 2012). The decline in structural integrity caused by large extracellular ice crystals during freeze-thaw cycles disrupts cell membranes and tissue structure, ultimately leading to myofibril protein degradation and significant enhanced tenderness (Qi et al., 2012; Wan et al., 2023). Enzymolysis also contributes to myofibrillar degradation, and Whipple and Koohmaraie (1992) claim that freeze-thawing increases calpain proteolytic activity in meat. Additionally, the repeated formation and melting of ice crystals accelerate the release of mitochondrial and lysosomal enzymes (Leygonie et al., 2012; Zhang et al., 2017), potentially contributing to myofibrillar protein degradation, thereby promoting meat tenderization.

3.3. Impacts of freeze-thaw cycles on lipid and protein oxidation of pork meat

Freeze-thaw cycles significantly impacted TBARS, carbonyl and sulfhydryl content of pork meat ($P < 0.001$, Fig. 2). Higher TBARS levels indicate greater lipid oxidation in meat. A significant increase in TBARS with freeze-thaw cycles was found ($P < 0.01$, Fig. 2a). Freezing and thawing ice crystals break down muscle tissue structure and cell membrane integrity, which may result in the release of pro-oxidants like heme iron, mitochondrial and lysosomal enzymes, and other pro-oxidants (Benjakul & Bauer, 2001; Leygonie et al., 2012). Lipid oxidation in muscle foods is assumed to occur at the cellular membrane level (Thanonkaew, Benjakul, Visessanguan, & Decker, 2006). Therefore, larger ice crystals formed by multiple freeze-thaw cycles could lead to more severe lipid oxidation through cell membrane disruption and the release of pro-oxidants. Freeze-thaw cycles significantly increased carbonyl content ($P < 0.001$, Fig. 2b) and decreased sulfhydryl content ($P < 0.001$, Fig. 2c). Generally, there is a positive correlation and mutual reinforcement between lipid and protein oxidation. Protein

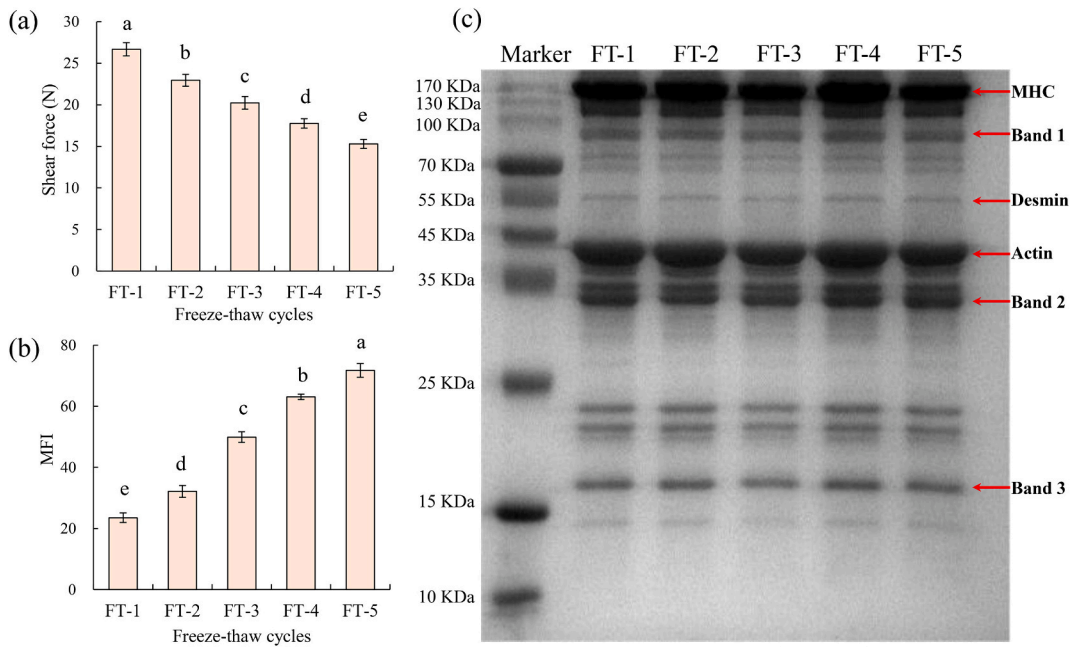

Fig. 1. Effects of repeated freeze-thaw on shear force (a), MFI (b), and myofibril protein SDS-PAGE of pork meat. Note: The results were expressed as mean with standard error. Different letters indicate significant differences ($P < 0.05$). MFI: myofibril fragmentation index. MHC: myosin heavy chain.

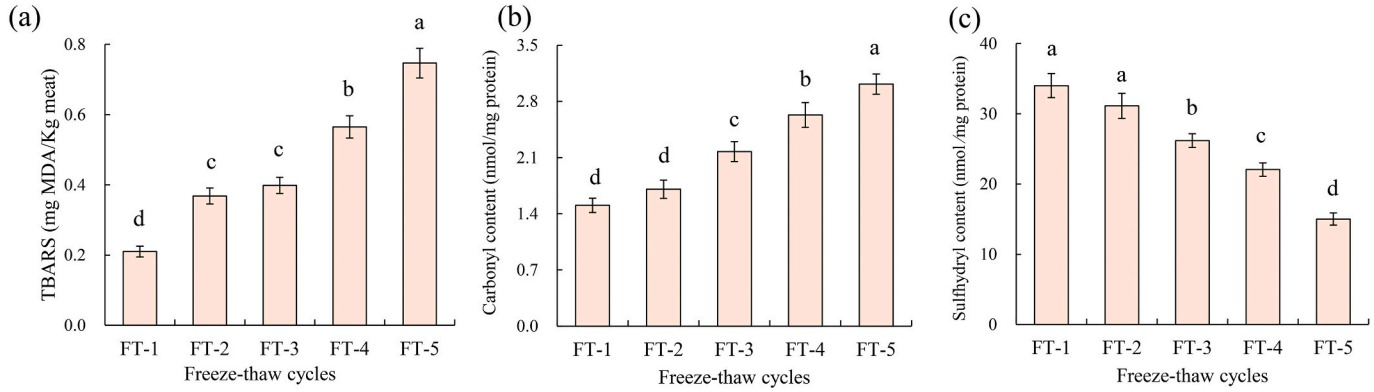

Fig. 2. Effects of repeated freeze-thaw on TBARS (a), carbonyl content (b), and sulfhydryl content (c) of pork meat. Note: The results were expressed as mean with standard error. Different letters indicate significant differences ($P < 0.05$).

carbonylation may potentially be initiated by radicals and hydroperoxides derived from unsaturated lipids (Estévez, 2011). Studies indicated that malondialdehyde and α,β-unsaturated aldehydes can stimulate the carbonylation of myofibrillar proteins and the autoxidation of myoglobin (Wang, He, Emara, Gan, & Li, 2019). These findings were consistent with several investigations that confirmed the increase in oxidation of proteins and lipids in repeated freeze-thaw meat (Ali et al., 2015; Wan et al., 2023).

3.4. Influences of freeze-thaw on color of pork meat

Freeze-thaw cycles significantly impacted the color attributes of pork meat (Table 2.) As the freeze-thaw cycles increased from FT-1 to FT-5, the L^* value of the meat significantly decreased, with a P value of 0.034. This reduction in lightness is possibly due to moisture loss or structural changes in the muscle fibers, which affect light reflection. Similarly, the a^* values showed a marked decline ($P < 0.001$) from 1.54 in FT-1 to 0.71 in FT-5. This decline in a^* value could be attributed to the oxidative degradation of myoglobin, the pigment responsible for the red color in meat, which is exacerbated by repeated freeze-thaw cycles.

Table 2
The effects of repeated freeze-thaw treatments on color attributes of pork meat.

Color attributes	FT-1	FT-2	FT-3	FT-4	FT-5	P value [1]
L^*	50.86 ± 0.80^{a}	50.02 ± 1.35^{ab}	48.41 ± 0.83^{abc}	46.86 ± 1.24^{bc}	46.64 ± 1.02^{c}	0.034
a^*	1.54 ± 0.05^{a}	1.32 ± 0.07^{b}	1.01 ± 0.06^{c}	0.84 ± 0.04^{d}	0.71 ± 0.05^{d}	<0.001
b^*	10.21 ± 0.37^{ab}	10.81 ± 0.46^{a}	10.18 ± 0.42^{ab}	9.23 ± 0.44^{b}	9.15 ± 0.37^{b}	0.040

Results are expressed as the mean ± standard error.
1 The P values were conducted by one-way ANOVA to evaluate the significance of freeze-thaw cycles on water related indicators of pork meat.
Means with different letters (a–d) in a row are different by Duncan's multiple range test at a threshold of $P < 0.05$.

Additionally, the temperature fluctuations during freeze-thaw cycles may promote the production of oxidants such as malondialdehyde and carbonyl compounds (reflected by the increased lipid and protein oxidations in Fig. 2), which may accelerate the autooxidation of myoglobin, leading to color deterioration (Wang et al., 2019).

3.5. Impacts of freeze-thaw on myofibrillar protein structures

The endogenous fluorescence of myofibrillar proteins reflects their structural integrity and the exposure of chromophores like tryptophan residues. This fluorescence is widely used to assess changes in protein tertiary structure. With an increase in FT cycles, a significant decrease in maximum fluorescence intensity was observed ($P < 0.01$, Fig. 3a). This decrease suggests that structural changes induced by repeated FT cycles may lead to denaturation or conformational alterations in the myofibrillar proteins. In native protein structures, tryptophan residues are buried in the core, contributing to the high fluorescence intensity. When proteins unfold, tryptophan residues gradually become exposed, leading to fluorescence quenching and a decrease in fluorescence intensity (Zhang et al., 2020). On the other hand, protein aggregation, folding, and tryptophan oxidation would also reduce fluorescence intensity (Zhang et al., 2020). The shift in maximum absorption wavelength from 331.5 nm to 333 nm (Fig. 3a) further supports this interpretation. This redshift indicates changes in the local environment of the chromophores, likely due to alterations in the protein's tertiary structure or the exposure of different residues. Similar results were found by Li, Wang, Kong, Shi, and Xia (2019), who also confirmed a decreased maximum fluorescence intensity and a redshift in the maximum absorption wavelength of myofibrillar proteins from mirror carp after repeated FT cycles. Moreover, the binding capacity of bromophenol blue in myofibrillar proteins increased with FT cycles ($P < 0.01$, Fig. 3b). This increase suggests greater exposure of hydrophobic sites on the protein surface, becoming more accessible as the protein structure unfolds and aggregates under repeated freeze-thaw conditions. This exposure likely results from the disruption of the native protein conformation, where previously buried hydrophobic amino acid residues become exposed and capable of binding with bromophenol blue. Simultaneously, a significant increase in dityrosine content with FT cycles ($P < 0.01$, Fig. 3b) indicated enhanced oxidative cross-linking, further supporting the notion that repeated freeze-thaw cycles promote protein oxidation and aggregation. These structural changes are critical, as they can alter the meat's texture and water-holding capacity, directly impacting product quality.

FTIR analysis was used to evaluate the secondary structural changes in myofibrillar proteins across different FT cycles. The spectra indicated alterations in the amide I and II bands (Fig. 3c), which correspond to the protein's secondary structure, including α-helices, β-sheets, β-turns, and random coils. The effect of FT cycles on the four kinds of secondary

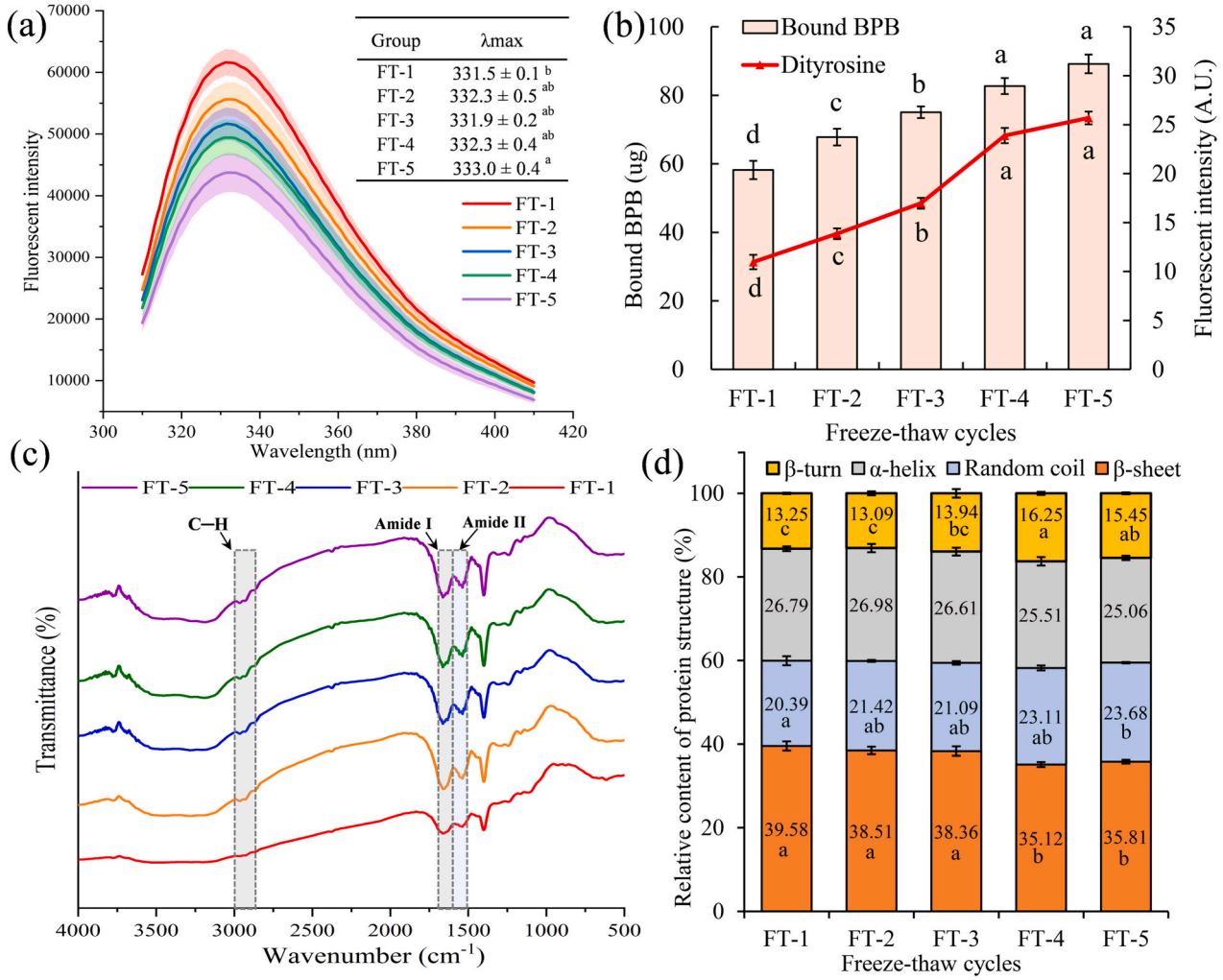

Fig. 3. Effects of repeated freeze-thaw on intrinsic fluorescence spectra of myofibril protein (a), surface hydrophobicity and dityrosine contents of myofibril protein (b), fourier transform infrared spectroscopy of myofibril protein (c), secondary structural changes in myofibrillar proteins (d). Note: The results were expressed as mean with standard error. Different letters indicate significant differences ($P < 0.05$).

structures of myofibrillar proteins was further quantified by the amide I region in the FTIR spectra. The proportion of secondary structure was shown in Fig. 3d, in which α-helix and β-sheet were the main secondary structures of myofibrillar proteins. The analysis revealed a significant reduction in β-sheet ($P < 0.05$) and a corresponding increase in β-turn ($P < 0.05$) and random coil ($P < 0.05$) structures with FT cycles. This structural shift towards more β-turn and random coils confirms the destabilization of the protein's conformation, likely due to unfolding and reaggregation processes induced by repeated freeze-thaw cycles. Proteins with more β-turn and random coil structures are usually are less stable and disordered (Yu et al., 2022). The disintegration of the secondary hydrogen bond structure is primarily responsible for the loss of structural stability in proteins and even protein denaturation. Repeated FT cycles may also cause the protein's secondary structure to become unstable by rupturing hydrogen bonds through physical processes such protein freezing damage, ice recrystallization, and water distribution (Wan et al., 2023). In the present study, the decreased total proportion of the β-sheet and α-helix structures with FT cycles possibly indicated that repeated freeze-thaw will induce more disordered flexible frameworks in myofibrillar proteins. These structural alterations can lead to reduced protein solubility and functionality, which are critical factors for meat processing and product quality.

3.6. Impacts of freeze-thaw on gel properties of myofibrillar proteins

The desired textural and water- and oil- holding qualities of gel-like muscle foods largely depend on the heat-induced gelling capabilities of myofibrillar proteins (Zhang et al., 2023). Freeze-thaw cycles significantly decreased the gels' strength ($P < 0.001$, Fig. 4c) and whiteness ($P < 0.05$, Fig. 4d), while increased cooking loss (Fig. 4a) and centrifugal loss ($P < 0.001$, Fig. 4b). Decreased WHC, strength, and whiteness of gels with freeze-thaw cycles were also reported (Xia, Kong, Xiong, & Ren, 2010). The reduction in gel whiteness may be attributed to non-enzymatic browning interactions between lipid oxidation products and the amines in proteins or in phospholipid head groups (Xia et al., 2010). Protein denaturation caused by freeze-thaw cycles would be the primary cause of the changes in the gel properties of myofibrillar proteins. Consensus mechanisms might include mechanical damage from ice crystal formation and the effect of solute content in unfrozen water (Zhang et al., 2023). Furthermore, it is commonly recognized that numerous reports of oxidative alterations of myofibrillar proteins exist, including increased dityrosine (Zhang et al., 2017), large molecular-weight protein aggregates (Pan et al., 2022), as well as decreased total and free sulfhydryl content (Hu & Xie, 2021). Zhang et al. (2023) proposed a potential mechanism for myofibrillar protein gelation impairment caused by denaturation/oxidation during freeze-thaw. Myofibrillar protein oxidation and denaturation cause protein molecules to overaggregate during freezing and thawing, interfering with myosin molecule salt dissociation and resulting in the coexistence of monomers and undissociated aggregates (Zhang et al., 2023). Consequently, some amorphous cross-links formed during heating would damage the gel network with large clusters, resulting in reduced gel strength and WHC.

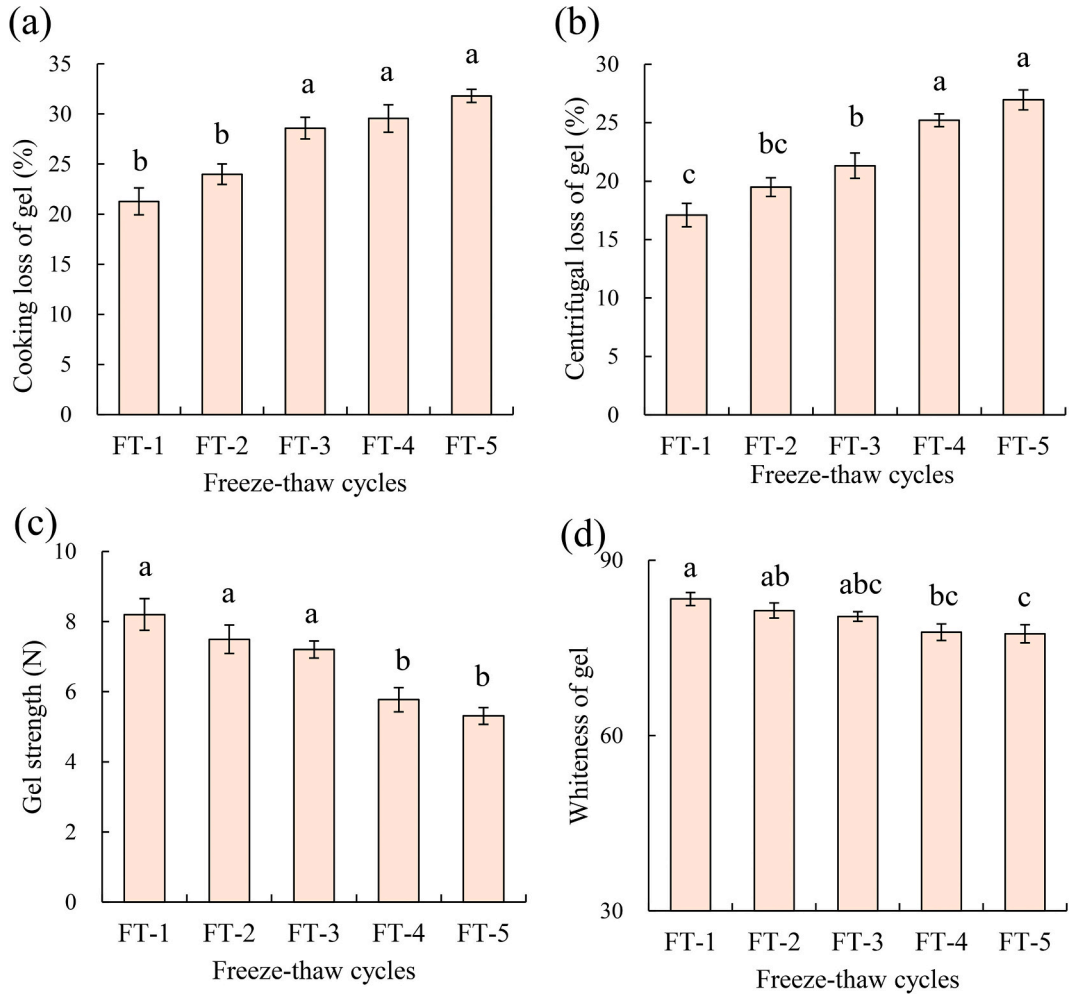

Fig. 4. Effects of repeated freeze-thaw on cooking loss (a), centrifugal loss (b), strength (c), and whiteness (d) of heat-induced gel of myofibril protein. Note: The results were expressed as mean with standard error. Different letters indicate significant differences ($P < 0.05$).

3.7. Impacts of freeze-thaw on emulsion of myofibrillar proteins

Myofibrillar protein, being an amphiphilic macromolecule, serve as an excellent emulsifier in meat products. The protein's capacity to adsorb at the oil-water interface is indicated by its emulsifying property, evaluated using ESI and EAI. Higher EAI and ESI values suggest that myofibrillar protein is advantageous for creating and stabilizing emulsions. With freeze-thaw cycles, the myofibrillar protein's EAI drastically decreased ($P < 0.001$, Fig. 5a), and the ESI likewise showed a downward trend ($P = 0.417$, Fig. 5b). Oxidation and denaturation of myofibrillar protein would lead to the creation of protein aggregates, causing decreased EAI and ESI. These aggregates diminish the protein's adsorption ability at the oil-water interface, decreasing emulsifying performance (Pan et al., 2022). In terms of microstructure, droplets from sample FT-1 were relatively small and homogeneous, whereas droplets from FT-4 and FT-5 showed fragmented dispersion, irregular size distribution, and irregular shape (Fig. 5c). Furthermore, the emulsion showed different degrees of stratification after 6 h storage (Fig. 5d), and the height of the serum layer after stratification significantly increased over storage periods. Stratification of the emulsion during storage was more severe with the freeze-thaw cycles. Combined with the microstructure results, myofibrillar protein emulsions with more freeze-thaw cycles showed larger oil droplets and irregular shapes, leading to poorer emulsion storage stability.

3.8. Effects of freeze-thaw cycles on metabolome changes of pork exudates

A total of 501 metabolites were tentatively identified in pork exudates from FT-1, FT-3 and FT-5 samples (6 biological replicates × 3 freeze-thaw treatments). These metabolites included 81 peptides, 49 amino acids and derivatives, 46 fatty acyls, 42 carboxylic acids and derivatives, 34 fatty acids, and 10 nucleosides. KEGG enrichment analysis was performed, and 16 significantly enriched pathways ($P < 0.05$) were revealed (Fig. 6). Nine biochemical pathways involved in amino acid metabolism/ biosynthesis were significantly enriched, mainly involved in beta-alanine metabolism, arginine biosynthesis, histidine metabolism, cysteine and methionine metabolism, arginine and proline metabolism, glycine, serine and threonine metabolism, and phenylalanine metabolism. Other pathways involved the glyoxylate and dicarboxylate metabolism, glutathione metabolism, pantothenate and CoA biosynthesis, and purine metabolism. For pairwise comparison, 67 differentially abundant metabolites (fold change >1.5 and $P < 0.05$) were identified in FT-3 vs. FT-1, FT-5 vs. FT-1, and FT-5 vs. FT-3, and these metabolites were listed in the supplemental materials (Table S1). Specifically, FT-5 vs. FT-1 showed the highest number of differentially abundant metabolites, 21 of which were down-regulated and the other 36 were up-regulated (Fig. S1). Twenty differentially abundant metabolites were identified in FT-3 vs. FT-1 (7 down-regulated and 13 up-regulated), only 7 were detected in FT-5 vs. FT-3 (4 down-regulated and 3 up-regulated) (Fig. S2). Most up-regulated metabolites were peptides and/or amino acids, and were found to be overabundant in the meat exudates from FT-5 samples. Enzyme-induced proteolysis is one of the most significant metabolic processes occurring in postmortem meat following slaughter. The enzymatic breakdown of muscle proteins produces polypeptide fragments, small peptides, and free amino acids. Repeated freeze-thawing increases proteolytic activity of calpain and disrupts the structure of the muscle cell, causing the release of mitochondrial and lysosomal enzymes, all of which facilitate the degradation of the myofibrillar protein and the production of more small molecules (peptides, amino acids). These compounds would gradually accumulate in the meat and may be released in exudates. Thus, it was reasonable that most enriched pathways involve amino acid metabolism/biosynthesis (Fig. 6), and more of these peptides/animo acids were detected in

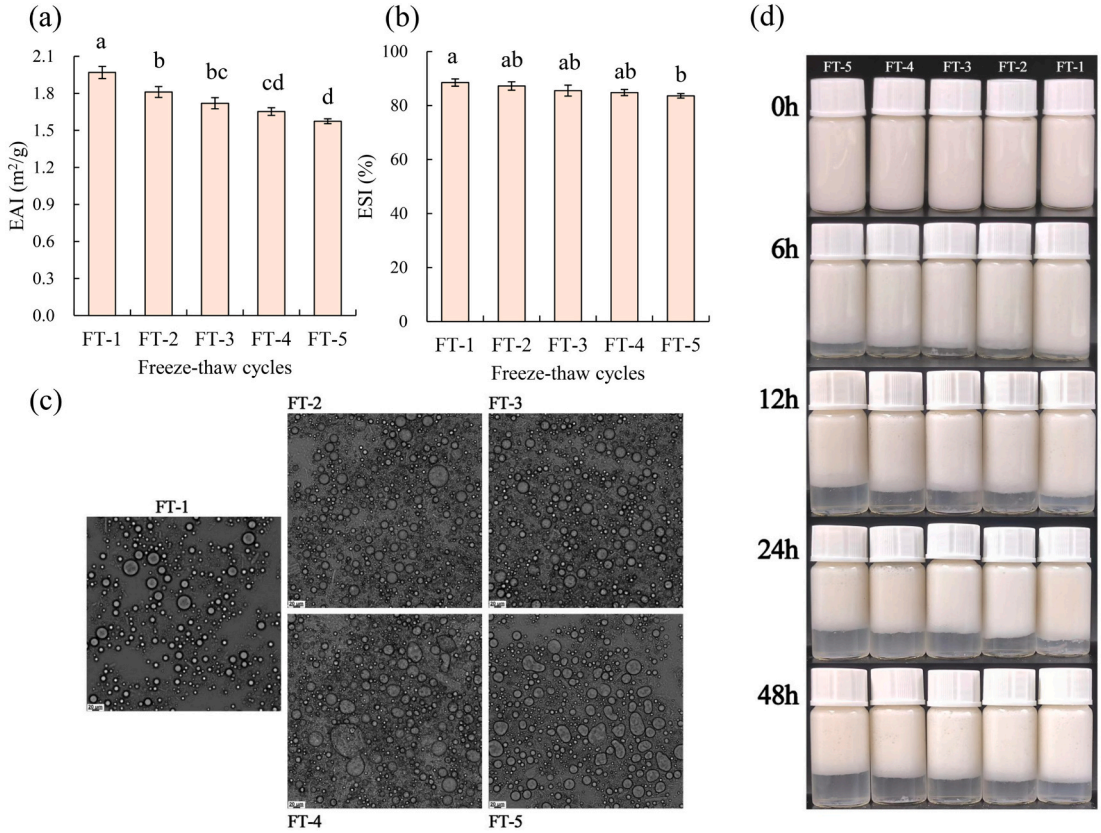

Fig. 5. Effects of repeated freeze-thaw on EAI (a), ESI (b), microstructure (c), and storage stability (d) of myofibril protein emulsion. Note: The results were expressed as mean with standard error. Different letters indicate significant differences ($P < 0.05$). EAI: emulsifying activity index. ESI: emulsifying stability index.

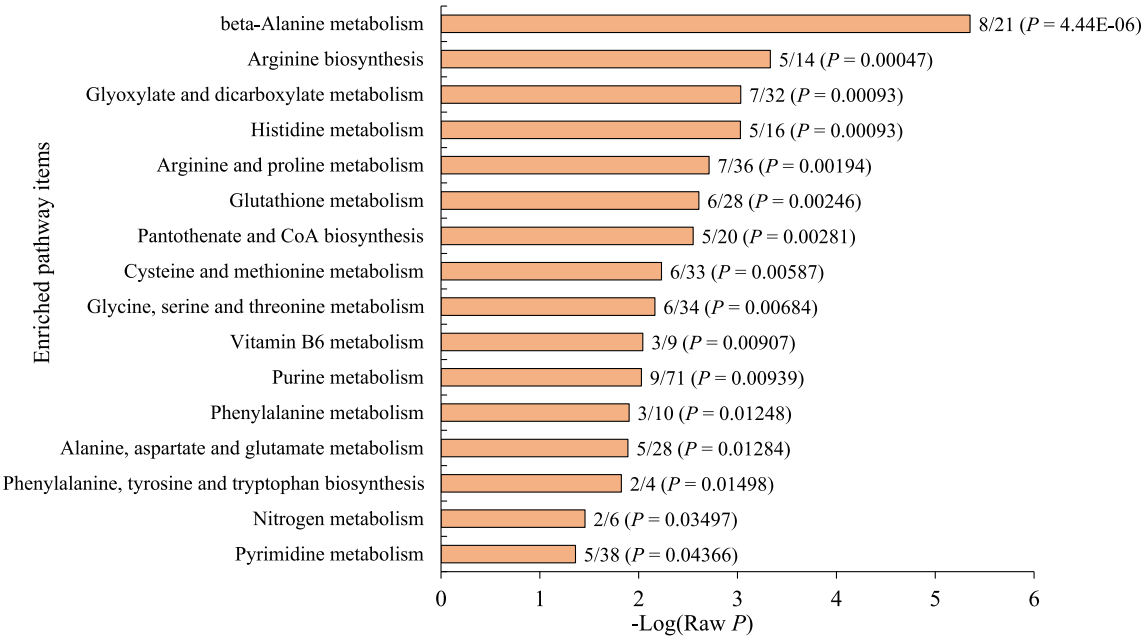

Fig. 6. KEGG enrichment analysis of metabolites that identified in pork meat exudates.

FT-5 exudates (Fig. S1). High-throughput metabolomic technologies have revealed the biochemical components of meat exudates, suggesting their valuable use for providing homogeneous information about the whole meat and their relevance to meat quality (Castejón, García-Segura, Escudero, Herrera, & Cambero, 2015; Setyabrata et al., 2023; Yu et al., 2021). However, no relevant studies have reported so far to reveal changes in the metabolome of meat exudates during repeated freeze-thawing. According to our previous research, quite a few peptides were found in pork exudates and significantly increased with postmortem wet aging time (Yu et al., 2021).

3.9. Characteristic metabolites finding

PLS-DA, a supervised discriminant analysis approach, models the relationship between variable expression levels and sample categories to facilitate discrimination among these categories. Therefore, the above 67 differentially abundant metabolites were used in PLS-DA analysis to screen the characteristic metabolites contributing most to different freeze-thaw cycle classifications. The results were visualized in PLS-DA score plots (Fig. 7a). In particular, exudate samples from three different freeze-thaw cycles could be distinguished into two clusters by principal component 1 (PC1), where FT-1 group and multiple freeze-thaw cycles (FT-3 and FT-5) were separately located in the left and right quadrants of PC1. Q^2, an estimate of the model's predictive power, is determined through cross-validation. Good predictions will have a high Q^2. In the present study, 5-fold cross-validation was performed. The accuracy of the model was 0.667, and the Q^2 was 0.786, indicating strong predictive accuracy and ability. These suggested the potential feasibility of using exudate metabolites to discriminate or predict the degree of freeze-thaw on meat.

Variable importance in projection (VIP), derived from PLS-DA, is commonly used to demonstrate the explanatory power of each variable's expression pattern in group classification and discrimination. Typically, a VIP value >1.0 is employed as a screening criterion. Thirty variables with a VIP > 1 were chosen as potentially significant metabolites in this investigation (Fig. 7b). A higher VIP score usually indicates a greater classification contribution of the metabolite among FT groups. Pearson's correlation analyses were conducted between these 30 metabolites and meat quality attributes. Twenty-one metabolites significantly correlated ($P < 0.05$, and $r > 0.6$) with at least one meat quality attribute and had VIP scores >1 from PLS-DA at the same time (Fig. 7c). Since there were no significant impacts on drip loss, centrifugal loss, and cooking loss of pork meat with freeze-thaw cycles, no interesting metabolites were found to be significantly correlated with these attributes. As expected, five peptides (Ile-Met, isoleucyl-arginine, glycylvaline, Glu-Val-Phe, and alanylnorvaline) were overabundant in the FT-5 group. They were positively correlated with freeze-thaw loss, MFI, TBARS, carbonyl content, bound BPB content of myofibrillar protein, dityrosine content, centrifugal loss of gel, and cooking loss of gel, and negatively correlated with water content, shear force, sulfhydryl content, a^* value of meat, and EAI of gel (Fig. 7c). Vitamin K1, scyllo-inositol, schweinfurthin G, rhododendrol, (+/−)-, *N*-methylisoleucine, lariciresinol, indole-3-lactic acid, and 2-hydroxy-3-(2-hydroxyphenyl) propanoic acid decreased with freeze-thaw cycles, and showed negative correlations with freeze-thaw loss, MFI, TBARS, carbonyl content, bound BPB content of myofibrillar protein, dityrosine content, gel centrifugal loss, and gel cooking loss, while positive correlations with water content, shear force, sulfhydryl content, a^* value of meat, and EAI of gel (Fig. 7c). Although the significant correlation results could not fully explain the underlying associations between exudate metabolites and pork quality attributes during freeze-thaw process, these characteristic metabolites and their abundance changes could act as potential biomarkers for freeze-thaw cycles or meat traits indications at least.

4. Conclusion

In conclusion, the impact of freeze-thaw on pork meat quality, myofibrillar protein gel and emulsion properties, and meat exudate metabolomics were investigated. Multiple repeated freeze-thaws significantly decreased the WHC, color attributes, and oxidative stability of pork meat, and altered secondary and tertiary structures of myofibrillar protein, adversely affecting gel WHC, gel strength, EAI, and ESI of myofibrillar protein. The study results comprehensively elucidated the valuable metabolite information of pork exudates with freeze-thaw and indicated the characteristic changes of freeze-thaw-induced exudate metabolites. A total of 30 variables with VIP > 1 were selected as potentially important metabolites with greater classification contribution on freeze-thaw cycles, and 21 of them showed significant correlations with at least one meat quality traits. These findings suggest the potential feasibility of using metabolomic information from

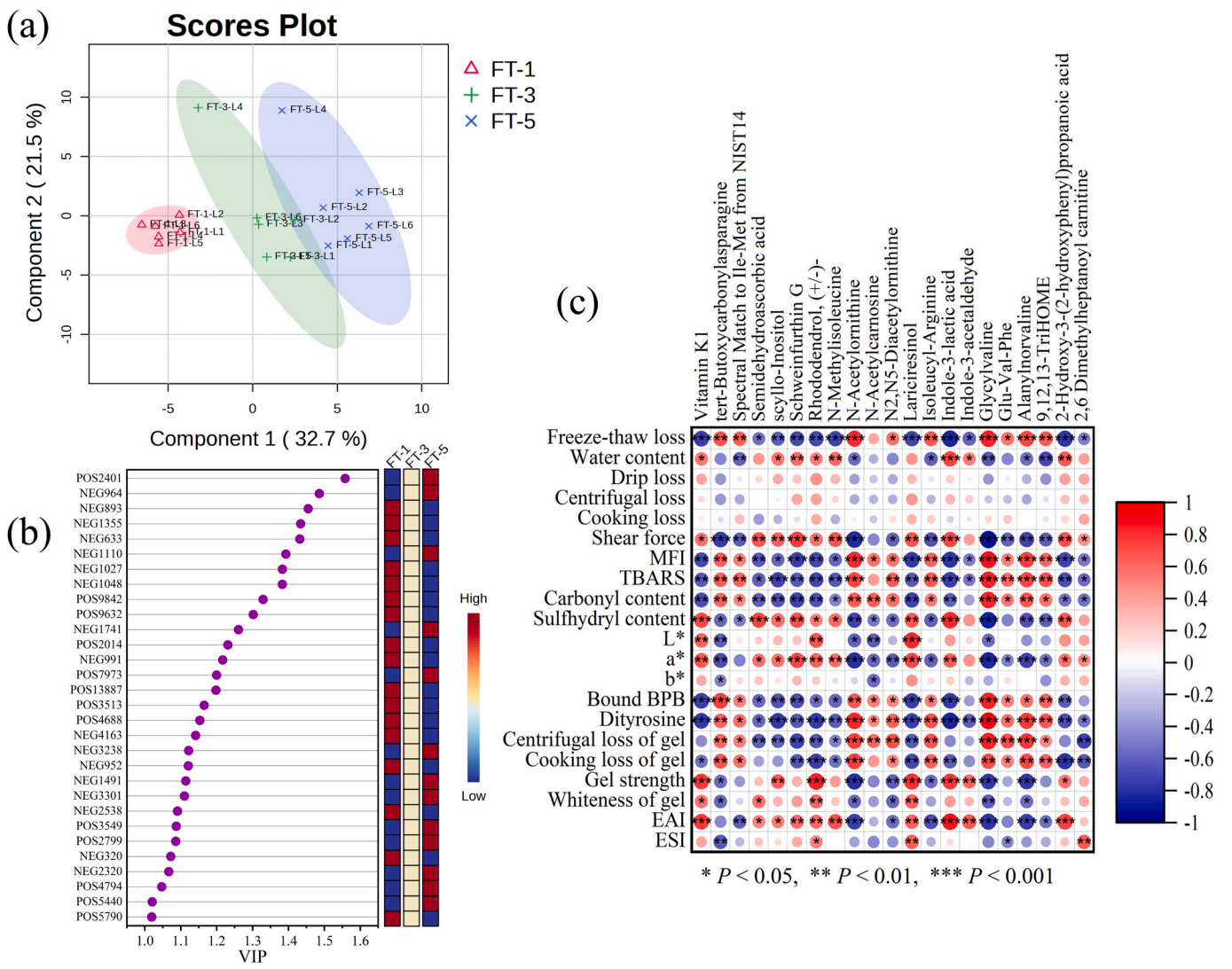

Fig. 7. The score plot of PLS-DA (a), thirty exudate metabolites with their VIP scores >1 from PLS-DA (b), and twenty-one metabolites significantly correlated ($P < 0.05$, and $r > 0.6$) with at least one of the meat quality attributes (c).

exudates to elaborate on or even predict the degree of freeze-thaw or meat quality attributes.

CRediT authorship contribution statement

Shuo Liu: Data curation. **Qianqian Liu:** Writing – original draft, Conceptualization. **Rongxin Wen:** Writing – review & editing. **Chengfeng Sun:** Project administration, Investigation.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101804.

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