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## Impact of sampling location and aging on the *Longissimus thoracis et lumborum* muscle proteome of dry-aged beef

Sara Álvarez <sup>a,b</sup>, Anne Maria Mullen <sup>a</sup>, Carlos Álvarez <sup>a</sup>, Ruth M. Hamill <sup>a</sup>, Eileen O'Neill <sup>b</sup> and Mohammed Gagaoua <sup>c,\*</sup>

<sup>a</sup> Dept. of Food Quality and Sensory Science, Teagasc Food Research Centre Ashtown, Dublin, D15 DY05, Ireland

<sup>b</sup> School of Food and Nutritional Sciences, University College, Cork, Western Road, Cork T12 YN60, Ireland

<sup>c</sup> PEGASE, INRAE, Institut Agro, 35590 Saint-Gilles, France

\* Correspondence: [mohammed.gagaoua@inrae.fr](mailto:mohammed.gagaoua@inrae.fr)

### Abstract

This study aimed to explore the differences in the proteome and molecular pathways between two sampling locations (external, internal) of bovine *Longissimus thoracis et lumborum* (*LTL*) muscles at 0, 21, and 28 days of dry-aging (i.e. 3, 24, and 31 days *post-mortem*). It further assessed the impact of aging on the proteome changes and the biological processes at interplay. Proteins related to defence response to bacterium and regulation of viral entry into host cell were identified to be more abundant on the external location before dry-aging, which may be associated to the oxidative conditions and microbial activity to which *post-mortem* muscle is exposed during dressing, chilling, and/or quartering of the carcasses. This highlights the relevance of sampling from interior tissues when searching for meat quality biomarkers. As dry-aging progressed, the proteome and related biological processes changed differently between sampling locations; proteins related to cell-cell adhesion and ATP metabolic processes pathways were revealed in the external location at 21 and 28 days, respectively. On the other hand, the impact of aging on the proteome of the interior samples, evidenced that muscle contraction and structure together with energy metabolism were the major pathways driving dry-aging. Additionally, aging impacted other pathways in the interior tissues, such as regulation of calcium import, neutrophil activation, and regeneration. Overall, the differences in the proteome allowed discriminating the three dry-aging times, regardless of the sampling location. Several proteins were proposed for validation as robust biomarkers to monitor the aging process (tenderization) of dry-aged beef: TTN, GRM4, EEF1A1, LDB3, CILP2, TNNT3, GAPDH, SERPINI1, and OMD.

**Keywords:** Dry-aging; Beef; Proteomics; Biochemical pathways; Enrichment analysis; SWATH-MS

## 1. Introduction

The muscle proteome is dynamic; it undergoes continual changes during the life of the animal and after slaughter, hence playing a pivotal role in the conversion of muscle into meat and its quality determination (Hollung et al., 2007). In this sense, proteomics provide powerful tools which can be used to reveal the underlying mechanisms and biochemical pathways that dictate the structural and metabolic changes observable in early *post-mortem* muscle and/or during meat aging/processing (Gagaoua et al., 2022). In recent years, proteomics has been used to advance our knowledge and understanding of the *post-mortem* changes occurring in fresh meat during meat aging under various conditions (della Malva et al., 2022; Gagaoua, Troy, et al., 2021; Sierra et al., 2021). While proteomics tools have been used to evidence degradation of muscle proteins and peptides during the production of dry-cured meats (Mora et al., 2015; Mora et al., 2010), to the best of our knowledge, there are no available studies on the application of proteomics to study the proteome changes and related mechanisms involved in the dry-aging of beef.

Dry-aging is a *post-mortem* processing technique where meat is exposed to controlled conditions of temperature (0-4 °C), relative humidity (70-80%), and airflow (0.5-2.5 m/s) for 14-35 days (typical restaurants and retailer conditions are 1-4°C, 75-85% and 21-35 days) (Koutsoumanis et al., 2023). In contrast to wet-aging, where beef is vacuum-packed, traditional dry-aging of beef is characterized by the absence of packaging. Although both strategies improve meat tenderness, the absence of this barrier during dry-aging results in increased water evaporation and surface dehydration which increases yield loss (weight and trim losses) and surface colour changes, when compared to wet-aging (Savell & Gehring, 2018). Muscle dehydration together with lipid oxidation and microbial activity have been confirmed to play a key role in the development of the signature dry-aged beef flavour (Zhang et al., 2023; Zhang et al., 2022).

Research efforts have improved our understanding on the role of microbial populations during dry-aging of beef (da Silva Bernardo et al., 2021; Ryu et al., 2018), and the meat quality and sensory acceptability of dry-aged beef (Berger et al., 2018; Li et al., 2014). In addition, in recent years, there has been a particular interest in deciphering the metabolome of dry-aged beef with the aim of identifying the diverse flavour-related metabolites responsible for its signature flavour (Bischof et al., 2021; Kim et al., 2016; Setyabrata et al., 2021; Setyabrata et al., 2022). Many of these metabolites are likely derived from lipid oxidation,

metabolism of nucleotides, and proteolysis during dry-aging (Zhang et al., 2022). In particular, the action of endogenous muscle enzymes and the microbial enzymes on the surface of beef has an impact on metabolome and the proteome of dry-aged beef (Greta Bischof et al., 2022). Indeed, proteolysis of structural and cytoskeletal proteins occurs during dry-aging of beef, and results in the increase of concentrations of peptides and free amino acids with aging time (Zhang, Yoo, et al., 2021), changing the beef proteome as aging advances. Whereas research has been done to characterize the metabolome of dry-aged beef, to the best of our knowledge, there are no available studies investigating the changes occurring in the proteome of this product. Therefore, proteomics research is warranted as these data will reveal the pathways and biological processes underlying the dry-aging of beef, and will further complement the available information in metabolomics. All this information together may ultimately assist processors to optimize dry-aging conditions in order to obtain the desirable dry-aged product, while attempting to minimize yield loss.

Therefore, the aim of this study was to perform for the first time an in-depth exploration of the changes occurring in the beef muscle proteome during dry-aging, and investigate the interconnectedness of the various biological processes that are involved in the production of this meat product. Furthermore, this work investigated if there are differences in protein abundances and related pathways between the external and internal locations of the meat cuts during the dry-aging process.

## 2. Material and Methods

### 2.1 Animals and muscle/dry-aged meat biopsies

As part of a larger experiment (not publicly available yet), striploins (*M. longissimus thoracis et lumborum*, cut from the 10<sup>th</sup> rib) from the right- and left-hand sides of six cross breed (Hereford x Limousin) bovine carcasses (4 steers and 2 heifers - no significant gender effect as per Gagaoua, Troy, et al. (2021); conformation score U= to O=; fat score 2= to 4=; age: 24-36 months; and average weight of 180.6 kg  $\pm$  19.8 kg), were collected from the slaughterhouse (Kepak, Ireland) at 2 days *post-mortem*. At 3 days *post-mortem*, the striploins were cut into 4 equal sections, and each randomly distributed to one of the dry-aging times: 0, 21, and 28 days (i.e. 3, 24, and 31 days *post-mortem*, respectively). Sections assigned to 21 and 28 days of dry-aging, were placed inside the DRY AGER DX 1000® (DRY AGER®, Germany) with set conditions at 2.0 °C, 75% relative humidity and air flow of 0.5-2.0 m/s. Subcutaneous fat was left on the sections, and these were dry-aged on racks with the fat side

facing up. To control for location, racks inside the cabinet were randomly rotated every week. At each time point, the assigned sections were cut into steaks, and muscle samples (approx. 2 g) were collected for proteomic analyses from both internal and external (i.e. lean surface prior to dry-aging, and crust as aging progressed) locations using aseptic methods (**Figure 1**). Samples were stored at  $-80^{\circ}\text{C}$  for further analysis.

## **2.2 Protein extraction and quantification**

Frozen muscle tissue samples (150-200 mg) were homogenized in 3 mL of fresh extraction buffer consisting of 8.3 M urea, 2 M thiourea, 1% DL-Dithiothreitol (DTT), 2% CHAPS, and Pharmalyte® 3-10 (Bouley et al., 2004) using a T18 digital ULTRA-TURRAX® (IKA®, Staufen, Germany) which was run at 20,000 rpm for 60 s (2 x 30 s). The homogenates were incubated on ice for 30 min, and subsequently, centrifuged for 30 min at 10000 x g at  $4^{\circ}\text{C}$ . The supernatant was transferred into Eppendorf tubes and stored at  $-80^{\circ}\text{C}$  until protein quantification.

Protein concentrations were determined using the Bradford method (Bradford, 1976), and bovine serum albumin (Thermo Scientific, Rockford, IL, USA) was employed as a standard to build the calibration curve. To determine protein concentrations in the protein extracts, absorbance was measured at 595 nm using a spectrophotometer (UV-1700, Pharmaspec, SHIMADZU).

## **2.3 One dimensional (1D) Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for shotgun proteomics**

The protein concentrations of the muscle extracts were adjusted with MilliQ water and an equal volume of Laemmli buffer was added (Sigma-Aldrich, St. Louis, MO, USA) to ensure a final protein mass of 40  $\mu\text{g}$  in each well. The samples were heated in a standard block heater (SBH130D/3, Bibby Scientific Limited, Staffordshire, UK) at  $80^{\circ}\text{C}$  for 15 minutes. A volume of 15  $\mu\text{L}$  of each sample were loaded in freshly prepared 12% resolving and 4% stacking gels for 1D SDS-PAGE using a Mini-PROTEAN® Tetra Cell System (Bio-Rad, Hercules, CA, USA) with a run time of 15 minutes at 4W to concentrate the proteins. Protein bands for shotgun proteomics were prepared as described by Lamri, della Malva, Djenane, López-Pedrouso, et al. (2023). Subsequent digestion of the protein bands and mass spectrometry analysis were performed following the protocol described by Chantada-Vázquez et al. (2021). Modified porcine trypsin (Promega, Madison, WI, USA) was used for

digestion of the dried gel bands at a concentration of 20 ng/ $\mu$ L in 20mM ammonium bicarbonate at 37 °C for 16h. The resulting peptides were extracted with 60% acetonitrile in 0.5% formic acid, and then, vacuum-concentrated in a SpeedVac and stored at -20 °C for further LC-MS/MS analysis.

#### ***2.4 Generation of the reference spectral library and protein quantification by SWATH-MS***

To generate the MS/MS spectral libraries, and obtain a good representation of the peptides and proteins in all samples, vials from each group (i.e. dry aged condition) were prepared, and analysed by a shotgun data-dependent acquisition (DDA) approach using a micro-LC-MS/MS. Briefly, peptides mixture of 4  $\mu$ L per condition, was separated on a micro-LC system Ekspert nLC425 (Eksigent, Dublin, CA, USA) using an Eksigent C18 column (150  $\times$  0.30 mm, 3 mm particle size and 120 Å pore size) (Eksigent Sciex). The eluting peptides were directly injected into a hybrid quadrupole-TOF mass spectrometer coupled with a Triple TOF 6600 (Sciex, Redwood City, CA, USA), which operated with a data-dependent acquisition system in positive ion mode. The MS raw files were used for peptides and proteins identification employing the Protein Pilot software (version 5.0.1, Sciex) and the bovine-specific Uniprot database (<https://www.uniprot.org/>). The false discovery rate (FDR) was set to 1% for both peptides and proteins.

For relative quantification of peptides and proteins, 4  $\mu$ L of peptides from each individual sample were analysed using a data-independent acquisition (IDA) method, known as SWATH (Sequential Window Acquisition of all Theoretical Mass Spectra)-MS method. Samples were analysed using the LC-MS equipment and LC gradient. A total of 36 samples from 6 treatments (6 biological replicates per group) were analysed. The data extraction from the SWATH runs was performed by PeakView v.2.2 (Sciex, Framingham, MA, USA) employing the SWATH Acquisition MicroApp v.2.0. This application processed the data based on the spectral library created, and used the following parameters: 10 peptides /protein and 7 fragments/peptide and FDR below 1%. Protein quantification was calculated by summing the peak areas of the corresponding peptides

#### ***2.5 Statistical analysis***

SWATH-MS analysis allowed the identification and quantification of 684 proteins and 1908 peptides at an FDR of 1%. Data processing and statistical analysis were performed using MetaboAnalyst platform (<https://www.metaboanalyst.ca/>). Proteins with > 50 %

missing values were removed. For the remaining proteins, missing values were estimated using the k-nearest neighbour (KNN) algorithm. Data was normalized using a  $\log_{10}$  transformation and Pareto scaling approach. Pairwise (two groups) and multiple (three groups) comparisons were performed to analyse the proteomic data. In the case of pairwise comparisons, a volcano plot with a 1.5-fold change (FC) and  $P$ -value of 0.05 was used. For multiple comparisons, a partial least square discriminant analysis (PLS-DA) with fixed VIP (Variable Importance in Projection) threshold  $\geq 2$  was employed.

## 2.6 Bioinformatics analysis

To benefit from the most complete annotation available, the bovine Uniprot IDs of the 684 proteins were converted into the orthologous human Entrez Gene ID (Gagaoua, Terlouw, et al., 2021), using the UniprotKB database (<https://www.uniprot.org/>). Both bovine and human Uniprot IDs were indexed in the databases and used for bioinformatics analysis. Metascape® tool (<https://metascape.org/>) was used to perform the Gene Ontology (GO) enrichment analysis and render the most significant and enriched GO terms, which allowed exploring the potential functions and biological processes of the differential abundant proteins (DAPs) identified in the proteome of dry-aged beef. Bioinformatics analysis were performed as described by Gagaoua, Terlouw, et al. (2021) with some brief modifications. In the current study, the GO terms with  $P$ -value  $< 0.05$ , a minimum overlap of 2, and an enrichment factor  $> 1.5$  (ratio between the observed counts and the counts expected by chance) were considered. Representative terms with a similarity score  $> 0.3$  were clustered together based on their membership similarities and visualized in networks layouts. In addition, Cytoscape v.3.9.1 was used to better visualize and explore the enriched networks, and Venn diagrams were generated to show the common proteins between the compared protein lists.

## 3. Results

A total of 684 proteins were identified and quantified by SWATH-MS analysis. In the following sections, first, we investigate the impact of sampling location on the proteome of dry-aged beef. To do this, we identify the differential proteins between external and internal locations of the aged beef at 0, 21, and 28 days of dry-aging day (i.e. 3, 24 and 31 days *post-mortem*). Second, we explore the impact of aging on the proteome of dry-aged beef. To do this, we investigate the changing proteins in the internal location during dry-aging. Furthermore, we study the potential molecular and biological pathways associated to the identified proteins. Lastly, we discriminate the three aging times based on the proteome

profile, and propose putative biomarkers of dry-aged beef tenderization (i.e. the degradation of muscle structure as aging progresses).

### ***3.1 Differentially abundant proteins (DAPs) between external and internal sampling locations during dry-aging***

#### ***3.1.1 Day 0 of dry-aging (3 days post-mortem)***

At day 0 of dry-aging, 18 DAPs were identified to differ between external and internal locations (**Figure 2A and Table S1**), from which 11 were more abundant in the external location: P4HB, JSRP1, F5, LYZF5, CATHL5, CHGA, SYNCRIP, CPN2, C4A, NECTIN2 and, ITIH2. Seven proteins were more abundant in the internal location: CRYL1, CILP2, CAVIN1, SMYD1, HSPB2, PIK3IP1 and, WFDC18. The biological pathways and cluster enrichment analysis performed on the 18 DAPs (**Figure 2B,C**), revealed 6 significant enriched GO terms, from which 5 were specific for the external location: “defence response to Gram-negative bacterium (GO: 0050829)”, “regulation of viral entry into host cell (GO: 0046596)”, “cellular response to cytokine stimulus (GO: 0071345)”, “muscle system process (GO: 0003012)”, and “negative regulation of endopeptidase activity (GO: 0010951)”. The term “muscle structure development GO: 0061061” was specific for internal location. The GO network between the representative enriched GO terms (**Figure 2B**), illustrates the extent of the enrichment of the clusters contributing to each term.

#### ***3.1.2 Day 21 of dry-aging (24 days post-mortem)***

At day 21 of dry-aging, 28 DAPs were identified between external and internal locations (**Figure 2D and Table S2**), from which 21 proteins were more abundant in the external location: PREP, ANXA4, JSRP1, CA2, MUC1, HEG1, MPST, PTPRG, WFDC2, PCDHGC3, CMBL, ARHGDI1, OTOR, GYG1, ST13, SCUBE1, DBI, ANG2, CD14, COL6A3 and, AZGP1. Six proteins were more abundant in the internal location: FBLN5, PDXP, LRRC15, IGFBP2, CDH11, and MELTF. One non-identified protein, A0A3Q1LJT1, was also found to be more abundant in the internal location. The bioinformatics enrichment analysis of the 28 DAPs (**Figure 2E,F**), revealed 8 significantly enriched GO terms, from which 7 GO terms were specific for the external location: “antibacterial humoral response (GO: 0019731)”, “endothelial cell differentiation (GO: 0045446)”, “negative regulation of catalytic activity (GO: 0043086)”, “regulation of interleukin-8 production (GO: 0032677)”, “RNA phosphodiester bond hydrolysis (GO: 0090501)”, “cellular nitrogen compound

catabolic process (GO: 0044270)", and "negative regulation of cell adhesion (GO: 0007162)".

### 3.1.3 Day 28 of dry-aging (31 days post-mortem)

At day 28, 23 DAPs were identified between external and internal locations (**Figure 2G and Table S3**), of which 9 were more abundant in the external location: ALB, HSPA8, FBLN2, PGK1, LDHB, EIF6, CD276, Hsp70, and ALDOA. A non-identified protein, A0A3Q1LWV8, was also found to be more abundant in the external location. The other 13 proteins were more abundant in the internal location: GNS, CD58, FBLN5, HGFAC, A1BG, VTN, LGALS3, DNASE1, C8G, FKBP4, ANGPTL2, CD55, and GC. The bioinformatics enrichment analysis with the 23 DAPS (**Figure 2H,I**), revealed 8 significant enriched GO terms, from which 3 were specific for external location: "ATP metabolic process (GO: 0046034)", "chaperone-mediated protein folding (GO: 0061077)", and "cellular response to starvation (GO: 0009267)". Whereas 2 GO terms were specific for the internal location: "regulation of T cell proliferation (GO: 0042127)" and "extracellular matrix organization (GO: 0030198)".

The comparison of the significantly enriched GO terms based on the three DAPs lists (i.e. external vs internal at 0, 21, and 28 days) revealed (**Figure 3A**) "humoral immune response (GO: 0006959)" as the only common term among the three lists. This term seemed to be more enriched at day 0. In addition, the heatmap evidenced that there were specific enriched GO terms for each list: "defense response to Gram-negative bacterium (GO: 0050829)" is most enriched at day 0, "calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules (GO: 0016339)" is at day 21, and "ATP metabolic process (GO: 0046034)" at day 28. Taking the above together, the overlap analysis identified only JSRP1 to be common between 0 and 21 days, and FBLN5 between 21 and 28 days (**Figure 3B**).

## 3.2 Impact of aging on the proteome of dry-aged beef: Changes in the differentially abundant proteins (DAPs) in the internal location during dry-aging

### 3.2.1 Day 0 (3 days post-mortem) vs day 21 (24 days post-mortem) of dry-aging

The comparison of day 0 and day 21, revealed 18 DAPs (**Figure 4A and Table S4**). From these, 15 proteins were more abundant at day 0: CILP2, MPST, TPPP3, CTSC, LDB3, PGLYRP2, PREP, ST13, RDX, CKM, FBN1, CD14, CSTB, ANG2, and FGG. On the other hand, 3 proteins were more abundant at day 21: CRISP3, DLAT, and SERPINI1. Seven

enriched GO terms (**Figure 4B**) were identified and most enriched at day 0: “negative regulation of homotypic cell-cell adhesion (GO: 0034111)”, “material process involved in female pregnancy (GO: 0060135)”, “kidney development GO: 0001822”, “negative regulation of hydrolase activity (GO: 0051346)”, “regulation of interferon-gamma production (GO:0032649)”, “sulphur compound biosynthetic process (GO:0044272)”, and “negative regulation of cell activation (GO:0050866)”.

### 3.2.2 Day 0 (3 days post-mortem) vs day 28 (31 days post-mortem) of dry-aging

The comparison of day 0 and day 28, revealed that the number of DAPs increased in comparison to those observed between 0 and 21 days. Thus, 33 DAPs were identified (**Figure 4C and Table S5**), from which 13 proteins were more abundant at day 0: TTN, CILP2, MYOZ1, ANKRD2, LDB3, AKR1B1, GRM4, EEF1A1, DEFA1, PGK1, NDRG2, Tnnt3, and ACY1. Whereas 20 proteins were more abundant at day 28: OMD, A1BG, MGC137099, CYB5R3, FGB, SPARCL1, UFD1, NHLRC3, C4BPA, DNASE1, SERPINI1, ARHGDI1, CPB2, FBLN5, ITIH2, SOD3, LCNL1, CSPG4, BTLA, and NECTIN4. The bioinformatics enrichment analysis carried out using the 33 DAPs revealed 10 significantly enriched GO terms (**Figure 4D,E**), from which 6 were specific at day 0: “sarcomere organization (GO: 0045214)”, “hexose biosynthetic process (GO: 0019319)”, “skeletal muscle organ development (GO: 0060538)”, “regulation of system process (GO: 0044057)”, “regulation of MAPK cascade (GO: 0043408)”, and “regulation of kinase activity (GO: 0043549)”. Whereas 5 GO terms were specific at day 28: “fibrinolysis (GO: 0042730)”, “negative regulation of proteolysis (GO: 0045861)”, “cell-cell adhesion (GO: 0098609)”, “protein catabolic process (GO: 0030163)”, and “regulation of leukocyte mediated immunity (GO: 0002703)”.

### 3.2.3 Day 21 (24 days post-mortem) vs day 28 (31 days post-mortem) of dry-aging

The comparison of day 21 and day 28, revealed an increase in the number of DAPs when compared to those observed between 0 and 21 days, but similar to those between 0 and 28 days. Thus, 34 DAPs, corresponding to 33 unique proteins, were identified (**Figure 4F and Table S6**), from which 8 proteins were more abundant at day 21: NDRG2, PGK1, PCYOX1, IDH3A, TTN, CPN2, ANKRD2, and AKR1B1. Whereas 25 proteins were more abundant at day 28: CP, ALDH6A1, CATHL1, PREP, ANGPTL2, ANXA3, NHLRC3, JSRP1, BTLA, TNC, CYB5R3, LCNL1, PTGR2, PRADC1, PDGFRB, CD27, ETFA, CPB2, ARHGDI1, EIF4A2, PCDHGC3, LGALS3, LGALS4, SH3BGR, and VAPA. The bioinformatics

enrichment analysis carried out using the 33 unique DAPs, revealed 10 significantly enriched GO terms (**Figure 4 G,H**), from which 2 GO terms were specific at day 21: “hexose biosynthetic process (GO: 0019319)”, and “muscle contraction (GO: 006936)”. Whereas 8 enriched GO terms were specific at day 28: “positive regulation of calcium ion import (GO: 0090280)”, “neutrophil activation (GO: 0042119)”, “regeneration (GO: 0031099)”, “regulation of T cell apoptotic process (GO: 0070232)”, “cellular amino acid catabolic process (GO: 0009063)”, “biological process involved in interaction with symbiont (GO: 0051702)”, “response to wounding (GO: 0009611)”, and “amide biosynthetic process (GO:0043604)”.

The comparison of the significantly enriched GO terms on the above three DAPs lists revealed 20 enriched terms (**Figure 5A**), among which “sarcomere organization (GO: 0045214)” was most enriched for ‘0d vs 28d’ list; “negative regulation of homotypic cell-cell adhesion (GO:0034111)” for ‘0d vs 21d’ list, and “positive regulation of calcium ion import (GO:0090280)” for ‘21d vs 28d’. No common enriched terms were found for all the three DAPs lists. The overlap analysis (**Figure 5B**) identified 3 proteins in common between the ‘0d vs 21d’ and the ‘0d vs 28d’ DAPs lists: CLP2, LDB3, SERPINI1. Eleven proteins were shared between the ‘0d vs 28d’ and the ‘21d vs 28d’ DAPs lists: LCNL1, NHLRC3, NDRG2, BTLA, TTN, CYB5R3, CPB2, ANKRD2, PGK1, ARHGDI1, and AKR1B1. One protein was common between ‘0d vs 21d’ and the ‘21d vs 28d’ DAPs lists: PREP. No common proteins were found among all three DAPs lists.

### 3.3 Identification of putative biomarkers for discriminating the three dry-aging times

Partial least squares-discriminant analysis (PLS-DA) allowed to discriminate the three dry-aging times irrespective of the sampling location: internal (**Figure 6A**) or external (**Figure 6B**). Thus, the molecular mechanisms and biological processes differ at every stage during dry-aging, irrespective of the location. The top 35 proteins with the highest VIP scores contributing to the separation in each group are depicted in **Figure 6C,D**. The proteins with  $VIP \geq 2$  were considered as good predictors, and can be suggested as putative biomarkers of dry-aged beef tenderization. In this regard, 33 proteins were identified to be the most explicative for the internal location (**Table S7**); whereas 31 proteins corresponding to 30 unique proteins were identified for the external location (**Table S8**). The comparison of the significantly enriched GO terms by means of heatmap using the two protein lists with  $VIP \geq 2$ , revealed 20 enriched terms (**Figure 6E**), among which, three were common between the

two sampling locations external and internal; and two of them were most enriched in the internal location: “cellular component assembly involved in morphogenesis (GO:0010927) and “humoral immune response (GO:0006959). The overlap analysis (**Figure 6F**) identified 4 proteins in common between external and internal location based on the two protein lists with  $VIP \geq 2$ : LDB3, PSMB7, LYZF5, and PGK1. Overall, in **Figure 6C**, those proteins with a  $VIP \geq 2.5$  are proposed as robust biomarkers for future validation as good predictors of dry-aged beef tenderization: TTN, GRM4, EEF1A1, LDB3, CILP2, TNNT3, GAPDH, SERPINI1, and OMD. Two proteins, TNNT3 and GAPDH, were identified as robust biomarkers of beef tenderness in an integromics study (Gagaoua, Terlouw, et al., 2021).

#### 4. Discussion

This study aimed to explore the changes occurring at the proteome level during dry-aging of beef by considering two important aspects, these being sampling location and aging time. We further aimed to investigate the biochemical pathways related to the dry-aged beef tenderization.

##### *4.1 Differences in the muscle proteome between external and internal sampling locations during beef dry-aging*

The proteome characterization of dry-aged beef revealed notable differences in the proteomes of internal and external sampling locations at each dry-aging time. Interestingly, proteins related to defence response to bacterium and regulation of viral entry into host cell were more abundant on the external location at 0 days of dry-aging (3 days *post-mortem*). These biochemical processes may result from a response of the muscle surface tissues to external stimuli, which may be explained by the oxidative conditions and the microbial activity to which the surfaces of meat cuts were subjected during the dressing of the carcass, during chilling storage, or later on, during the quartering and cutting. Early *post-mortem*, when muscle metabolism is still highly active and trying to cope with the oxygen depletion, the external surface may face further challenges relative to the inner areas and we speculate that muscle tissue on the external location may activate specific response pathways in an attempt to protect the muscle cells against microorganism infection and/or virus entry. This may partly explain the higher abundance of certain proteins, such as CATHL5 and CHGA, which are peptides presenting antimicrobial activity (Hoq et al., 2011; Whelehan et al., 2014); as well as LYZF5 which is a lysozyme that exerts antibacterial activity against a number of bacterial species (Leśniewski & Yang, 2021); and NECTIN2, which mediates

cell-cell adhesion, particularly by interacting with immune receptors, and further acts as an entry receptor for certain virus (Samanta & Almo, 2015).

More generally, hypoxia after slaughter and exsanguination, triggers molecular mechanisms in an attempt to protect cells against stress conditions and/or apoptosis, and preserve normal cell functions (Ouali et al., 2013). Physiological stress derived from adverse environmental conditions (oxidative conditions and microbial activity) together with the lack of oxygen supply in early *post-mortem* muscle, may also be associated with the expression of chaperone proteins. At day 0 of dry-aging (3 days *post-mortem*), differences in the abundance of P4HB and HSPB2 between sampling locations were observed. P4HB is an enzyme that catalyses disulfide bond formation between the cysteines of folding proteins, and inhibits protein aggregation through its chaperone activity (Wilkinson & Gilbert, 2004). In contrast, small Heat Shock Proteins (sHSPs), like HSPB2, exert a protective role during physiological stress by binding partially denatured proteins, and preventing proteins from irreversible aggregation (energy independent-process) until normal cell activity is restored (Gagaoua, Terlouw, et al., 2021). Indeed, protein refolding can take place by ATP-dependent chaperones such as Hsp70 (Sun & Macrae, 2005).

On the other hand, pathways related to muscle structure development were more enriched in the internal location of meat at day 0 of dry-aging (3 days *post-mortem*). Apoptosis and proteolysis early *post-mortem*, with continued protein breakdown during aging, result in the alteration and weakening of myofibrillar and structural proteins, and therefore, contribute to meat tenderization (Kemp & Parr, 2012). This has been evidence in this study by differences in the abundance of SMYD1 between sampling locations early *post-mortem*, as this protein appears to have an essential role in myosin thick filament assembly and muscle development (Just et al., 2011; Li et al., 2009). Nonetheless, it is also worth highlighting the possible involvement of the earlier mentioned chaperone proteins in the muscle structure pathways through their protective roles. Indeed, Bernard et al. (2007), investigating the transcriptome of bovine *Longissimus thoracis* muscle, suggested that down-regulation of small HSPs (HSPB1 and CRYAB) could be related to increased tenderness.

After 21 days of dry-aging (24 days *post-mortem*), terms related to cell-cell adhesion *via* plasma membrane adhesion molecules were the most enriched, and particularly, proteins related to antibacterial response were more abundant in the external location compared to the internal location. The abundances of these proteins may be enhanced on the external location

early *post-mortem* due to the attempt of muscle cells to maintain cell homeostasis, and cope with hypoxia and the entry of microorganisms into cells – as previously suggested. Furthermore, dehydration, microbial activity, and oxidation during dry-aging, which were proposed as the major mechanisms driving dry-aged beef flavour (Zhang et al., 2022), may further contribute to the differences in protein abundance between external and internal locations during dry-aging. For instance, proteolytic microbial activity was proposed to increase the peptide and amino acid flavour precursors in the crust of 21 days dry-aged (in moisture permeable bag) beef striploins (Zhang, Ross, et al., 2021) and in the inner part of 28 days dry-aged beef rump (*Middle gluteal*) (Lee, Yoon, et al., 2019). In an earlier study, Lee, Choe, et al. (2019) observed that dehydration increased the concentration of most identified free amino acids when moisture content was significantly reduced in beef striploins after 28 days of dry-aging. Oxygen, however, may inhibit the proteolytic activity of muscle endogenous enzymes such as  $\mu$ -calpain during early *post-mortem* period (Rowe et al., 2004), hence this may contribute to the difference in protein abundance between both sampling locations. The current study revealed differences in the abundances of CDH11, PCDHGC3, ANG, and WFDC2 between sampling locations. CDH11 is a glycoprotein and an integral component of the adherence junctions, which in live cells is involved in skeletal architecture, osteoblast differentiation and osteoid matrix mineralization (Leal-Gutiérrez et al., 2018). PCDHGC3 belongs to the cadherin family subgroup known as protocadherins (PCDHs), which are involved in cell-cell contacts and adhesion (Frank & Kemler, 2002). The ribonuclease ANG is involved in various physiological processes in living cells, such as, new blood vessel formation, antibacterial and antiviral activities, among others (Sheng & Xu, 2016); whereas WFDC2 is a secretory protein that may be involved in the host defence (Bingle et al., 2006).

After 28 days of dry-aging (31 days *post-mortem*), the proteome differences occurring between internal and external location contrasted with those observed at earlier dry-aging time. For example, proteins related to ATP metabolic process and chaperone-mediated protein folding were more abundant on the external location of meat. These proteins may be related to the development of the signature dry-aged flavour. In fact, aging changes the metabolome of beef, and hence, its taste and tenderness through the degradation of adenosine 5'-triphosphate (ATP) and protein degradation into free amino acids (Koutsidis et al., 2008a, 2008b), which are important flavour precursors. In particular, ATP degradation results in the generation of taste-related compounds, such as, adenosine 5'-monophosphate (AMP) and

inosine 5'-monophosphate (IMP); then, IMP can be metabolized to hypoxanthine (Hyp). These compounds are generated as a result of the *post-mortem* energy metabolism in the muscle (G. Bischof et al., 2022), and changes in their concentration may contribute to the enrichment of dry-aged flavour (Dashdorj et al., 2015; Dashdorj et al., 2016). These results may suggest a pivotal role of the energy metabolism pathways, in particular early *post-mortem* when the muscle cell metabolism is still active, on the levels of these flavour-related compounds across both internal and external location in the final dry-aged product. This study showed differences in the abundances of two glycolytic proteins, ALDOA and PGK1, and two large 70 kDa HSPs (HSPA1A and HSPA8), between the two sampling locations (higher in the external). These inducible HSPs may protect muscle cell proteins from *post-mortem* cellular stress, intervene in cell signalling pathways, and help maintaining the mitochondrial membrane potential and ATP levels in *post-mortem* muscle (Gagaoua, Terlouw, et al., 2021). We speculate that HSPs, through their anti-apoptotic role, may also contribute in determining the final levels of the taste-related compounds AMP, IMP, and Hyp in the final dry-aged product. Interestingly, Zhu et al. (2023) reported significant correlations between the abundancies of some energy metabolism proteins (e.g. STBD1 and PHKA1) and HSPs (e.g. HSPB6 and HSPA5) with the sensory flavour trait in wet-aged beef after 14 days *post-mortem*. It is clear that research combining metabolomics and proteomics approaches are needed to clarify the role of the energy metabolism pathways on the final levels of ATP degradation products, and to reveal the correlation of these flavour-related metabolites with the generation of the dry-aged flavour.

To the best of our knowledge, this is the first study to characterize such biologically significant differences in muscle proteome between external and internal locations, either prior or during dry-aging of beef. Taking a holistic view, the hierarchical heatmap demonstrated a different pattern for each dry-aging time when external and internal locations were compared (Figure 3A). These results re-emphasize the dynamic changes occurring in the proteome of dry-aged beef, and also evidence how important is to consider the location of sampling, especially when investigating the proteome of meat for identification of putative biomarkers. This is particularly relevant for dry-aging, where meat is exposed to the external atmosphere throughout the aging period.

## ***4.2 Effect of aging time on the muscle proteome of dry-aged beef***

### ***4.2.1 Significant role of muscle structure in the tenderization of beef during dry-aging***

The integrity of muscle structure and related biological pathways have been earlier reported to play a key role during the progression of meat tenderization (Gagaoua, Terlouw, et al., 2021). Indeed, breakdown of key myofibrillar and cytoskeletal proteins due to the action of endogenous proteolytic systems are central to meat tenderization (Ertbjerg, 2022). The comparisons of day 0 to day 28 of dry-aged beef revealed, as expected, sarcomere organization and muscle development to be significantly enriched terms (intact at day 0 and degraded at day 28). Likewise, the comparison of day 21 and day 28 revealed the enrichment of muscle contraction terms (specific at day 21 and degraded at day 28). In depth investigation of these results, evidenced that both TNNT3 and TTN were significantly more abundant at day 0, and their abundance decreased after 28 days of dry-aging possibly due to proteolytic activity. The troponin complex, including TNNT3 subunit, interacts with other thin filament proteins in the I-band and is involved in the regulation of actin-myosin interactions in the sarcomere, thus, its degradation *post-mortem* has been strongly implicated in development of meat tenderness (Huff Lonergan et al., 2010). TNNT3 proteolytic breakdown products appearing over 14 days of *post-mortem* aging (wet-aging) in *Longissimus thoracis* muscles was identified, among another 12 proteins, to compose the 30 kDa proteolytic fragment band (Gagaoua, Toy, et al., 2021) which has been extensively used to monitor the extent of proteolysis during aging (Koohmaraie et al., 1984; Macbride & Parrish JR., 1977). Furthermore, TNNT3 was identified as an indicator beef tenderization (Marino et al., 2015), and a good biomarker of beef tenderness (Gagaoua, Terlouw, et al., 2021). TTN, involved in maintaining sarcomere alignment during contraction, is degraded in *post-mortem* muscle and contributes to the weakening of sarcomere structure, and ultimately, tenderization of meat (Huff Lonergan et al., 2010). TTN degradation products have been reported in the literature (Fritz & Greaser, 1991; Fritz et al., 1993; Huff-Lonergan et al., 1995; Rowe et al., 2004; Wu et al., 2014). TTN has not yet been identified as a differential abundant protein using proteomic approaches for beef tenderness, but it has been shown to be involved in beef colour (Gagaoua et al., 2018; Hughes et al., 2019). Further investigations are needed to validate these proteins as biomarkers of dry-aged beef.

In earlier studies, Z-disk destabilization by the degradation of proteins close to this region was proposed as one of the major events contributing to tenderization of meat (Hopkins & Thompson, 2002; Taylor et al., 1995). LDB3 is located in the sarcomere and plays a key role in maintaining Z line integrity and muscle structure (Zhou et al., 2001), thus, the degradation of this protein may play a role in the tenderization of meat during aging. Indeed, LDB3 has

been previously shown to undergo significant degradation with increasing aging period (wet aging up to 14 days) as a result of protease activity in bovine *Longissimus lumborum* and *Psoas major* muscles (Song et al., 2022). This observation is in line with the results of the current study, wherein LDB3 is more abundant at day 0 when compared to 21 and 28 days of dry-aging. Thus, we speculate that a decrease in the abundance of LDB3 during dry-aging may be related to the breakdown of muscle skeletal structure due to endogenous proteolytic activity. MYOZ1 is also located in the Z-disk (Faulkner et al., 2001), and its proteolysis may be likely involved in the Z-disk destabilization, and therefore, tenderization during dry-aging. Furthermore, MYOZ1 and MYOZ3 were less abundant in the most tender loins and revealed as promising candidate biomarkers for meat tenderness prediction in heifers (Boudon et al., 2020), and MYOZ3 was confirmed as a robust biomarker of tenderness in bulls (Zhu et al., 2021). Tenderization of meat is also influenced by the disruption of the myofibrils in the I-band region in the *post-mortem* bovine muscle (Taylor et al., 1995). ANKRD2 is found in the I band (Pallavicini et al., 2001), and the results showed that this protein decreased with dry-aging time. ANKRD2 was proposed to be involved in *rigor mortis* of beef (Ding et al., 2022), being part of the muscle stress response pathway which stabilizes the myofibrillar structure under stressing cell conditions (Laville et al., 2009). In addition, this protein was proposed as a biomarker for beef tenderness by Picard and Gagaoua (2020), and confirmed in a later study (Boudon et al., 2020). In our study, TTN3, TTN, LDB3, MYOZ1 and ANKRD2 were detected at each time point and decreased with aging time, thus, these proteins may be useful to monitor tenderization or aging progression in the context of beef dry-aging.

#### 4.2.2 The role of energy metabolism during dry-aging of beef

Earlier studies in meat proteomics have revealed the importance of energy metabolism pathways during early *post-mortem* (Zhai et al., 2020), and aging (López-Pedrouso et al., 2021; Sierra et al., 2021), and their role in beef tenderness determination (Gagaoua, Terlouw, et al., 2021; Peter P. Purslow et al., 2021). In particular, energy metabolism pathways are strictly linked with *post-mortem* aging (Ouali et al., 2013; Peter P. Purslow et al., 2021). The expression of enzymes related to glycolytic pathways, and to some extent those associated to tricarboxylic acid cycle and oxidative phosphorylation pathways, increase early *post-mortem* to respond to the energy demand of the muscle under hypoxic conditions (Gagaoua, Terlouw, et al., 2021; Jia et al., 2007; Lamri, della Malva, Djenane, Albenzio, et al., 2023; Lamri, della Malva, Djenane, López-Pedrouso, et al., 2023; Ouali et al., 2013), however, the activity and abundances of these proteins decrease with long *post-mortem* aging period (Polati et al.,

2012). The current work showed that pathways related to hexose biosynthetic processes were enriched at both 0 and 21 days when compared to 28 days of dry-aging, and that the relative abundances of PGK1 and AKR1B1 proteins from the energy metabolism and ATP metabolic processes, decreased with aging. In an earlier study, PGK1 (glycolytic enzyme) was reported to significantly decrease after 14 days of wet-aging in bovine *longissimus* muscle (Silva et al., 2019). However, it was found to be negatively correlated with Warner-Bratzler shear force in bovine muscle (Gagaoua et al., 2020). AKR1B1, however, was only identified for the first time by Picard and Gagaoua (2020) in their meta-proteomics study, who proposed that it may act as a biomarker of beef tenderness. Thus, the results of this work suggest that energy metabolism pathways may be related to the tenderization of beef during dry-aging. Nevertheless, studies combining meat quality, metabolomics and proteomics in dry-aging are warranted for unravelling the biological pathways determining the quality and sensory traits, and in particular, the contribution of the energy metabolism pathway to the signature of dry-aged beef flavour.

#### 4.2.3 Other pathways: regulation of calcium import, neutrophil activation, and regeneration

In muscle, the sarcoplasmic reticulum (SR) releases calcium ions *via* calcium channels for regulating myofibrillar filament sliding. Calcium binds the troponin-tropomyosin complex after activation of the muscle (ATP-dependent), allowing myosin head to bind to actin for muscle contraction (after ATP hydrolysis into ADP and Pi) (P. P. Purslow et al., 2021). Relaxation of muscle occurs when calcium is pumped back into the SR, and troponin-tropomyosin complex blocks myosin from binding to actin (P. P. Purslow et al., 2021). This process occurs over age as while the ATP levels are maintained in the muscle. In early *post-mortem* muscle, when ATP consumption exceeds its synthesis, failure of ATP-dependent calcium pumps occurs and calcium ions accumulate into the sarcoplasm. Once ATP levels are depleted, the interaction formed between myosin and actin cannot be released, hence establishing *rigor mortis* (Huang et al., 2014). The results in this study show that biological processes related to positive regulation of calcium ion were most enriched after 28 days of dry-aging with increased in the abundance of related proteins, JSRP1 and ANXA3, possibly due to the *post-mortem* degradation of the cellular structures (e.g. membranes) where these proteins are normally located (Laville et al., 2009). JSRP1 is an integral protein of the skeletal muscle sarcoplasmic reticulum, co-localizes with its  $\text{Ca}^{2+}$  release channel (the ryanodine receptor), and interacts with calsequestrin and dihydropyridine receptor Cav1 in the skeletal muscle (Anderson et al., 2006), being involved in muscle contraction. ANXA3, a

calcium-dependent membrane phospholipid-binding protein, is involved in multiple cellular activities such as: vesicular transport, exocytosis and endocytosis; interaction with cytoskeletal proteins; formation of calcium ion channels; apoptosis, inflammation and cellular signalling (Ouali et al., 2013; Yang et al., 2021).

Other pathways were also enriched after 28 days of dry-aging, such as, neutrophil activation and regeneration, and proteins related to these biological processes were identified. In live cell muscles, CATHL1 acts as an antimicrobial peptide when released from the secretory granules of neutrophils and macrophages upon leukocyte activation (Kościuczuk et al., 2012). In living organisms, CPB2 regulates blood clot stability and limits fibrinolysis (blood clots breaking down process) (Tawara et al., 2016); and TNC, an extracellular matrix glycoprotein synthesized during damage repair and inflammatory processes, is essential for angiogenesis during wound healing (Wang et al., 2022).

The hierarchical heatmap demonstrated a different pattern between each aging period, and showed no shared GO terms among them (Figure 5A). Overall, changes in the relative abundance of proteins as aging time increases may result from various events. First, better extractability as aging progresses (della Motta et al., 2022), since the degradation of cellular compartments (e.g. membranes and myofibrillar networks) can release these proteins from the cell structures where they are normally contained (Laville et al., 2009). Second, degradation of these proteins by *post-mortem* proteolysis (Laville et al., 2009), resulting in the accumulation of smaller protein fragments. Third, either the solubilisation of proteins from the myofibrillar fraction or the aggregation of proteins from the sarcoplasmic fraction (Marino et al., 2014; Marino et al., 2013), may influence their relative abundance during aging. Due to these events the relative abundance of proteins is dynamic during dry-aging, as observed in this study. This continuous evolution in the proteome is also reflected on the various enriched biological processes at each aging time. Proteins involved in muscle contraction and structure, as well as, energy metabolism pathways were the main impacted during dry-aging. However, other proteins related to biological processes such as regulation of calcium import, neutrophil activation, and regeneration were also impacted.

#### 4. Conclusion

This study has evidenced, for the first time, the differential changes occurring at the proteome level between the external and internal sampling locations of beef striploins during dry-aging. Before dry-aging, the differential abundant proteins in the external location appear

to be related to biological pathways associated with response to external stimuli, which may be explained by the oxidative conditions and microbial activity to which muscle surfaces are exposed during the dressing of the carcass, the chilling storage, or later on, during the quartering and cutting. Furthermore, oxidative conditions, dehydration and microbial activity during dry-aging may have also contributed to the differences between sampling locations as aging progressed. These outcomes highlight the relevance of sampling location when investigating the proteome of meat for the identification of beef biomarkers for monitoring the tenderization or aging progression – in particular, for products like dry-aged beef..

In addition, this study has characterized the proteome of dry-aged beef for the first time, and demonstrates that dynamics and unique changes occur at each stage of the dry-aging period. Muscle structure and metabolism related pathways seem to be the major biological processes impacted during dry-aging, but interestingly, other pathways such as regulation of calcium import, neutrophil activation, and regeneration were also impacted. The current work also evidences the need for research combining metabolomics and proteomics tools to better our understanding on the role of energy metabolism pathways on the final levels of ATP degradation products, and how/if these derived flavour-related metabolites are major contributors to the dry-aged flavour. Characterizing the proteome of dry-aged beef allow us to build up our knowledge and understanding on the molecular mechanisms underlying the development of the unique dry-aged products. Furthermore, this research may establish a future basis for proposing putative biomarkers to predict and tailor the unique dry-aged quality traits, as well as, for dry-aged beef authentication. Several proteins were identified as putative biomarkers for future validation that have potential as good predictors of dry-aged beef tenderization: TIMM4, CRM4, EEF1A1, LDB3, CILP2, TNNT3, GAPDH, SERPINI1, and OMD.

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### **Declaration of Competing Interest**

No potential conflict of interest was reported by the authors.

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## Impact of sampling location and aging on the *Longissimus thoracis et lumborum* muscle proteome of dry-aged beef

Sara Álvarez <sup>a,b</sup>, Anne Maria Mullen <sup>a</sup>, Carlos Álvarez <sup>a</sup>, Ruth M. Hamill <sup>a</sup>, Eileen O'Neill <sup>b</sup> and Mohammed Gagaoua <sup>c,\*</sup>

<sup>a</sup> Dept. of Food Quality and Sensory Science, Teagasc Food Research Centre Ashtown, Dublin, D15 DY05, Ireland

<sup>b</sup> School of Food and Nutritional Sciences, University College, Cork, Western Road, Cork T12 YN60, Ireland

<sup>c</sup> PEGASE, INRAE, Institut Agro, 35590 Saint-Gilles, France

\* Correspondence: [mohammed.gagaoua@inrae.fr](mailto:mohammed.gagaoua@inrae.fr)

### Abstract

This study aimed to explore the differences in the proteome and molecular pathways between two sampling locations (external, internal) of bovine *Longissimus thoracis et lumborum* (*LTL*) muscles at 0, 21, and 28 days of dry-aging (i.e. 3, 24, and 31 days *post-mortem*). It further assessed the impact of aging on the proteome changes and the biological processes at interplay. Proteins related to defence response to bacterium and regulation of viral entry into host cell were identified to be more abundant on the external location before dry-aging, which may be associated to the oxidative conditions and microbial activity to which *post-mortem* muscle is exposed during dressing, chilling, and/or quartering of the carcasses. This highlights the relevance of sampling from interior tissues when searching for meat quality biomarkers. As dry-aging progressed, the proteome and related biological processes changed differently between sampling locations; proteins related to cell-cell adhesion and ATP metabolic processes pathways were revealed in the external location at 21 and 28 days, respectively. On the other hand, the impact of aging on the proteome of the interior samples, evidenced that muscle contraction and structure together with energy metabolism were the major pathways driving dry-aging. Additionally, aging impacted other pathways in the interior tissues, such as regulation of calcium import, neutrophil activation, and regeneration. Overall, the differences in the proteome allowed discriminating the three dry-aging times, regardless of the sampling location. Several proteins were proposed for validation as robust biomarkers to monitor the aging process (tenderization) of dry-aged beef: TTN, GRM4, EEF1A1, LDB3, CILP2, TNNT3, GAPDH, SERPINI1, and OMD.

**Keywords:** Dry-aging; Beef; Proteomics; Biochemical pathways; Enrichment analysis; SWATH-MS

## 1. Introduction

The muscle proteome is dynamic; it undergoes continual changes during the life of the animal and after slaughter, hence playing a pivotal role in the conversion of muscle into meat and its quality determination (Hollung et al., 2007). In this sense, proteomics provide powerful tools which can be used to reveal the underlying mechanisms and biochemical pathways that dictate the structural and metabolic changes observable in early *post-mortem* muscle and/or during meat aging/processing (Gagaoua et al., 2022). In recent years, proteomics has been used to advance our knowledge and understanding of the *post-mortem* changes occurring in fresh meat during meat aging under various conditions (della Malva et al., 2022; Gagaoua, Troy, et al., 2021; Sierra et al., 2021). While proteomics tools have been used to evidence degradation of muscle proteins and peptides during the production of dry-cured meats (Mora et al., 2015; Mora et al., 2010), to the best of our knowledge, there are no available studies on the application of proteomics to study the proteome changes and related mechanisms involved in the dry-aging of beef.

Dry-aging is a *post-mortem* processing technique where meat is exposed to controlled conditions of temperature (0-4 °C), relative humidity (70-80%), and airflow (0.5-2.5 m/s) for 14-35 days (typical restaurants and retailer conditions are 1-4°C, 75-85% and 21-35 days) (Koutsoumanis et al., 2023). In contrast to wet-aging, where beef is vacuum-packed, traditional dry-aging of beef is characterized by the absence of packaging. Although both strategies improve meat tenderness, the absence of this barrier during dry-aging results in increased water evaporation and surface dehydration which increases yield loss (weight and trim losses) and surface colour changes, when compared to wet-aging (Savell & Gehring, 2018). Muscle dehydration together with lipid oxidation and microbial activity have been confirmed to play a key role in the development of the signature dry-aged beef flavour (Zhang et al., 2023; Zhang et al., 2022).

Research efforts have improved our understanding on the role of microbial populations during dry-aging of beef (da Silva Bernardo et al., 2021; Ryu et al., 2018), and the meat quality and sensory acceptability of dry-aged beef (Berger et al., 2018; Li et al., 2014). In addition, in recent years, there has been a particular interest in deciphering the metabolome of dry-aged beef with the aim of identifying the diverse flavour-related metabolites responsible for its signature flavour (Bischof et al., 2021; Kim et al., 2016; Setyabrata et al., 2021; Setyabrata et al., 2022). Many of these metabolites are likely derived from lipid oxidation,

metabolism of nucleotides, and proteolysis during dry-aging (Zhang et al., 2022). In particular, the action of endogenous muscle enzymes and the microbial enzymes on the surface of beef has an impact on metabolome and the proteome of dry-aged beef (Greta Bischof et al., 2022). Indeed, proteolysis of structural and cytoskeletal proteins occurs during dry-aging of beef, and results in the increase of concentrations of peptides and free amino acids with aging time (Zhang, Yoo, et al., 2021), changing the beef proteome as aging advances. Whereas research has been done to characterize the metabolome of dry-aged beef, to the best of our knowledge, there are no available studies investigating the changes occurring in the proteome of this product. Therefore, proteomics research is warranted as these data will reveal the pathways and biological processes underlying the dry-aging of beef, and will further complement the available information in metabolomics. All this information together may ultimately assist processors to optimize dry-aging conditions in order to obtain the desirable dry-aged product, while attempting to minimize yield loss.

Therefore, the aim of this study was to perform for the first time an in-depth exploration of the changes occurring in the beef muscle proteome during dry-aging, and investigate the interconnectedness of the various biological processes that are involved in the production of this meat product. Furthermore, this work investigated if there are differences in protein abundances and related pathways between the external and internal locations of the meat cuts during the dry-aging process.

## 2. Material and Methods

### 2.1 Animals and muscle/dry-aged meat biopsies

As part of a larger experiment (not publicly available yet), striploins (*M. longissimus thoracis et lumborum*, cut from the 10<sup>th</sup> rib) from the right- and left-hand sides of six cross breed (Hereford x Limousin) bovine carcasses (4 steers and 2 heifers - no significant gender effect as per Gagaoua, Troy, et al. (2021); conformation score U= to O=; fat score 2= to 4=; age: 24-36 months; and average weight of 180.6 kg  $\pm$  19.8 kg), were collected from the slaughterhouse (Kepak, Ireland) at 2 days *post-mortem*. At 3 days *post-mortem*, the striploins were cut into 4 equal sections, and each randomly distributed to one of the dry-aging times: 0, 21, and 28 days (i.e. 3, 24, and 31 days *post-mortem*, respectively). Sections assigned to 21 and 28 days of dry-aging, were placed inside the DRY AGER DX 1000® (DRY AGER®, Germany) with set conditions at 2.0 °C, 75% relative humidity and air flow of 0.5-2.0 m/s. Subcutaneous fat was left on the sections, and these were dry-aged on racks with the fat side

facing up. To control for location, racks inside the cabinet were randomly rotated every week. At each time point, the assigned sections were cut into steaks, and muscle samples (approx. 2 g) were collected for proteomic analyses from both internal and external (i.e. lean surface prior to dry-aging, and crust as aging progressed) locations using aseptic methods (**Figure 1**). Samples were stored at  $-80^{\circ}\text{C}$  for further analysis.

## **2.2 Protein extraction and quantification**

Frozen muscle tissue samples (150-200 mg) were homogenized in 3 mL of fresh extraction buffer consisting of 8.3 M urea, 2 M thiourea, 1% DL-Dithiothreitol (DTT), 2% CHAPS, and Pharmalyte® 3-10 (Bouley et al., 2004) using a T18 digital ULTRA-TURRAX® (IKA®, Staufen, Germany) which was run at 20,000 rpm for 60 s (2 x 30 s). The homogenates were incubated on ice for 30 min, and subsequently, centrifuged for 30 min at 10000 x g at  $4^{\circ}\text{C}$ . The supernatant was transferred into Eppendorf tubes and stored at  $-80^{\circ}\text{C}$  until protein quantification.

Protein concentrations were determined using the Bradford method (Bradford, 1976), and bovine serum albumin (Thermo Scientific, Rockford, IL, USA) was employed as a standard to build the calibration curve. To determine protein concentrations in the protein extracts, absorbance was measured at 595 nm using a spectrophotometer (UV-1700, Pharmaspec, SHIMADZU).

## **2.3 One dimensional (1D) Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for shotgun proteomics**

The protein concentrations of the muscle extracts were adjusted with MilliQ water and an equal volume of Laemmli buffer was added (Sigma-Aldrich, St. Louis, MO, USA) to ensure a final protein mass of 40  $\mu\text{g}$  in each well. The samples were heated in a standard block heater (SBH130D/3, Bibby Scientific Limited, Staffordshire, UK) at  $80^{\circ}\text{C}$  for 15 minutes. A volume of 15  $\mu\text{L}$  of each sample were loaded in freshly prepared 12% resolving and 4% stacking gels for 1D SDS-PAGE using a Mini-PROTEAN® Tetra Cell System (Bio-Rad, Hercules, CA, USA) with a run time of 15 minutes at 4W to concentrate the proteins. Protein bands for shotgun proteomics were prepared as described by Lamri, della Malva, Djenane, López-Pedrouso, et al. (2023). Subsequent digestion of the protein bands and mass spectrometry analysis were performed following the protocol described by Chantada-Vázquez et al. (2021). Modified porcine trypsin (Promega, Madison, WI, USA) was used for

digestion of the dried gel bands at a concentration of 20 ng/ $\mu$ L in 20mM ammonium bicarbonate at 37 °C for 16h. The resulting peptides were extracted with 60% acetonitrile in 0.5% formic acid, and then, vacuum-concentrated in a SpeedVac and stored at -20 °C for further LC-MS/MS analysis.

#### ***2.4 Generation of the reference spectral library and protein quantification by SWATH-MS***

To generate the MS/MS spectral libraries, and obtain a good representation of the peptides and proteins in all samples, vials from each group (i.e. dry aged condition) were prepared, and analysed by a shotgun data-dependent acquisition (DDA) approach using a micro-LC-MS/MS. Briefly, peptides mixture of 4  $\mu$ L per condition, was separated on a micro-LC system Ekspert nLC425 (Eksigent, Dublin, CA, USA) using an Eksigent C18 column (150  $\times$  0.30 mm, 3 mm particle size and 120 Å pore size) (Eksigent Sciex). The eluting peptides were directly injected into a hybrid quadrupole-TOF mass spectrometer coupled with a Triple TOF 6600 (Sciex, Redwood City, CA, USA), which operated with a data-dependent acquisition system in positive ion mode. The MS raw files were used for peptides and proteins identification employing the Protein Pilot software (version 5.0.1, Sciex) and the bovine-specific Uniprot database (<https://www.uniprot.org/>). The false discovery rate (FDR) was set to 1% for both peptides and proteins.

For relative quantification of peptides and proteins, 4  $\mu$ L of peptides from each individual sample were analysed using a data-independent acquisition (IDA) method, known as SWATH (Sequential Window Acquisition of all Theoretical Mass Spectra)-MS method. Samples were analysed using the LC-MS equipment and LC gradient. A total of 36 samples from 6 treatments (6 biological replicates per group) were analysed. The data extraction from the SWATH runs was performed by PeakView v.2.2 (Sciex, Framingham, MA, USA) employing the SWATH Acquisition MicroApp v.2.0. This application processed the data based on the spectral library created, and used the following parameters: 10 peptides /protein and 7 fragments/peptide and FDR below 1%. Protein quantification was calculated by summing the peak areas of the corresponding peptides

#### ***2.5 Statistical analysis***

SWATH-MS analysis allowed the identification and quantification of 684 proteins and 1908 peptides at an FDR of 1%. Data processing and statistical analysis were performed using MetaboAnalyst platform (<https://www.metaboanalyst.ca/>). Proteins with > 50 %

missing values were removed. For the remaining proteins, missing values were estimated using the k-nearest neighbour (KNN) algorithm. Data was normalized using a  $\log_{10}$  transformation and Pareto scaling approach. Pairwise (two groups) and multiple (three groups) comparisons were performed to analyse the proteomic data. In the case of pairwise comparisons, a volcano plot with a 1.5-fold change (FC) and  $P$ -value of 0.05 was used. For multiple comparisons, a partial least square discriminant analysis (PLS-DA) with fixed VIP (Variable Importance in Projection) threshold  $\geq 2$  was employed.

## 2.6 Bioinformatics analysis

To benefit from the most complete annotation available, the bovine Uniprot IDs of the 684 proteins were converted into the orthologous human Entrez Gene ID (Gagaoua, Terlouw, et al., 2021), using the UniprotKB database (<https://www.uniprot.org/>). Both bovine and human Uniprot IDs were indexed in the databases and used for bioinformatics analysis. Metascape® tool (<https://metascape.org/>) was used to perform the Gene Ontology (GO) enrichment analysis and render the most significant and enriched GO terms, which allowed exploring the potential functions and biological processes of the differential abundant proteins (DAPs) identified in the proteome of dry-aged beef. Bioinformatics analysis were performed as described by Gagaoua, Terlouw, et al. (2021) with some brief modifications. In the current study, the GO terms with  $P$ -value  $< 0.05$ , a minimum overlap of 2, and an enrichment factor  $> 1.5$  (ratio between the observed counts and the counts expected by chance) were considered. Representative terms with a similarity score  $> 0.3$  were clustered together based on their membership similarities and visualized in networks layouts. In addition, Cytoscape v.3.9.1 was used to better visualize and explore the enriched networks, and Venn diagrams were generated to show the common proteins between the compared protein lists.

## 3. Results

A total of 684 proteins were identified and quantified by SWATH-MS analysis. In the following sections, first, we investigate the impact of sampling location on the proteome of dry-aged beef. To do this, we identify the differential proteins between external and internal locations of the aged beef at 0, 21, and 28 days of dry-aging day (i.e. 3, 24 and 31 days *post-mortem*). Second, we explore the impact of aging on the proteome of dry-aged beef. To do this, we investigate the changing proteins in the internal location during dry-aging. Furthermore, we study the potential molecular and biological pathways associated to the identified proteins. Lastly, we discriminate the three aging times based on the proteome

profile, and propose putative biomarkers of dry-aged beef tenderization (i.e. the degradation of muscle structure as aging progresses).

### ***3.1 Differentially abundant proteins (DAPs) between external and internal sampling locations during dry-aging***

#### ***3.1.1 Day 0 of dry-aging (3 days post-mortem)***

At day 0 of dry-aging, 18 DAPs were identified to differ between external and internal locations (**Figure 2A and Table S1**), from which 11 were more abundant in the external location: P4HB, JSRP1, F5, LYZF5, CATHL5, CHGA, SYNCRIP, CPN2, C4A, NECTIN2 and, ITIH2. Seven proteins were more abundant in the internal location: CRYL1, CILP2, CAVIN1, SMYD1, HSPB2, PIK3IP1 and, WFDC18. The biological pathways and cluster enrichment analysis performed on the 18 DAPs (**Figure 2B,C**), revealed 6 significant enriched GO terms, from which 5 were specific for the external location: “defence response to Gram-negative bacterium (GO: 0050829)”, “regulation of viral entry into host cell (GO: 0046596)”, “cellular response to cytokine stimulus (GO: 0071345)”, “muscle system process (GO: 0003012)”, and “negative regulation of endopeptidase activity (GO: 0010951)”. The term “muscle structure development GO: 0061061” was specific for internal location. The GO network between the representative enriched GO terms (**Figure 2B**), illustrates the extent of the enrichment of the clusters contributing to each term.

#### ***3.1.2 Day 21 of dry-aging (24 days post-mortem)***

At day 21 of dry-aging, 28 DAPs were identified between external and internal locations (**Figure 2D and Table S2**), from which 21 proteins were more abundant in the external location: PREP, ANXA4, JSRP1, CA2, MUC1, HEG1, MPST, PTPRG, WFDC2, PCDHGC3, CMBL, ARHGDI1, OTOR, GYG1, ST13, SCUBE1, DBI, ANG2, CD14, COL6A3 and, AZGP1. Six proteins were more abundant in the internal location: FBLN5, PDXP, LRRC15, IGFBP2, CDH11, and MELTF. One non-identified protein, A0A3Q1LJT1, was also found to be more abundant in the internal location. The bioinformatics enrichment analysis of the 28 DAPs (**Figure 2E,F**), revealed 8 significantly enriched GO terms, from which 7 GO terms were specific for the external location: “antibacterial humoral response (GO: 0019731)”, “endothelial cell differentiation (GO: 0045446)”, “negative regulation of catalytic activity (GO: 0043086)”, “regulation of interleukin-8 production (GO: 0032677)”, “RNA phosphodiester bond hydrolysis (GO: 0090501)”, “cellular nitrogen compound

catabolic process (GO: 0044270)", and "negative regulation of cell adhesion (GO: 0007162)".

### 3.1.3 Day 28 of dry-aging (31 days post-mortem)

At day 28, 23 DAPs were identified between external and internal locations (**Figure 2G and Table S3**), of which 9 were more abundant in the external location: ALB, HSPA8, FBLN2, PGK1, LDHB, EIF6, CD276, Hsp70, and ALDOA. A non-identified protein, A0A3Q1LWV8, was also found to be more abundant in the external location. The other 13 proteins were more abundant in the internal location: GNS, CD58, FBLN5, HGFAC, A1BG, VTN, LGALS3, DNASE1, C8G, FKBP4, ANGPTL2, CD55, and GC. The bioinformatics enrichment analysis with the 23 DAPS (**Figure 2H,I**), revealed 8 significant enriched GO terms, from which 3 were specific for external location: "ATP metabolic process (GO: 0046034)", "chaperone-mediated protein folding (GO: 0061077)", and "cellular response to starvation (GO: 0009267)". Whereas 2 GO terms were specific for the internal location: "regulation of T cell proliferation (GO: 0042127)" and "extracellular matrix organization (GO: 0030198)".

The comparison of the significantly enriched GO terms based on the three DAPs lists (i.e. external vs internal at 0, 21, and 28 days) revealed (**Figure 3A**) "humoral immune response (GO: 0006959)" as the only common term among the three lists. This term seemed to be more enriched at day 0. In addition, the heatmap evidenced that there were specific enriched GO terms for each list: "defense response to Gram-negative bacterium (GO: 0050829)" is most enriched at day 0, "calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules (GO: 0016339)" is at day 21, and "ATP metabolic process (GO: 0046034)" at day 28. Taking the above together, the overlap analysis identified only JSRP1 to be common between 0 and 21 days, and FBLN5 between 21 and 28 days (**Figure 3B**).

## 3.2 Impact of aging on the proteome of dry-aged beef: Changes in the differentially abundant proteins (DAPs) in the internal location during dry-aging

### 3.2.1 Day 0 (3 days post-mortem) vs day 21 (24 days post-mortem) of dry-aging

The comparison of day 0 and day 21, revealed 18 DAPs (**Figure 4A and Table S4**). From these, 15 proteins were more abundant at day 0: CILP2, MPST, TPPP3, CTSC, LDB3, PGLYRP2, PREP, ST13, RDX, CKM, FBN1, CD14, CSTB, ANG2, and FGG. On the other hand, 3 proteins were more abundant at day 21: CRISP3, DLAT, and SERPINI1. Seven

enriched GO terms (**Figure 4B**) were identified and most enriched at day 0: “negative regulation of homotypic cell-cell adhesion (GO: 0034111)”, “material process involved in female pregnancy (GO: 0060135)”, “kidney development GO: 0001822”, “negative regulation of hydrolase activity (GO: 0051346)”, “regulation of interferon-gamma production (GO:0032649)”, “sulphur compound biosynthetic process (GO:0044272)”, and “negative regulation of cell activation (GO:0050866)”.

### 3.2.2 Day 0 (3 days post-mortem) vs day 28 (31 days post-mortem) of dry-aging

The comparison of day 0 and day 28, revealed that the number of DAPs increased in comparison to those observed between 0 and 21 days. Thus, 33 DAPs were identified (**Figure 4C and Table S5**), from which 13 proteins were more abundant at day 0: TTN, CILP2, MYOZ1, ANKRD2, LDB3, AKR1B1, GRM4, EEF1A1, DEB, PGK1, NDRG2, Tnnt3, and ACY1. Whereas 20 proteins were more abundant at day 28: OMD, A1BG, MGC137099, CYB5R3, FGB, SPARCL1, UFD1, NHLRC3, C4BPA, DNASE1, SERPINI1, ARHGDI, CPB2, FBLN5, ITIH2, SOD3, LCNL1, CSPG4, BTLA, and NECTIN4. The bioinformatics enrichment analysis carried out using the 33 DAPs revealed 10 significantly enriched GO terms (**Figure 4D,E**), from which 6 were specific at day 0: “sarcomere organization (GO: 0045214)”, “hexose biosynthetic process (GO: 0019319)”, “skeletal muscle organ development (GO: 0060538)”, “regulation of system process (GO: 0044057)”, “regulation of MAPK cascade (GO: 0043408)”, and “regulation of kinase activity (GO: 0043549)”. Whereas 5 GO terms were specific at day 28: “fibrinolysis (GO: 0042730)”, “negative regulation of proteolysis (GO: 0045861)”, “cell-cell adhesion (GO: 0098609)”, “protein catabolic process (GO: 0031163)”, and “regulation of leukocyte mediated immunity (GO: 0002703)”.

### 3.2.3 Day 21 (24 days post-mortem) vs day 28 (31 days post-mortem) of dry-aging

The comparison of day 21 and day 28, revealed an increase in the number of DAPs when compared to those observed between 0 and 21 days, but similar to those between 0 and 28 days. Thus, 34 DAPs, corresponding to 33 unique proteins, were identified (**Figure 4F and Table S6**), from which 8 proteins were more abundant at day 21: NDRG2, PGK1, PCYOX1, IDH3A, TTN, CPN2, ANKRD2, and AKR1B1. Whereas 25 proteins were more abundant at day 28: CP, ALDH6A1, CATHL1, PREP, ANGPTL2, ANXA3, NHLRC3, JSRP1, BTLA, TNC, CYB5R3, LCNL1, PTGR2, PRADC1, PDGFRB, CD27, ETFA, CPB2, ARHGDI, EIF4A2, PCDHGC3, LGALS3, LGALS4, SH3BGR, and VAPA. The bioinformatics

enrichment analysis carried out using the 33 unique DAPs, revealed 10 significantly enriched GO terms (**Figure 4 G,H**), from which 2 GO terms were specific at day 21: “hexose biosynthetic process (GO: 0019319)”, and “muscle contraction (GO: 006936)”. Whereas 8 enriched GO terms were specific at day 28: “positive regulation of calcium ion import (GO: 0090280)”, “neutrophil activation (GO: 0042119)”, “regeneration (GO: 0031099)”, “regulation of T cell apoptotic process (GO: 0070232)”, “cellular amino acid catabolic process (GO: 0009063)”, “biological process involved in interaction with symbiont (GO: 0051702)”, “response to wounding (GO: 0009611)”, and “amide biosynthetic process (GO:0043604)”.

The comparison of the significantly enriched GO terms on the above three DAPs lists revealed 20 enriched terms (**Figure 5A**), among which “sarcomere organization (GO: 0045214)” was most enriched for ‘0d vs 28d’ list; “negative regulation of homotypic cell-cell adhesion (GO:0034111)” for ‘0d vs 21d’ list, and “positive regulation of calcium ion import (GO:0090280)” for ‘21d vs 28d’. No common enriched terms were found for all the three DAPs lists. The overlap analysis (**Figure 5B**) identified 3 proteins in common between the ‘0d vs 21d’ and the ‘0d vs 28d’ DAPs lists: CLP2, LDB3, SERPINI1. Eleven proteins were shared between the ‘0d vs 28d’ and the ‘21d vs 28d’ DAPs lists: LCNL1, NHLRC3, NDRG2, BTLA, TTN, CYB5R3, CPB2, ANKRD2, PGK1, ARHGDI1, and AKR1B1. One protein was common between ‘0d vs 21d’ and the ‘21d vs 28d’ DAPs lists: PREP. No common proteins were found among all three DAPs lists.

### 3.3 Identification of putative biomarkers for discriminating the three dry-aging times

Partial least squares-discriminant analysis (PLS-DA) allowed to discriminate the three dry-aging times irrespective of the sampling location: internal (**Figure 6A**) or external (**Figure 6B**). Thus, the molecular mechanisms and biological processes differ at every stage during dry-aging, irrespective of the location. The top 35 proteins with the highest VIP scores contributing to the separation in each group are depicted in **Figure 6C,D**. The proteins with  $VIP \geq 2$  were considered as good predictors, and can be suggested as putative biomarkers of dry-aged beef tenderization. In this regard, 33 proteins were identified to be the most explicative for the internal location (**Table S7**); whereas 31 proteins corresponding to 30 unique proteins were identified for the external location (**Table S8**). The comparison of the significantly enriched GO terms by means of heatmap using the two protein lists with  $VIP \geq 2$ , revealed 20 enriched terms (**Figure 6E**), among which, three were common between the

two sampling locations external and internal; and two of them were most enriched in the internal location: “cellular component assembly involved in morphogenesis (GO:0010927) and “humoral immune response (GO:0006959). The overlap analysis (**Figure 6F**) identified 4 proteins in common between external and internal location based on the two protein lists with  $VIP \geq 2$ : LDB3, PSMB7, LYZF5, and PGK1. Overall, in **Figure 6C**, those proteins with a  $VIP \geq 2.5$  are proposed as robust biomarkers for future validation as good predictors of dry-aged beef tenderization: TTN, GRM4, EEF1A1, LDB3, CILP2, TNNT3, GAPDH, SERPINI1, and OMD. Two proteins, TNNT3 and GAPDH, were identified as robust biomarkers of beef tenderness in an integromics study (Gagaoua, Terlouw, et al., 2021).

#### 4. Discussion

This study aimed to explore the changes occurring at the proteome level during dry-aging of beef by considering two important aspects, these being sampling location and aging time. We further aimed to investigate the biochemical pathways related to the dry-aged beef tenderization.

##### *4.1 Differences in the muscle proteome between external and internal sampling locations during beef dry-aging*

The proteome characterization of dry-aged beef revealed notable differences in the proteomes of internal and external sampling locations at each dry-aging time. Interestingly, proteins related to defence response to bacterium and regulation of viral entry into host cell were more abundant on the external location at 0 days of dry-aging (3 days *post-mortem*). These biochemical processes may result from a response of the muscle surface tissues to external stimuli, which may be explained by the oxidative conditions and the microbial activity to which the surfaces of meat cuts were subjected during the dressing of the carcass, during chilling storage, or later on, during the quartering and cutting. Early *post-mortem*, when muscle metabolism is still highly active and trying to cope with the oxygen depletion, the external surface may face further challenges relative to the inner areas and we speculate that muscle tissue on the external location may activate specific response pathways in an attempt to protect the muscle cells against microorganism infection and/or virus entry. This may partly explain the higher abundance of certain proteins, such as CATHL5 and CHGA, which are peptides presenting antimicrobial activity (Hoq et al., 2011; Whelehan et al., 2014); as well as LYZF5 which is a lysozyme that exerts antibacterial activity against a number of bacterial species (Leśnierowski & Yang, 2021); and NECTIN2, which mediates

cell-cell adhesion, particularly by interacting with immune receptors, and further acts as an entry receptor for certain virus (Samanta & Almo, 2015).

More generally, hypoxia after slaughter and exsanguination, triggers molecular mechanisms in an attempt to protect cells against stress conditions and/or apoptosis, and preserve normal cell functions (Ouali et al., 2013). Physiological stress derived from adverse environmental conditions (oxidative conditions and microbial activity) together with the lack of oxygen supply in early *post-mortem* muscle, may also be associated with the expression of chaperone proteins. At day 0 of dry-aging (3 days *post-mortem*), differences in the abundance of P4HB and HSPB2 between sampling locations were observed. P4HB is an enzyme that catalyses disulfide bond formation between the cysteines of folding proteins, and inhibits protein aggregation through its chaperone activity (Wilkinson & Gilbert, 2004). In contrast, small Heat Shock Proteins (sHSPs), like HSPB2, exert a protective role during physiological stress by binding partially denatured proteins, and preventing proteins from irreversible aggregation (energy independent-process) until normal cell activity is restored (Gagaoua, Terlouw, et al., 2021). Indeed, protein refolding can take place by ATP-dependent chaperones such as Hsp70 (Sun & Macrae, 2005).

On the other hand, pathways related to muscle structure development were more enriched in the internal location of meat at day 0 of dry-aging (3 days *post-mortem*). Apoptosis and proteolysis early *post-mortem*, with continued protein breakdown during aging, result in the alteration and weakening of myofibrillar and structural proteins, and therefore, contribute to meat tenderization (Kemp & Parr, 2012). This has been evidence in this study by differences in the abundance of SMYD1 between sampling locations early *post-mortem*, as this protein appears to have an essential role in myosin thick filament assembly and muscle development (Just et al., 2011; Li et al., 2009). Nonetheless, it is also worth highlighting the possible involvement of the earlier mentioned chaperone proteins in the muscle structure pathways through their protective roles. Indeed, Bernard et al. (2007), investigating the transcriptome of bovine *Longissimus thoracis* muscle, suggested that down-regulation of small HSPs (HSPB1 and CRYAB) could be related to increased tenderness.

After 21 days of dry-aging (24 days *post-mortem*), terms related to cell-cell adhesion *via* plasma membrane adhesion molecules were the most enriched, and particularly, proteins related to antibacterial response were more abundant in the external location compared to the internal location. The abundances of these proteins may be enhanced on the external location

early *post-mortem* due to the attempt of muscle cells to maintain cell homeostasis, and cope with hypoxia and the entry of microorganisms into cells – as previously suggested. Furthermore, dehydration, microbial activity, and oxidation during dry-aging, which were proposed as the major mechanisms driving dry-aged beef flavour (Zhang et al., 2022), may further contribute to the differences in protein abundance between external and internal locations during dry-aging. For instance, proteolytic microbial activity was proposed to increase the peptide and amino acid flavour precursors in the crust of 21 days dry-aged (in moisture permeable bag) beef striploins (Zhang, Ross, et al., 2021) and in the inner part of 28 days dry-aged beef rump (*Middle gluteal*) (Lee, Yoon, et al., 2019). In an earlier study, Lee, Choe, et al. (2019) observed that dehydration increased the concentration of most identified free amino acids when moisture content was significantly reduced in beef striploins after 28 days of dry-aging. Oxygen, however, may inhibit the proteolytic activity of muscle endogenous enzymes such as  $\mu$ -calpain during early *post-mortem* period (Rowe et al., 2004), hence this may contribute to the difference in protein abundance between both sampling locations. The current study revealed differences in the abundances of CDH11, PCDHGC3, ANG, and WFDC2 between sampling locations. CDH11 is a glycoprotein and an integral component of the adherence junctions, which in live cells is involved in skeletal architecture, osteoblast differentiation and osteoid matrix mineralization (Leal-Gutiérrez et al., 2018). PCDHGC3 belongs to the cadherin family subgroup known as protocadherins (PCDHs), which are involved in cell-cell contacts and adhesion (Frank & Kemler, 2002). The ribonuclease ANG is involved in various physiological processes in living cells, such as, new blood vessel formation, antibacterial and antiviral activities, among others (Sheng & Xu, 2016); whereas WFDC2 is a secretory protein that may be involved in the host defence (Bingle et al., 2006).

After 28 days of dry-aging (31 days *post-mortem*), the proteome differences occurring between internal and external location contrasted with those observed at earlier dry-aging time. For example, proteins related to ATP metabolic process and chaperone-mediated protein folding were more abundant on the external location of meat. These proteins may be related to the development of the signature dry-aged flavour. In fact, aging changes the metabolome of beef, and hence, its taste and tenderness through the degradation of adenosine 5'-triphosphate (ATP) and protein degradation into free amino acids (Koutsidis et al., 2008a, 2008b), which are important flavour precursors. In particular, ATP degradation results in the generation of taste-related compounds, such as, adenosine 5'-monophosphate (AMP) and

inosine 5'-monophosphate (IMP); then, IMP can be metabolized to hypoxanthine (Hyp). These compounds are generated as a result of the *post-mortem* energy metabolism in the muscle (G. Bischof et al., 2022), and changes in their concentration may contribute to the enrichment of dry-aged flavour (Dashdorj et al., 2015; Dashdorj et al., 2016). These results may suggest a pivotal role of the energy metabolism pathways, in particular early *post-mortem* when the muscle cell metabolism is still active, on the levels of these flavour-related compounds across both internal and external location in the final dry-aged product. This study showed differences in the abundances of two glycolytic proteins, ALDOA and PGK1, and two large 70 kDa HSPs (HSPA1A and HSPA8), between the two sampling locations (higher in the external). These inducible HSPs may protect muscle cell proteins from *post-mortem* cellular stress, intervene in cell signalling pathways, and help maintaining the mitochondrial membrane potential and ATP levels in *post-mortem* muscle (Gagaoua, Terlouw, et al., 2021). We speculate that HSPs, through their anti-apoptotic role, may also contribute in determining the final levels of the taste-related compounds AMP, IMP, and Hyp in the final dry-aged product. Interestingly, Zhu et al. (2023) reported significant correlations between the abundancies of some energy metabolism proteins (e.g. STBD1 and PHKA1) and HSPs (e.g. HSPB6 and HSPA5) with the sensory flavour trait in wet-aged beef after 14 days *post-mortem*. It is clear that research combining metabolomics and proteomics approaches are needed to clarify the role of the energy metabolism pathways on the final levels of ATP degradation products, and to reveal the correlation of these flavour-related metabolites with the generation of the dry-aged flavour.

To the best of our knowledge, this is the first study to characterize such biologically significant differences in muscle proteome between external and internal locations, either prior or during dry-aging of beef. Taking a holistic view, the hierarchical heatmap demonstrated a different pattern for each dry-aging time when external and internal locations were compared (Figure 3A). These results re-emphasize the dynamic changes occurring in the proteome of dry-aged beef, and also evidence how important is to consider the location of sampling, especially when investigating the proteome of meat for identification of putative biomarkers. This is particularly relevant for dry-aging, where meat is exposed to the external atmosphere throughout the aging period.

## ***4.2 Effect of aging time on the muscle proteome of dry-aged beef***

### ***4.2.1 Significant role of muscle structure in the tenderization of beef during dry-aging***

The integrity of muscle structure and related biological pathways have been earlier reported to play a key role during the progression of meat tenderization (Gagaoua, Terlouw, et al., 2021). Indeed, breakdown of key myofibrillar and cytoskeletal proteins due to the action of endogenous proteolytic systems are central to meat tenderization (Ertbjerg, 2022). The comparisons of day 0 to day 28 of dry-aged beef revealed, as expected, sarcomere organization and muscle development to be significantly enriched terms (intact at day 0 and degraded at day 28). Likewise, the comparison of day 21 and day 28 revealed the enrichment of muscle contraction terms (specific at day 21 and degraded at day 28). In depth investigation of these results, evidenced that both TNNT3 and TTN were significantly more abundant at day 0, and their abundance decreased after 28 days of dry-aging possibly due to proteolytic activity. The troponin complex, including TNNT3 subunit, interacts with other thin filament proteins in the I-band and is involved in the regulation of actin-myosin interactions in the sarcomere, thus, its degradation *post-mortem* has been strongly implicated in development of meat tenderness (Huff Lonergan et al., 2010). TNNT3 proteolytic breakdown products appearing over 14 days of *post-mortem* aging (wet-aging) in *Longissimus thoracis* muscles was identified, among another 12 proteins, to compose the 30 kDa proteolytic fragment band (Gagaoua, Toy, et al., 2021) which has been extensively used to monitor the extent of proteolysis during aging (Koohmaraie et al., 1984; Macbride & Parrish JR., 1977). Furthermore, TNNT3 was identified as an indicator beef tenderization (Marino et al., 2015), and a good biomarker of beef tenderness (Gagaoua, Terlouw, et al., 2021). TTN, involved in maintaining sarcomere alignment during contraction, is degraded in *post-mortem* muscle and contributes to the weakening of sarcomere structure, and ultimately, tenderization of meat (Huff Lonergan et al., 2010). TTN degradation products have been reported in the literature (Fritz & Greaser, 1991; Fritz et al., 1993; Huff-Lonergan et al., 1995; Rowe et al., 2004; Wu et al., 2014). TTN has not yet been identified as a differential abundant protein using proteomic approaches for beef tenderness, but it has been shown to be involved in beef colour (Gagaoua et al., 2018; Hughes et al., 2019). Further investigations are needed to validate these proteins as biomarkers of dry-aged beef.

In earlier studies, Z-disk destabilization by the degradation of proteins close to this region was proposed as one of the major events contributing to tenderization of meat (Hopkins & Thompson, 2002; Taylor et al., 1995). LDB3 is located in the sarcomere and plays a key role in maintaining Z line integrity and muscle structure (Zhou et al., 2001), thus, the degradation of this protein may play a role in the tenderization of meat during aging. Indeed, LDB3 has

been previously shown to undergo significant degradation with increasing aging period (wet aging up to 14 days) as a result of protease activity in bovine *Longissimus lumborum* and *Psoas major* muscles (Song et al., 2022). This observation is in line with the results of the current study, wherein LDB3 is more abundant at day 0 when compared to 21 and 28 days of dry-aging. Thus, we speculate that a decrease in the abundance of LDB3 during dry-aging may be related to the breakdown of muscle skeletal structure due to endogenous proteolytic activity. MYOZ1 is also located in the Z-disk (Faulkner et al., 2001), and its proteolysis may be likely involved in the Z-disk destabilization, and therefore, tenderization during dry-aging. Furthermore, MYOZ1 and MYOZ3 were less abundant in the most tender loins and revealed as promising candidate biomarkers for meat tenderness prediction in heifers (Boudon et al., 2020), and MYOZ3 was confirmed as a robust biomarker of tenderness in bulls (Zhu et al., 2021). Tenderization of meat is also influenced by the disruption of the myofibrils in the I-band region in the *post-mortem* bovine muscle (Taylor et al., 1995). ANKRD2 is found in the I band (Pallavicini et al., 2001), and the results showed that this protein decreased with dry-aging time. ANKRD2 was proposed to be involved in *rigor mortis* of beef (Ding et al., 2022), being part of the muscle stress response pathway which stabilizes the myofibrillar structure under stressing cell conditions (Laville et al., 2009). In addition, this protein was proposed as a biomarker for beef tenderness by Picard and Gagaoua (2020), and confirmed in a later study (Boudon et al., 2020). In our study, TTN3, TTN, LDB3, MYOZ1 and ANKRD2 were detected at each time point and decreased with aging time, thus, these proteins may be useful to monitor tenderization or aging progression in the context of beef dry-aging.

#### 4.2.2 The role of energy metabolism during dry-aging of beef

Earlier studies in meat proteomics have revealed the importance of energy metabolism pathways during early *post-mortem* (Zhai et al., 2020), and aging (López-Pedrouso et al., 2021; Sierra et al., 2021), and their role in beef tenderness determination (Gagaoua, Terlouw, et al., 2021; Peter P. Purslow et al., 2021). In particular, energy metabolism pathways are strictly linked with *post-mortem* aging (Ouali et al., 2013; Peter P. Purslow et al., 2021). The expression of enzymes related to glycolytic pathways, and to some extent those associated to tricarboxylic acid cycle and oxidative phosphorylation pathways, increase early *post-mortem* to respond to the energy demand of the muscle under hypoxic conditions (Gagaoua, Terlouw, et al., 2021; Jia et al., 2007; Lamri, della Malva, Djenane, Albenzio, et al., 2023; Lamri, della Malva, Djenane, López-Pedrouso, et al., 2023; Ouali et al., 2013), however, the activity and abundances of these proteins decrease with long *post-mortem* aging period (Polati et al.,

2012). The current work showed that pathways related to hexose biosynthetic processes were enriched at both 0 and 21 days when compared to 28 days of dry-aging, and that the relative abundances of PGK1 and AKR1B1 proteins from the energy metabolism and ATP metabolic processes, decreased with aging. In an earlier study, PGK1 (glycolytic enzyme) was reported to significantly decrease after 14 days of wet-aging in bovine *longissimus* muscle (Silva et al., 2019). However, it was found to be negatively correlated with Warner-Bratzler shear force in bovine muscle (Gagaoua et al., 2020). AKR1B1, however, was only identified for the first time by Picard and Gagaoua (2020) in their meta-proteomics study, who proposed that it may act as a biomarker of beef tenderness. Thus, the results of this work suggest that energy metabolism pathways may be related to the tenderization of beef during dry-aging. Nevertheless, studies combining meat quality, metabolomics and proteomics in dry-aging are warranted for unravelling the biological pathways determining the quality and sensory traits, and in particular, the contribution of the energy metabolism pathway to the signature of dry-aged beef flavour.

#### 4.2.3 Other pathways: regulation of calcium import, neutrophil activation, and regeneration

In muscle, the sarcoplasmic reticulum (SR) releases calcium ions *via* calcium channels for regulating myofibrillar filament sliding. Calcium binds the troponin-tropomyosin complex after activation of the muscle (ATP-dependent), allowing myosin head to bind to actin for muscle contraction (after ATP hydrolysis into ADP and Pi) (P. P. Purslow et al., 2021). Relaxation of muscle occurs when calcium is pumped back into the SR, and troponin-tropomyosin complex blocks myosin from binding to actin (P. P. Purslow et al., 2021). This process occurs over age as while the ATP levels are maintained in the muscle. In early *post-mortem* muscle, when ATP consumption exceeds its synthesis, failure of ATP-dependent calcium pumps occurs and calcium ions accumulate into the sarcoplasm. Once ATP levels are depleted, the interaction formed between myosin and actin cannot be released, hence establishing *rigor mortis* (Huang et al., 2014). The results in this study show that biological processes related to positive regulation of calcium ion were most enriched after 28 days of dry-aging with increased in the abundance of related proteins, JSRP1 and ANXA3, possibly due to the *post-mortem* degradation of the cellular structures (e.g. membranes) where these proteins are normally located (Laville et al., 2009). JSRP1 is an integral protein of the skeletal muscle sarcoplasmic reticulum, co-localizes with its  $\text{Ca}^{2+}$  release channel (the ryanodine receptor), and interacts with calsequestrin and dihydropyridine receptor Cav1 in the skeletal muscle (Anderson et al., 2006), being involved in muscle contraction. ANXA3, a

calcium-dependent membrane phospholipid-binding protein, is involved in multiple cellular activities such as: vesicular transport, exocytosis and endocytosis; interaction with cytoskeletal proteins; formation of calcium ion channels; apoptosis, inflammation and cellular signalling (Ouali et al., 2013; Yang et al., 2021).

Other pathways were also enriched after 28 days of dry-aging, such as, neutrophil activation and regeneration, and proteins related to these biological processes were identified. In live cell muscles, CATHL1 acts as an antimicrobial peptide when released from the secretory granules of neutrophils and macrophages upon leukocyte activation (Kościuczuk et al., 2012). In living organisms, CPB2 regulates blood clot stability and limits fibrinolysis (blood clots breaking down process) (Tawara et al., 2016); and TNC, an extracellular matrix glycoprotein synthesized during damage repair and inflammatory processes, is essential for angiogenesis during wound healing (Wang et al., 2022).

The hierarchical heatmap demonstrated a different pattern between each aging period, and showed no shared GO terms among them (Figure 5A). Overall, changes in the relative abundance of proteins as aging time increases may result from various events. First, better extractability as aging progresses (della Motta et al., 2022), since the degradation of cellular compartments (e.g. membranes and myofibrillar networks) can release these proteins from the cell structures where they are normally contained (Laville et al., 2009). Second, degradation of these proteins by *post-mortem* proteolysis (Laville et al., 2009), resulting in the accumulation of smaller protein fragments. Third, either the solubilisation of proteins from the myofibrillar fraction or the aggregation of proteins from the sarcoplasmic fraction (Marino et al., 2014; Marino et al., 2013), may influence their relative abundance during aging. Due to these events the relative abundance of proteins is dynamic during dry-aging, as observed in this study. This continuous evolution in the proteome is also reflected on the various enriched biological processes at each aging time. Proteins involved in muscle contraction and structure, as well as, energy metabolism pathways were the main impacted during dry-aging. However, other proteins related to biological processes such as regulation of calcium import, neutrophil activation, and regeneration were also impacted.

#### 4. Conclusion

This study has evidenced, for the first time, the differential changes occurring at the proteome level between the external and internal sampling locations of beef striploins during dry-aging. Before dry-aging, the differential abundant proteins in the external location appear

to be related to biological pathways associated with response to external stimuli, which may be explained by the oxidative conditions and microbial activity to which muscle surfaces are exposed during the dressing of the carcass, the chilling storage, or later on, during the quartering and cutting. Furthermore, oxidative conditions, dehydration and microbial activity during dry-aging may have also contributed to the differences between sampling locations as aging progressed. These outcomes highlight the relevance of sampling location when investigating the proteome of meat for the identification of beef biomarkers for monitoring the tenderization or aging progression – in particular, for products like dry-aged beef..

In addition, this study has characterized the proteome of dry-aged beef for the first time, and demonstrates that dynamics and unique changes occur at each stage of the dry-aging period. Muscle structure and metabolism related pathways seem to be the major biological processes impacted during dry-aging, but interestingly, other pathways such as regulation of calcium import, neutrophil activation, and regeneration were also impacted. The current work also evidences the need for research combining metabolomics and proteomics tools to better our understanding on the role of energy metabolism pathways on the final levels of ATP degradation products, and how/if these derived flavour-related metabolites are major contributors to the dry-aged flavour. Characterizing the proteome of dry-aged beef allow us to build up our knowledge and understanding on the molecular mechanisms underlying the development of the unique dry-aged products. Furthermore, this research may establish a future basis for proposing putative biomarkers to predict and tailor the unique dry-aged quality traits, as well as, for dry-aged beef authentication. Several proteins were identified as putative biomarkers for future validation that have potential as good predictors of dry-aged beef tenderization: TIMM4, CRM4, EEF1A1, LDB3, CILP2, TNNT3, GAPDH, SERPINI1, and OMD.

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### **Declaration of Competing Interest**

No potential conflict of interest was reported by the authors.

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**Declaration of Competing Interest**

No potential conflict of interest was reported by the authors.

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**CRedit authorship contribution statement**

**Sara Álvarez:** Conceptualization, Methodology / Study design, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization.

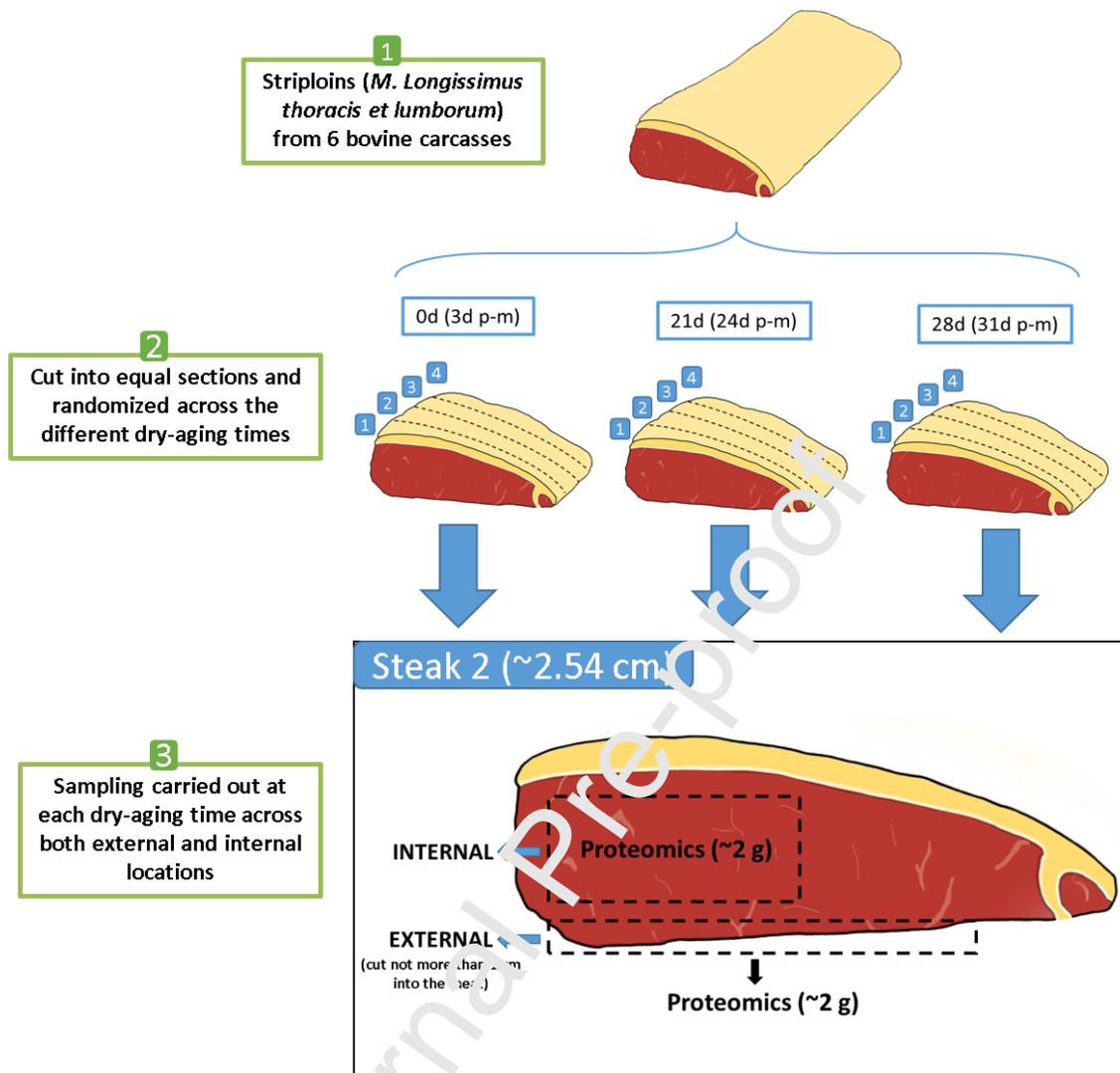
**Anne Maria Mullen:** Conceptualization, Methodology / Study design, Resources, Writing – review and editing, Supervision, Project administration, Funding acquisition.

**Carlos Álvarez:** Methodology / Study design, Resources, Writing – review and editing, Supervision, Funding acquisition.

**Ruth M. Hamill:** Writing – review and editing, Supervision.

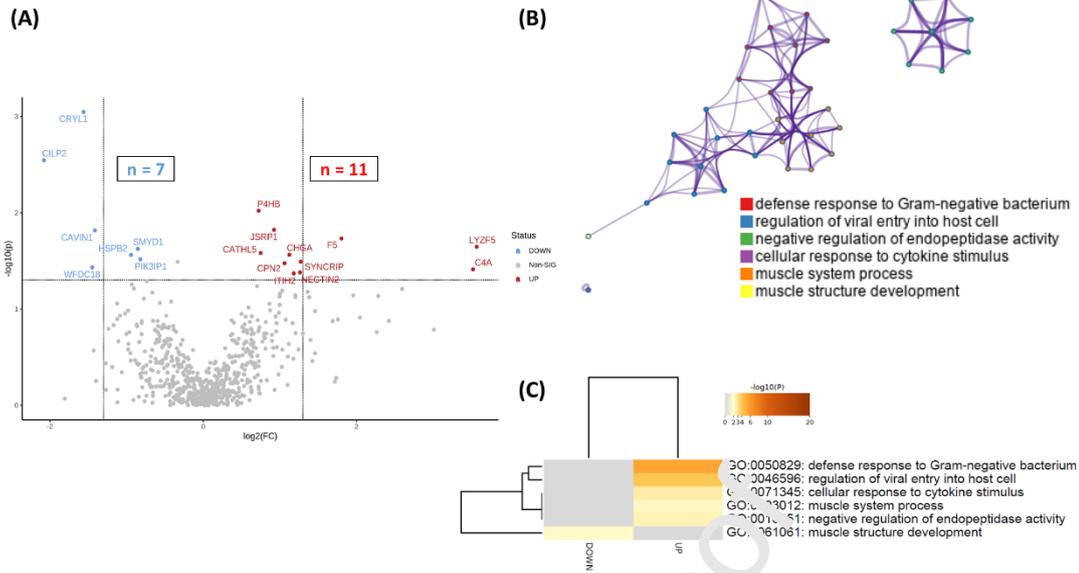
**Eileen O'Neill:** Resources, Writing – review and editing, Supervision, Project administration, Funding acquisition.

**Mohammed Gagaoua:** Conceptualization, Methodology / Study design, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review and editing, Visualization, Supervision.

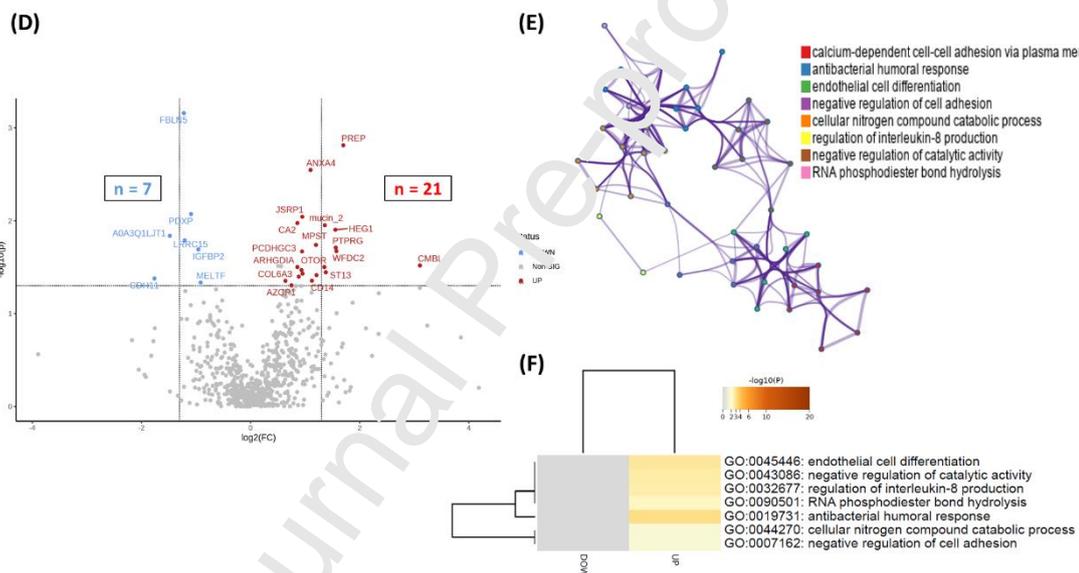


**Figure 1.** Representative diagram describing the sampling plan. Samples for proteomic analysis were collected from “steak 2”, from both external (i.e. lean surface prior to dry-aging, and crust as aging progressed) and internal locations at 0, 21, 28 days of dry-aging (i.e. 3, 24, and 31 days p-m).

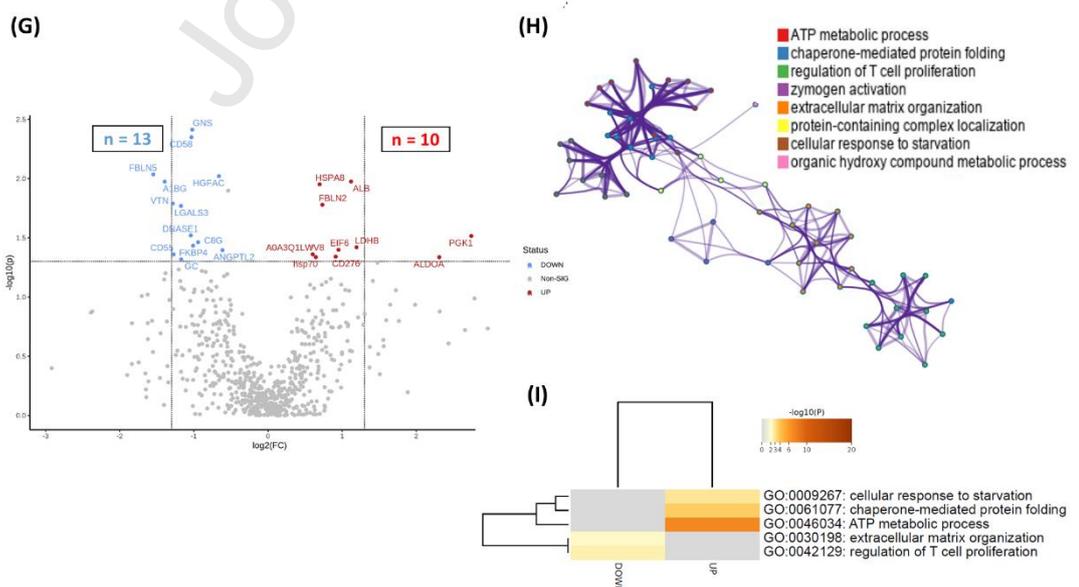
External vs Internal (Day 0)



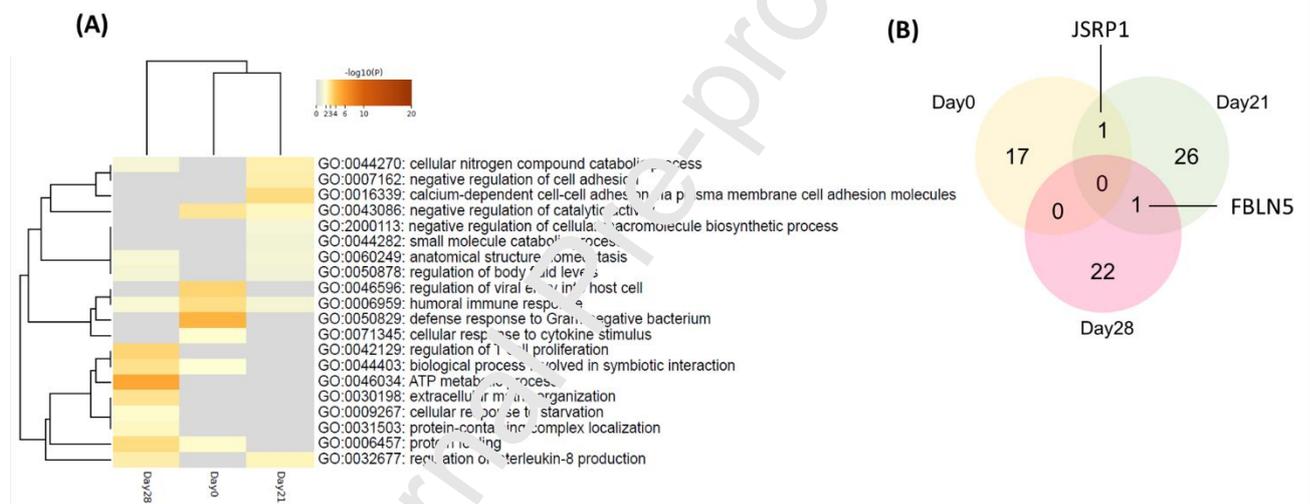
External vs Internal (Day 21)



External vs Internal (Day 28)



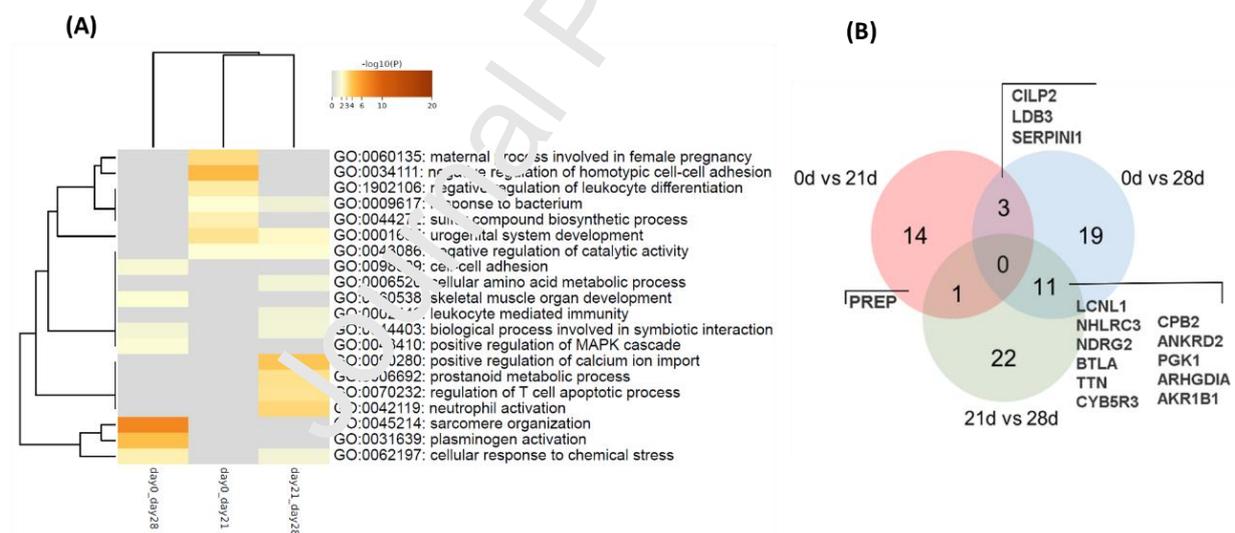
**Figure 2.** Differentially abundant proteins (DAPs) and, biological pathways and process enrichment analysis of the DAPs as affected by location (external, internal) at 0, 21, and 28 days of dry-aging (i.e. 3, 24, and 31 days p-m). **(A, D, G)** Volcano plots showing the DAPs for each comparison. For each dry-aging time, the points with red colour represent proteins that are more abundant in the external location; whereas the points with blue colour are proteins which are more abundant in the internal location. **(B, E, H)** Cluster process enrichment analysis based on the DAPs, where nodes that share the same cluster are close to each other and share a unique colour and ID (both used to differentiate each cluster). The cluster is named after the node showing the most enriched Gene Ontology (GO) term, however, each node displays a different GO term. **(C, F, I)** Heatmaps based on the significantly enriched GO terms using the DAPs lists from each comparison. GO terms that are “UP” at external are “DOWN” at internal location, and viceversa.



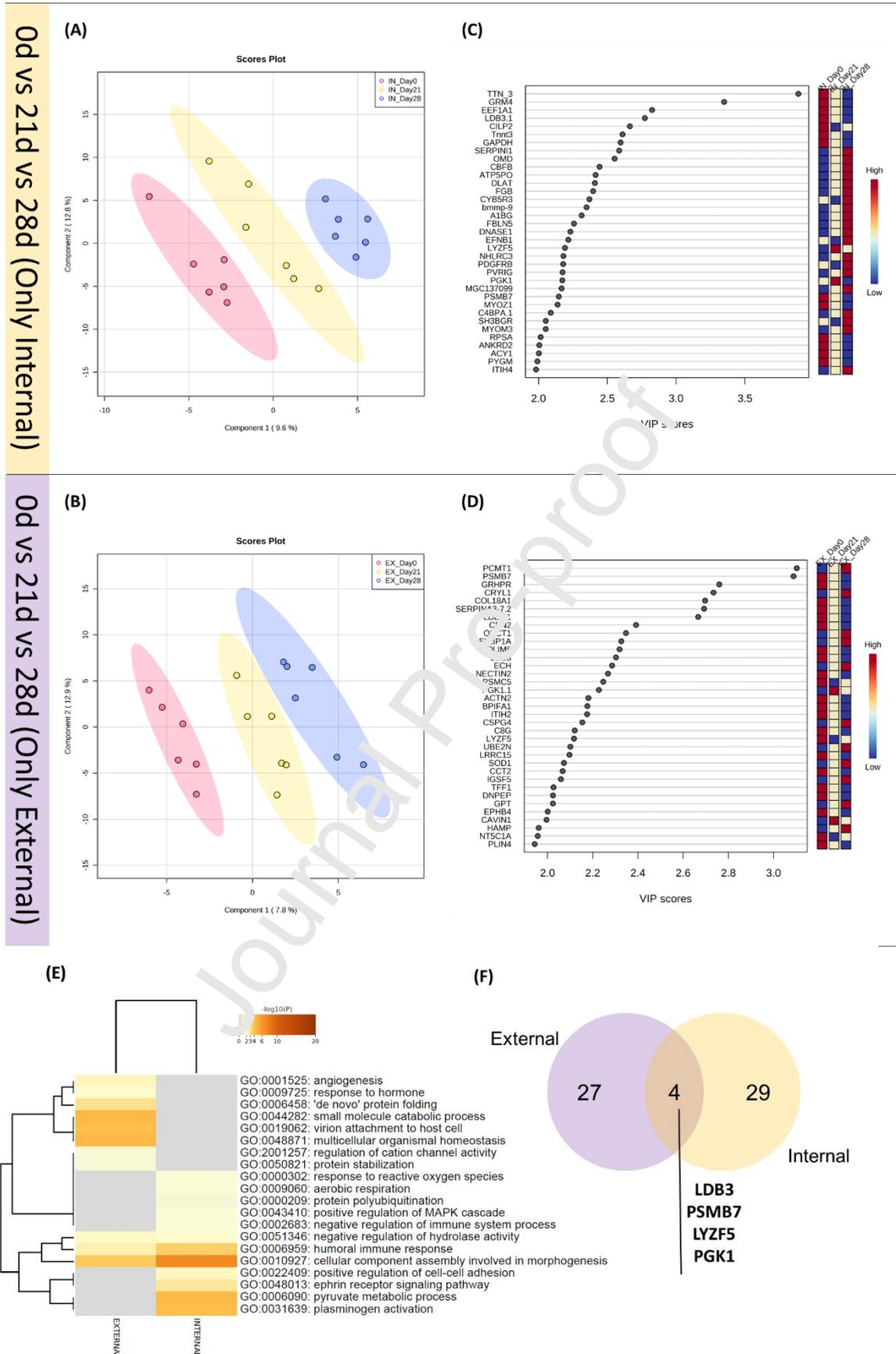
**Figure 3.** Bioinformatics analysis using the protein lists obtained when investigating the differential abundant proteins (DAPs) between external and internal location at 0, 21, and 28 days of dry-aging (i.e. 3, 24, and 31 days p-m). **(A)** Heatmap based on significantly enriched Gene Ontology (GO) terms using the three DAPs lists **(B)** Venn Diagram showing the common proteins between these DAPs lists.



**Figure 4.** Differentially abundant proteins (DAPs) and, biological pathways and process enrichment analysis of the DAPs as affected by aging in the internal location of dry-aged beef. **(A, C, F)** Volcano plots showing the DAPs for each comparison. From the top figure and moving down, the points with red colour represent proteins that are more abundant: **(A)** at day 0 (down at 21 days); **(C)** at day 0 (down at 28 days); and **(F)** at day 21 (down at 28 days), respectively. For the blue points, the opposite is true. **(B, D, G)** Cluster process enrichment analysis based on DAPs, where nodes that share the same cluster are close to each other and share a common colour. A unique colour and ID are used to differentiate each cluster. **(E, H)** Heatmaps based on significantly enriched Gene Ontology (GO) terms using the DAPs lists from each comparison. GO terms that are “UP” at day 0 are “DOWN” at day 28, and viceversa. Likewise, GO terms that are “UP” at day 21 are “DOWN” at day 28, and vice versa.



**Figure 5.** Bioinformatics analysis using the protein lists obtained when investigating the differential abundant proteins (DAPs) as affected by aging time in the internal location during dry-aging. **A)** Heatmap based on significantly enriched Gene Ontology (GO) terms using the three DAPs lists. **B)** Venn Diagram showing the common proteins between these DAPs lists.



**Figure 6.** Partial least squares-discriminant analysis (PLS-DA) showing that three different groups are discriminated based on the dry-aging times (0, 21, and 28 days) at both locations internal and

external. **(A,B)** Score Plots obtained by PLS-DA analysis. **(C,D)** Variable Importance in the Projection (VIP) obtained from PLS-DA analysis. Displayed are the 35 proteins with the highest VIP scores. Those proteins with  $VIP \geq 2$  were selected as good predictors for efficiently separating the three aging times.  $VIP \geq 2.5$  is fixed as a robust criteria for the validation of biomarkers. **(E)** Bioinformatics heatmap based on significantly enriched Gene Ontology (GO) terms using the protein lists with  $VIP \geq 2$  obtained by PLS-DA. **(F)** Venn Diagram showing the common proteins between the two lists of putative biomarkers.

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