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Abstract

Beef sensory quality comprises a suite of traits, each of which manifests its ultimate phenotype through interaction of muscle physiology with environment, both in vivo and post-mortem. Understanding variability in meat quality remains a persistent challenge, but omics studies to uncover biological connections between natural variability in proteome and phenotype could provide validation for exploratory studies and offer new insights. Multivariate analysis of proteome and meat quality data from *Longissimus thoracis et lumborum* muscle samples taken early post-mortem from 34 Limousin-sired bulls was conducted. Using for the first-time label-free shotgun proteomics combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS), 85 proteins were found to be related with tenderness, chewiness, stringiness and flavour sensory traits. The putative biomarkers were classified in five interconnected biological pathways; i) muscle contraction, ii) energy metabolism, iii) heat shock proteins, iv) oxidative stress, v) regulation of cellular processes and binding. Among the proteins, PHKA1 and STBD1 correlated with all four traits, as did the GO biological process 'generation of precursor metabolites and energy'. Optimal regression models explained a high level (58 – 71%) of phenotypic variability with proteomic data for each quality trait. The results of this study propose several regression equations and biomarkers to explain the variability of multiple beef eating quality traits. Thanks to annotation and network analyses, they further suggest protein interactions and mechanisms underpinning the physiological processes regulating these key quality traits.

Keywords: Regression models; Interactome; Beef eating quality; Biomarkers; Shotgun proteomics.

Significance

The proteomic profiles of animals with divergent quality profiles have been compared in numerous studies; however, a wide range of phenotypic variation is required to better understand the mechanisms underpinning the complex biological pathways correlated with beef quality and protein interactions. We used multivariate regression analyses and bioinformatics to analyse shotgun proteomics data to decipher the molecular signatures involved in beef texture and flavour variations with a focus on multiple quality traits. We developed multiple regression equations to explain beef texture and flavour. Additionally, potential candidate biomarkers correlated with multiple beef quality traits are suggested, which could have utility as indicators of beef overall sensory quality. This study explained the biological process responsible for determining key quality traits such as tenderness, chewiness, stringiness, and flavour in beef, which will provide support for future beef proteomics studies.

1. Introduction

Beef palatability encompasses a suite of complementary sensory experiences including colour, texture, juiciness, flavour and mouthfeel. While texture has long been considered the key driver of meat sensory quality [1], as this attribute has improved perhaps due to improved post-mortem management of beef in recent years, flavour attributes are coming more into focus as another driving factor that influences consumer appreciation of beef [1-4]. Both texture and flavour are now considered to be of primary importance in ensuring optimal and consistent product appeal [4]. Texture traits include initial bite tenderness, which can be defined as the ease of breaking down the fibres of meat when it is first bitten or cut [5], while chewiness and stringiness describe, respectively; the energy required to chew the beef before swallowing, and the extent of the sensation of strings in the mouth during later stages of chewing [6]. Texture is influenced by several intrinsic and extrinsic factors such as species, genotype, nutrition, age, slaughter procedure and post mortem treatment, storage conditions and ageing time [4,7]. Among these, the conversion of muscle to meat is the essential step for the beef texture development, which is triggered by apoptosis and mediated by a particular group of cysteine peptidases called caspases [8]. Flavour defined from a sensory perspective is the intensity of beef flavour perception from the inner core of the cooked meat [9,10] and is influenced by the amount of fat in the meat and the fatty acid profile, the profile of small soluble compounds that are released as volatile compounds during thermal processing and the metabolite profile of the muscle [11]. In a 12-member trained sensory panel assessment of more than 100 beef striploin samples [12], tenderness was found to be positively correlated with flavour, and negatively with chewiness and stringiness. Unravelling the complexities of the biological factors influencing these important traits [13], could unlock the ability of processors to ensure the delivery of a consistent high-quality product that is desired by consumers.

To better understand the underlying mechanisms, foodomics technologies such as transcriptomics, proteomics, metabolomics and lipidomics have been used to reveal the physiological pathways and biological processes that are influential on product quality in a range of animal products and species [14,15]. Beef sensory quality at point of consumption is influenced by the interaction between muscle cell physiology and the environment, both *in vivo* and at slaughter as well as during the post-mortem phase of meat maturation [16]. Omics technologies have also progressed our understanding of post-mortem muscle metabolism and how it relates to meat quality development [17–23]. Of the omics technologies, proteomics

has played a significant role during the discovery, evaluation and validation of biomarkers [24]. Many studies have sought to identify specific proteins present in muscle at slaughter that are characteristic of the biochemical status of muscle and whose abundance has potential to predict or explain ultimate meat quality at point of consumption [19,22,25]. Studies have identified markers of beef tenderness, colour, intramuscular fat and post-mortem pH decline [26–32]. Despite the fact that most of these studies examined animals with divergent or extreme phenotypes, the nature of the relationship between proteins and eating quality traits should not be overlooked. Consideration of this variability is required for a fuller understanding of the mechanisms by which biological variability relates to phenotypic outcome, and in this case, to establish the nature of the relationship between proteomic biomarkers abundance and meat quality traits of importance to the consumer.

Furthermore, in contrast to transcriptomics work [33], previous studies frequently focused on identifying individual proteins associated with meat quality. While this is important in order to identify biomarkers, the functions, processes and pathways influencing quality and specifically the interactions among biomarkers beyond their individual association with a given trait has not received adequate attention [17,23]. This is borne out by occasional contradictory findings on the direction of association of a given protein with a trait. For example, ACTA1 and MYH1 were identified as both positively or negatively correlated with beef tenderness in different population samples [19]. Functional proteomics attempts to elucidate the biological function of identified proteins and molecular cellular mechanisms that are shared between groups of proteins [34]. Compared to individual biomarkers, whose relationship with a trait may vary depending on the biological context, by identifying the underlying functions and pathways that increase or decrease the expression of a given phenotype we can exploit redundancies and can demonstrate more consistent and robust associations with a given trait [33]. Considering the above drawbacks, this study aims to identify molecular mechanisms contributing to the development of multiple and important sensory traits and examine the relationships between the protein abundances and sensory eating quality traits of beef from *Longissimus thoracis et lumborum* (LTL) muscle samples from Limousin-sired young bulls to shortlist robust biomarkers with relevance for predicting ultimate meat-eating quality. By means of shotgun proteomics and a multivariate regression analysis this work further proposed underlying mechanisms behind the development of tenderness, chewiness, stringiness and flavour.

2. Materials and methods

2.1. Animals handling and muscle sampling

Under standardised production conditions, 34 cross breed Limousin bull progeny of elite Irish beef artificial insemination bulls were finished at the Irish Cattle Breeders Federation Tully Progeny Test Centre (Tully, Kildare, Ireland). Information on how animals were reared and slaughtered as well as the sampling protocol have been previously described in Zhu et al. [12]. Specifically, all animals were finished to U- to E+ conformation score, fat score of 3- to 5 and at an average age of 487 days (±24 days) and live weight of 678 kg (±58 kg). The *Longissimus thoracis et lumborum* (LTL) muscle samples from the 10th rib of each carcass were collected at 1 h post-mortem and finely macerated in 5 mL RNAlater® for proteomics analyses [25]. At 48 h post-mortem, the loins were boned out and steaks were taken from the right-side LTL (2.54 cm for thickness), vacuum packaged and aged for 12 further days at 4 °C and then frozen at -20 °C until sensory analysis.

2.2. Sensory evaluation

Compusense 5.6 software was adopted to gather the scores from a 12-member trained sensory panel based on an amended version of AMSA (2015) guidelines [12]. Specifically, the steaks were allowed to come to room temperature for 60 minutes without any packaging before being cooked on a Velox CG-3 grill at 210 °C until reaching an internal temperature of 71°C. Then, steaks were rested in foil for 4 min, cut into approximately 2.5 cm × 4 cm pieces, wrapped individually in foil, and served. Members of a 12-member trained sensory panel conducted assessments on each sample. All panellists would cleanse their palates with water and plain crackers between each sample. Each panellist was instructed to bisect the steak piece along the cooked surface and assess the sample according to the same methodology (replicated) using the provided assessment sheet [35]. The 34 steak samples were evaluated and assigned average scores from 5- 7 trained assessors, based on the sensory traits upon which the panel had been trained, specifically focusing on tenderness, chewiness, stringiness, and flavour, utilizing a standardized scale of 0–100, where higher score equates to more tender, more intense beef flavour, more chewy, more stringy [36]. Summary data on 34 sample sensory evaluations are shown in **Table 1**.

2.3. Muscle protein extraction and quantification

Frozen muscle tissue samples (80 mg) were homogenised in 2 mL of 8.3 M urea, 2 M thiourea, 1% dithiothreitol, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 2% immobilised pH gradient (IPG) buffer pH 3–10 using a T 25 digital

ULTRA-TURRAX® following the protocol of Bouley et al. [37]. The protein homogenates were incubated for 30 min at 4 ◦C and then by a 30 min centrifugation at 10,000× g. The protein concentration was determined by the dye binding protocol described by Bradford [38]. The stand curve was prepared by using bovine serum albumin (BSA) and the absorbance of the sample was measuring at 595 nm using a spectrophotometer [38].

2.4. Shotgun proteomics and LC-MS/MS data processing

12 % polyacrylamide Mini-PROTEAN® TGX™ precast gels (8.6 × 6.7 × 0.1 cm, Bio-Rad Laboratories, Deeside, UK) were used to concentrate the protein extracts in stacking gel. The shotgun proteomics was performed according to the protocol of Zhu et al. [12], the details about the process of protein bands, as well as the operating parameters of LC-MS/MS has also been given in that paper. The raw data from the LC-MS/MS were then aligned against the *Bos taurus* database (i.e.ref_bos_taurus, 23970 sequences) with Mascot V.2.5.1 (http://www.matrixscience.com). The precursor and fragment mass tolerance were set up at 10 ppm and 0.02 Da, respectively. Carbamidomethylation (C), oxidation (M) and deamidation (NQ) were included in the variable modifications. Protein identification can be verified when at least two peptides derived from one protein showed statistically significant identity.

2.5. Statistical analyses

Statistical analyses were carried out with XLSTAT 2018.2 (AddinSoft, Paris, France). Data were examined for entry errors, missing data and outliers, and the keratin proteins were excluded from the raw database to avoid interference. Log2 transformation and mean normalization was performed on individual protein abundance.

Pearson correlations were computed between the individual tenderness, chewiness, stringiness and flavour values and the quantified protein abundances. Correlation analysis values were considered significant at $P < 0.05$. The proteins were further categorised manually into different biological pathways based on their annotation provided by UniProtKB (http://www.uniprot.org/). To get the best performance of the regression model built using the protein abundances for each quality traits, the limitation of four proteins, to respect the parsimony principle, was set up for the settings in each regression equation for each quality trait [39]. The absence of collinearity was systematically tested, specifically, the variable was identified as collinear if it possessed a high condition index > 10 [39].

2.6. Bioinformatics analyses

Proteins that significantly correlated with those four eating quality traits had been selected by overlap analysis and then by different biological pathways based on the information provided by UniProtKB (https://www.uniprot.org/). ProteQTL tool included in ProteINSIDE (http://www.proteinside.org/) was performed on the above proteins to identify their potential role as quantitative trait loci (QTL) of beef qualities. A digital library of published Animal QTL Database (https://www.animalgenome.org/QTLdb) was included in the ProteQTL engine which includes cattle QTL and association data curated from published scientific papers. Gene Ontology analysis was performed to investigate GO terms for potential functions and molecular mechanisms by using two web-based tools, ProteINSIDE (http://www.proteinside.org/) and Metascape® (https://metascape.org/) respectively. In the former tool, the top 30 significant GO enrichment terms (p-value, Benjamini–Hochberg < 0.05) for tenderness and flavour were considered and covered Biological Process (BP) and Molecular Function (MF) categories. Enrichment analyses for pathway and process were implemented on those proteins in Metascape® following previous procedures [40,41]. Statistically significant enriched ontology terms were identified based on the hypergeometric test and Benjamini–Hochberg P-value correction algorithm.

3. Results

3.1 Pearson correlation analysis and related biological pathway to the beef sensory traits

After removal of keratin and uncharacterized proteins, a total of 1281 proteins, were quantified in the 34 bovine muscle samples, under an FDR of 1% and with a minimum of 2 confirmed peptides. Among them, **Figure 1a,b** showed that thirty-eight, nine, ten, and fifty-nine proteins were significantly correlated with tenderness, stringiness, chewiness and flavour respectively, while a subset was correlated with multiple quality traits. As shown in **Table 2**, a total of 85 unique proteins were assigned to 5 main biological pathways which were muscle contraction and structure proteins (n=22); energy metabolism (n=24); heat shock proteins (n=5); oxidative stress proteins (n=3) and (v) regulation of cellular processes, binding, apoptosis and transport (n=31). Furthermore, the proteins that were correlated with at least 3 of the 4 sensory traits can be also observed in **Table 2**. The energy metabolism, muscle contraction and binding & apoptosis pathways were the most dominant pathways among the five pathways.

Seven proteins; CORO6 (Coronin 6), PHKB (Phosphorylase b kinase regulatory subunit), HSPB6 (Heat shock protein beta-6), CACNA1S (Voltage-dependent L-type calcium channel

subunit alpha), LGALS1 (Galectin-1), SYPL2 (Synaptophysin-like 2), TTN (Titin); had significant correlations with three quality traits (**Table 2 and Figure 1b**), while two (PHKA1 and STBD1) were significantly correlated with all four quality traits.

3.2. Regression equation model to explain the four beef eating quality traits

The linear regression models performed for each quality trait are presented in **Table 3**. The models explained between 51 and 71% of the phenotypic variability in the four quality traits ($P < 0.001$), with the highest explanatory value in the tenderness model (71%) and lowest in the chewiness model (51%). Specifically, with the inclusion of four proteins: CACNA2D1 (Voltage-dependent calcium channel subunit alpha-2/delta-1), EIF5A (Eukaryotic translation initiation factor 5A-1), STBD1 (Starch-binding domain-containing protein 1), WDR1 (WD repeat-containing protein 1) 71% of the variability of tenderness, could be explained. Therein, CACNA2D1 and STBD1 were positively correlated with tenderness while EIF5A and WDR1 were negatively correlated. It should be highlighted that CACNA2D1 alone explained 43 % of the variability which is the highest of all of the proteins across the four models. As for chewiness, SOD1 (Superoxide dismutase [Cu-Zn]), PHKA1 (Phosphorylase b kinase regulatory subunit alpha), ATP5F1C (ATP synthase subunit gamma, mitochondrial) were identified to explain 51% of the variability in this trait, with the positive (SOD1, ATP5F1C) and negative (PHKA1) relationship between the proteins and chewiness. Interestingly, SOD1 was the only common protein shared between different optimised models which explained 32% of the variability in chewiness and 14% of the variability in stringiness, being higher in more chewy and stringy samples. The other three proteins retained in the model for stringiness were TPT1 (Translationally-controlled tumour protein), TPM3 (Tropomyosin alpha-3 chain), HPX (Hemopexin). Those four proteins explained 58% of the variability in stringiness with three associating in the positive direction (TPT1, SOD1, TPM3) and one negatively associated (HPX).

Regarding models for beef flavour, variability in abundance of three proteins, i.e. PHKG1 (Phosphorylase kinase catalytic subunit gamma 1), CORO6 (Coronin), ATP5F1A (ATP synthase subunit alpha) accounted for 70% of the variability in flavour. PHKG1 was positively associated with beef flavour while CORO6 and ATP5F1A were negatively correlated. It is also notable that ATP5F1C and ATP5F1A are different isoforms of the same protein, ATP synthase subunit, the former explained 6% of the variability in chewiness (positive direction) and the latter explained 16 % of the variability in flavour (negative direction).

3.3. Quantitative trait loci (QTL) analysis of beef quality

A number of proteins that were associated with meat sensory traits are encoded by genes located in known QTL for various beef quality traits. Twenty proteins correlated with beef texture traits are encoded by genes located in known bovine QTLs for texture, which can be seen in **Table 4**. These include 16 genes located in QTLs for shear force (CKMT2, MYOZ3, ANXA6, VDAC1, OGN, FBP2, MYL2, MYL3, UQCRC1, SLC25A3, MB, PGM1, RTN4, VDAC3, CMBL, MYLK2) and 4 genes located in QTLs for sensory tenderness score (PREP, PTGR2, ACTN3, NDUFV1). As for meat flavour score, one gene (*MDH2*) is located in a known QTL for flavour. Moreover, 22 significant proteins are encoded by genes located in known QTLs for other quality traits that closely related to beef texture and flavour, for example, marbling score (n=1; APOBEC2), fat thickness at the 12th rib (n=6; SLC25A3, MB, MDH2, PHKG1, ATP2A1, MYLPF), intramuscular fat (n=4; CYCS, MYLK2, AHSG, EIF4A2), lignoceric acid content (n=5; ACTN3, NDUFV1, PGM1, TPM3, HADHB), myristic acid content (n=6; EIF5A, NPEPPS, ALDOC, MYH1, CLTC, CORO6). Interestingly, some proteins are encoded by genes located in QTL for multiple quality traits, specifically, MDH2 was located in a region on Chr.25 containing two overlapping QTL, one for meat flavour score and one for fat thickness at the 12th rib, while SLC25A3 and MB on Chr.5 were located in overlapping QTL for shear force and fat thickness at the 12th rib, MYLK2 on Chr.13 is located in a region of overlapping QTLs for shear force and intramuscular fat. In addition, genes encoding ACTN3 and NDUFV1 at Chr.29 were identified for lignoceric acid content and tenderness score, PGM1 at Chr.3 was located in a region with overlapping QTL for lignoceric acid content and shear force.

3.4. Pathway and process enrichment analysis

The Gene Ontology (GO) results for tenderness and flavour are given in **Table 5** and **Table 6,** respectively. Specifically, as for tenderness, muscle contraction (GO:0006936), sarcomere organization (GO:0045214) and cardiac muscle contraction (GO:0060048) were the top three GO enriched terms identified from the list of the 38 candidate proteins biomarkers in Biological Process (BP) (**Table 5**). In addition, protein binding (GO:0005515), actin filament binding (GO:0051015) and structural constituent of muscle (GO:0008307) were the most dominant three Molecular Function (MF) terms. As for the beef flavour, oxidative phosphorylation (GO:0006119), mitochondrial ATP synthesis coupled proton transport (GO:0042776) and regulation of the force of heart contraction (GO:0002026) were identified to be the top three GO terms among the list of 59 candidate protein biomarkers (**Table 6**). And

the most important three MF terms for flavour include protein binding (GO:0005515), RNA binding (GO:0003723) and structural constituent of muscle (GO:0008307).

A hierarchical heat map comparing the protein lists and pathways across four quality traits shows clustering of the top 20 significantly enriched terms identified by Metascape® (**Figure 1c**). Further details and relevant information about those 20 enriched terms were described in **Table S1**. The Gene ontology term called generation of precursor metabolites and energy (GO: 0006091) was common to tenderness, chewiness, stringiness and flavour. In addition, calcium signalling pathway (ko04020), metabolism of carbohydrates (R-HSA-71387) and response to an inorganic substance (GO: 0010035) pathways were common to flavour, chewiness and tenderness. The glycogen catabolic process (GO: 0005980) was common to all the traits except tenderness. Interestingly, carbohydrate derivative catabolic process (GO: 1901136) and smooth muscle contraction (R-HSA-445355) were specific to tenderness; purine ribonucleoside monophosphate metabolic process (GO: 0009167) and eight more terms were specific to flavour. It is evident that there are very many more GO Terms associated with flavour compared to the texture traits. Muscle contraction (GO:0006936), skeletal muscle contraction (GO: 0003009) and myofibril assembly (GO: 0030239) were shared by flavour and tenderness.

As for the individual quality traits, the process enrichment and cluster analysis pathways resulted in the identification of nine top and significantly enriched terms for tenderness, four for chewiness, two for stringiness and sixteen for flavour (**Figure 2a-2d**). The most two dominant terms for tenderness were muscle contraction (GO: 0006936) and muscle structure development (GO: 0061061) (**Figure 2a**). As the most significant GO term, glycogen catabolic process (GO: 0005980) was common between chewiness and stringiness (**Figure 2b and 2c**). The enrichment for flavour was ranked by the order of importance (i) generation of precursor metabolites and energy (GO: 0006091), (ii) muscle contraction (GO: 0006936), (iii) purine ribonucleoside monophosphate metabolic process (GO: 0009167), and (iv) carbohydrate metabolic process (GO: 0005975) (**Figure 2d**).

To further capture the relationships between the terms, a subset of enriched terms has been selected and rendered as a network plot (**Figure 3** and **Figure 4**), which highlighted the critical role of muscle contraction, generation of precursor metabolites and energy, purine ribonucleoside monophosphate metabolic process, metabolism of carbohydrates and calcium signalling pathway in beef quality determination. Specifically, generation of precursor metabolites and energy were shared between all the attributes, while purine ribonucleoside

monophosphate metabolic process and inorganic cation transmembrane transport were specific for flavour (**Figure 4**).

4. Discussion

Numerous studies have compared proteomic profiles between groups of animals that are extreme or divergent for quality, however, deciphering the mechanisms underpinning the complex biological pathways correlated with beef quality in natural populations, along with interactions between proteins, requires analysis of a wide range of phenotypic variation. The current study applies multivariate regression analyses and bioinformatics to shotgun proteomics data and meat quality data, in a large group of samples (n=34) which allows us to i) obtain deeper insights on the Gene Ontology and biological pathways associated with variability in beef texture and flavour; ii) propose explanatory models of beef texture and flavour using multiple regression approaches; and iii) identify novel protein biomarkers correlated with beef texture quality traits (tenderness, chewiness, stringiness) and flavour, focusing in particular on those that correlated with multiple beef quality traits, which could have utility as indicators of overall beef sensory quality evaluation. This will help us to have a better understanding of the relationships between the protein biomarkers and multiple quality traits from a macro viewpoint. In addition, the evaluation of biomarkers that are correlated with multiple quality traits will allow revealing the consistencies and divergencies in the underlying mechanisms for further monitoring through rearing and production systems practices, which are known to be impacted differently due to the synergies and antagonisms that exist. The ultimate goal is to identify robust biomarkers that can be representative of several quality traits while validated in a one quality objective.

4.1 Significant enriched Gene Ontology terms correlated with beef texture and flavour

One enriched term common to all three texture traits (tenderness, chewiness and stringiness) (**Figure 1c**), "generation of precursor metabolites and energy (GO: 0006091)", was also identified as a significant GO term for beef flavour in this study (**Figure 2d** and **Figure 3**). Fresh meat contains non-volatile components such as sugars, peptides, amino acids, inorganic salts, and organic acids, which contribute to the formation of volatile flavour components during thermal treatment [42]. A number of studies have demonstrated the importance of precursors in the development of beef flavour, which is in line with our results [11,43]. In addition, the appearance of precursors such as amino acids and peptides are

correlated with meat tenderization [44], which supports the involvement of this pathway with beef texture traits in this study.

It is also noteworthy that the "calcium signalling pathway (ko04020)" and "metabolism of carbohydrates (R-HSA-71387)" were common to two texture quality traits (tenderness and chewiness) and flavour. Calcium homeostasis is essential for skeletal muscle contraction and relaxation, controls a multitude of cellular processes and furthermore affects the tenderness of beef [43] . The enzymatic speed of several important enzymes in glycolysis is regulated by calcium ions, which triggers apoptosis onset through some signalling pathways in skeletal muscle [44] . Moreover, Yang et al. [45] have reported that there is a positive relationship between elevated calpain activity with calcium ion supplementation and the generation of volatile flavour compounds in the LTL beef muscles, which is consistent with our findings. The underlying mechanism can be attributed to the important role of calcium ions in the regulation of the myofibrillar proteolysis and the degradation of the Z-line proteins, producing the accumulation of small peptides and free amino acids which contribute to the enhancement of beef flavour [48].

The GO term "metabolism of carbohydrates (R-HSA-71387)", has been identified as one of the major biological pathways correlated with beef quality traits in numerous studies [13,19,23,46]. Energy metabolism is a crucial process to maintain homeostasis and permit muscle contraction and work *in vivo*. The metabolism of carbohydrate in muscle consists of a cascade of interconnected aerobic and anaerobic pathways that drive energy generation for muscle contraction [49]. After slaughter and following depletion of the oxygen in the cellular environment, aerobic metabolism including that of carbohydrate will quickly come to a halt, while anaerobic glycolysis continues for some time post-mortem. This switch to anaerobic metabolism is reported to be correlated with the phosphorylation of glycolytic enzymes [9]. Depending on the carbohydrate stores in the muscle, the regulation of carbohydrate metabolism via glycolysis in individuals drives differential rates and extents of post-mortem pH decline [20] and a consequent diversity of muscle biochemical states at the development of *rigor mortis* i.e. the point in time when there is a cessation of the ability of the myofibrillar complex to dissociate in the absence of ATP [40,50]. This has an important influence on the potential for tenderisation with endogenous enzymes over the beef maturation period, and additionally, depending on interactions with environmental temperature, the volume of subcellular carbohydrate reserves and the extent of utilisation of those reserves in the early post-mortem period defines the contractile state, and sarcomere length, which is influential on

ultimate tenderness, independent of proteolytic activity [51]. The Maillard reaction is a complex series of reactions between reducing sugars (carbohydrates) and amino acids during heat treatment, and plays a major role in development of beef flavour [52]. Glycolysis results in the production of a substantial number of non-volatile compounds including various reducing sugars and low-molecular-weight water-soluble compounds, which are essential components in the Maillard reaction which is responsible for the formation of roasted meat flavour [11]. Specific end-products of glycolysis such as inosine 5′-monophosphate were highlighted in the present study, which are well-known as important components of flavour development in beef [53]. Furthermore, synergistic effects of certain products of muscle protein post-mortem proteolysis e.g. certain amino acids which have umami properties occur with end-products of glycolysis monophosphates (e.g. IMP) which can boost umami flavour even further through the Maillard Reaction [53].

The GO term "muscle contraction (GO: 0006936)" pathway was highly significant for both tenderness and flavour which confirms its essential role in meat quality. Myofibrillar proteins, such as actin, myosin, and tubulin, are important structural proteins in muscle tissue [46]. The degradation of the Z-disc is a major feature of meat tenderization post-mortem [54]. Three major cytoskeletal structures were summarized as primary factors that affect the meat tenderness which including the Z- to Z-disc attachments, Z- and M-line attachments and the elastic filament protein titin [32,55,56].

4.2 Significant enriched Gene Ontology terms related to the proteins correlated with flavour

Beef flavour can be described as a complex sensation involving two aspects, its odour and the aroma and taste of cooked meat [43]. Interactions between proteins, lipids and carbohydrates are influential due to their capability of developing into important flavour precursors during heating through for instance the Maillard reaction [57]. Our study appears to highlight variability in the precursors and inputs to the Maillard reaction.

In this study, the GO terms "generation of precursor metabolites and energy (GO: 0006091)" and "purine ribonucleoside monophosphate metabolic process (GO: 0009167)" were identified as two of the most significant pathways for beef flavour (**Figure 2d**). Interestingly, this finding supports previous studies that showed essential flavour precursors, such as amino acids and nucleotides, play an important role in developing beef flavour during the Maillard reaction [44,58]. The GO terms "carbohydrate metabolic process (GO: 0005975)" and "glycogen catabolic process (GO: 0005980)" are the other two important

processes pointing towards the essential role of glycogen metabolism in the formation of beef flavour. It can be inferred that variation in the contents of reducing sugar influenced by glycogen metabolism can lead to changes in the content of volatile flavour-associated compounds generated by the Maillard reaction, altering beef flavour intensity and profile [59]. It is noteworthy that "muscle contraction (GO: 0006936)", "skeletal muscle contraction (GO: 0003009)", "supermolecular fiber organization (GO: 0097435)" and "class I MHC mediated antigen processing (R-HSA-983169)" were also highlighted in this study suggesting the relationship between muscle structural proteins and beef flavour. According to the study of Komiya et al. [59], the composition of muscle fiber type is thought to affect beef palatability which supports the findings of our study. Specifically, it indicates that high levels of slow MYH1, which is considered as a biomarker of muscle fiber type [61,62], can induce strong umami taste and richness in beef flavour [63]. In addition, the composition of MYH1 has been correlated with the levels of total free amino acid which in-turn are influential in beef flavour [64].

4.3 Candidate protein biomarkers from the energy metabolism pathway

It is interesting and important to note that, in all of the potential biomarkers of this study, PHKA1 and STBD1 were the only two candidates that were correlated with all four beef quality traits, both of which are involved in the energy metabolism pathway (**Table 3** and **Figure 1b**). Furthermore, PHKA1 and STBD1 were also retained in the chewiness and tenderness regression model respectively (**Table 3**). This also coincides with the result that the GO term "generation of precursor metabolites and energy (GO: 0006091)" is identified as the most important biological pathway associated with beef quality in this study (**Figure 1c**).

PHKA1 is the regulatory subunit of phosphorylase kinase (Phk) [65]. The PHKA1 gene encodes the alpha subunit, while PHKB on chromosome 16q12–q13 encodes the beta subunit, while the gamma subunit is encoded by the PHKG1 gene on chromosome 7p12–p21, while the delta subunit is also known as calmodulin [66]. Phosphorylase kinase can stimulate the degradation of glycogen in response to various neural and hormonal signals [65]. The crucial role of glycolysis in beef quality has been highlighted in many studies [7,19,67]. In the current study, PHKA1 was significantly positively correlated with beef tenderness and flavour, and negatively with chewiness and stringiness. In addition, PHKB was also negatively correlated with chewiness, stringiness and positively with flavour, furthermore PHKG1 is also retained as a positive biomarker for flavour in the regression equation (**Table 3**), which is further identified as the QTL for fat thickness at the 12th rib on Chr.25 (**Table 4**).

All three subunits of phosphorylase kinase were correlated with beef quality traits in the same direction, specifically, positively with tenderness and flavour and negatively to stringiness and chewiness. The underlying mechanism could be explained by the fact that the Phk enzyme may enhance glycolytic activity during the post-mortem period, resulting in faster pH decline, which may have an influence on meat tenderization and final texture of the meat [19].

STBD1 is identified as being encoded by GENX-3414 which is mainly distributed in skeletal muscle, T-tubules, and sarcoplasmic reticulum [68]. It makes an important contribution to glycogen metabolism and cellular localization by binding and anchoring the glycogen to membranes [68] . In addition, STBD1 has been identified as a novel biomarker of beef quality that is positively correlated with tenderness and negatively with chewiness in our previous study [12] , which is consistent with the result in the current research.

It is noteworthy that, ATP synthase subunit alpha (ATP5F1A), ATP synthase subunit beta (ATP5F1B), ATP synthase subunit gamma (ATP5F1C) and ATP synthase subunit f (ATP5MF) were all negatively correlated with beef flavour. Moreover, ATP5F1C was also negatively correlated with tenderness and included in the regression model of beef chewiness, while ATP5F1A was retained in the regression model of flavour. These proteins are the subunits of the highly conserved mitochondrial ATP synthase complex, which is localized in the inner membrane of mitochondrial where it is responsible for the synthesis of ATP from ADP by harnessing the energy generated by the proton gradient maintained by the electron transport chain [70]. The abundance of carbohydrate and the potential for ATP generation in the muscle is central to the ultimate phenotypic expression of many meat quality traits. ATP synthase has a very large importance in cellular metabolism, and variability in the end products of ATP degradation are well-known to be linked with flavour, for example is an ATP breakdown product, inosinic acid enhance the perceived umami sensation of glutamic acid, thus improving beef flavour [71]. This study is the first to observe a correlation between ATP5F1C and ATP5MF and beef quality. The observation of all three subunits of ATP synthase in negative association with flavour lends a robustness to the association and this could have arisen as a feature of the large sample size and high degree of phenotypic variability examined in the present study, providing useful complementary evidence to the available studies in the field [58]. Interestingly, ATP5F1B has been identified to be a putative biomarker of beef tenderness, although different studies have shown associations in both positive and negative directions for ATP5F1B with tenderness [19]. Due to the central importance of ATP metabolism on many aspects of meat quality, it is not surprising that effects on one trait may

mask that on others, emphasising the value of both the large sample sizes and multivariate approach utilised in the present study.

4.4 Candidate protein biomarkers from calcium signalling & binding and transport pathways

The most explanatory protein in the regression model of beef tenderness, CACNA2D1, belongs to a member of the alpha-2/delta subunit family that can enhance the density of voltage-gated calcium channels at the plasma membrane [72]. It could be speculated that the highly influential role of CACNA2D1 in the regression model for tenderness relates to the crucial function of the calcium signalling pathway in beef tenderness development [73]. *CACNA2D1* is a candidate gene for several carcass and meat quality traits due to its location within quantitative trait loci (QTLs), for marbling score [74], meat-to-bone ratio [75] and carcass weight [76]. A related protein, the voltage dependent L-type calcium channel subunit alpha (CACNA1S) was also positively correlated with tenderness and flavour, and negatively with chewiness [77]. It has also been reported to be involved in cell death [78]. Our study is the first to report this protein as a potential biomarker of beef quality.

Synaptophysin-like 2 (SYPL2) is a member of the synaptophysin family, which is mainly expressed in adipose tissue, brain and cerebellum [79]. This protein was positively correlated with beef tenderness and flavour while negatively with chewiness. Although few studies have shown the relationship between SYPL2 and beef quality, it has been reported that the protein plays a role in cellular calcium, which is regularly implicated in tenderness development [80–82].

Galectin-1 (LGALS1), recognized as negatively correlated with tenderness and positively with chewiness and stringiness, is a β-galactoside-binding protein that plays an important role in cell proliferation and skeletal muscle differentiation by acting as regulator of apoptosis [83]. In line with our results, LGALS1 was previously described as a negative biomarker of tenderness in bovine *Longissimus thoracis* muscle [83]. However, in the *Longissimus thoracis* muscle from Angus crossbred steers, LGALS1 is reported to be positively correlated with tenderness [84]. The mechanism behind the association between LGALS1 and meat tenderness is still not clear, but may depend on the specific breed and muscle type [46].

Elongation factor EIF5A is an mRNA-binding protein that is responsible for mRNA turnover and nucleocytoplasmic transport, and is associated with ribosomes and the cytoskeleton [85]. Although there is a limited number of studies that examine the relationship between EIF5A and tenderness [56], it is interestingly to note that EIF5A was found to change

post-mortem and was significantly positively related with Warner−Bratzler shear force in Danish porcine muscle [86] which is consistent with our result wherein EIF5A was negatively correlated with beef tenderness.

The Hemopexin (HPX) is a heme-binding glycoprotein that is retained as a negative contributor in the regression model of stringiness in this study. HPX acts as a driving factor of heme transport within the plasma, preventing oxidative damage caused by hemoglobin during intravascular hemolysis [87]. In addition, this protein is described as a negative biomarker of beef tenderness in heifers [17] and is correlated with water-holding capacity in porcine muscle [17].

Translationally-controlled tumour protein (TPT1), also called histamine-releasing factor (HRF) or fortilin, was retained in the regression model of stringiness (positive direction), while also being negatively correlated with beef flavour in this study. TPT1 is a multifunctional protein expressed in the majority of eukaryotic organisms, participating in various biological processes, including protein synthesis and degradation, apoptosis and cellular homeostasis [88]. Several studies have declared that TPT1 plays an important role in protecting the function of the anti-apoptotic protein Mcl-1 [89] and the antioxidant enzyme peroxiredoxin PRX1 [90], which possibly explains its positive correlation with beef stringiness. Moreover, TPT1 also acts as a survival factor in mammalian cells involved in cell protection and unfolded protein response under a variety of stress conditions [91].

4.5 Candidate protein biomarkers from muscle contraction and structure pathway

Coronin (CORO6) was identified as being negatively correlated with tenderness and flavour while positively with chewiness. Coronins are a family of actin-binding proteins that occurring in many eukaryotes [92] and are primarily located in the heart and skeletal muscle [93]. It is suggested they are involved in the promotion of cellular processes by rapid remodelling of the actin cytoskeleton, which is closely linked with endocytosis and cell motility [94]. As a newly identified member of the coronin family, Coronin 6 plays an important role in the regulation of the interaction between acetylcholine receptors and the actin cytoskeletal network, which is a crucial process during muscle contraction [95]. In addition, it has been reported as the QTL for CORO6 at Chr.19 (**Table 4**). Our study is the first to show a link between this protein and beef quality, which may partly be explained by its function in the modulating of the receptor-associated actin cytoskeleton and the dynamics of cellular actin networks [95].

Titin (TTN) identified as positively correlated with tenderness and flavour while negatively with chewiness, is a key protein in the myofibril assembly providing connections from Z discs to M lines across two halves of the sarcomere [96]. Titin was identified as a positive biomarker of beef tenderness and juiciness in the study of Gagaoua et al. [96] . It has been postulated in previous studies that the partial degradation of Titin after slaughter disrupts the myofibrillar network and through this mechanism can make a significant contribution to the improvement of beef tenderness [98,99]. In addition, titin has been associated with other phenotypes of beef quality such as beef colour [97] and water-holding capacity (WHC) [100], as well as other species such as chickens [101] and pigs [18].

Tropomyosin alpha-3 chain, as known as TPM3, is one of the major isoforms of Tropomyosin (TPM) that is mainly expressed in slow skeletal muscle whose function is to collaboratively work with the troponin complex (troponins T, C and I) to modulate calcium-sensitive interplay between actin and myosin heavy chain [102]. In the current study, TPM3 was identified as being negatively correlated with beef tenderness and was retained as the most explanatory protein in the stringiness regression model (positive direction), which is in accordance with the study of Zapata et al. [23]. In addition, TPM3 was identified on Chr.3 as QTL for lignoceric acid content. In contrast to our findings, however, TPM3 is previously reported as a positive biomarker of tenderness on *Longissimus* muscle from crossbred Charolais x Aubrac heifers [17]. This contradictory conclusion may reflect the effects of breed and muscle type on the biomarkers of beef tenderness.

WDR1 (WD repeat-containing protein 1), also known as Actin-interacting protein 1 (AIP1), is closely bound up with the dynamic reorganization of the actin cytoskeleton, which includes both assembly and disassembly of actin filaments [103]. In this study, WDR1 was found to be negatively correlated with beef tenderness which is consistent with the result of Chaze et al. [103]. This result may be explained by the fact that WDR1 has the ability to associate preferentially with actin filaments decorated with ADF/cofilin and promote filament disassembly [103].

4.6 Candidate protein biomarkers from heat shock & oxidative stress proteins

Heat shock protein beta-6 (HSPB6), was identified as being negatively correlated with tenderness and flavour and positively with stringiness. It belongs to small heat shock proteins (HSPs) that are widely considered as useful biomarkers of beef tenderness, colour, water-holding capacity and other quality traits [31,56,105,106]. Extrinsic stressors, such as pre-

slaughter or post-mortem management conditions are sources of the intensive production of small HSPs in the muscle which, like the larger HSPs, also play an important role in delaying the onset of apoptosis, maintaining the integrity of myofibrillar proteins, and also cellular homeostasis [46,106]. According to the integromics meta-analysis of Gagaoua et al. [18] , HSPB6 is reported as a robust biomarker of beef tenderness which is strongly enumerated as the top 5 biomarkers in that database. In the present study, HSPB6 was negatively correlated with beef tenderness, which may be due to its protective function against proteolysis in skeletal muscle. The relationship between tenderness and HSPB6 is not always straightforward though as a similar frequency of positive and negative associations have been discovered in previous studies. This may be explained by the influence of factors such as animal breed, gender, muscle type and pre-slaughter conditions [46,62,107].

Superoxide dismutase [Cu-Zn], also known as SOD1, is an antioxidant enzyme that is mainly expressed in the sarcoplasm and the inter membrane of mitochondria. SOD is engaged in the removal of surplus reactive oxygen species (ROS) to protect the muscle proteins from damage, and further maintaining cell homeostasis [108]. In the current study, SOD1 was retained in the regression model of chewiness and stringiness in a positive direction. It is noteworthy that this result is consistent with the previous conclusion of a meta-analysis that SOD1 is a negative biomarker for tenderness in bulls, steers and cows [19]. Nevertheless, two studies show a positive relationship between SOD1 and tenderness [109,110]. Further research should be undertaken to investigate the potential underlying mechanism and thereby decipher the different results shown in various studies. In addition, SOD1 is also reported to be correlated with beef colour in a previous proteomic study [31].

5. Conclusion

Interconnected proteins from several molecular pathways have been identified as putative biomarkers for beef tenderness, chewiness, stringiness and flavour in *Longissimus thoracis* et *Lumborum* (LTL) muscle of Limousin bulls. This study is the largest and first study to date on proteomics, using a label-free approach, to study beef flavour. Following regression analyses, 13 proteins were retained as of interest to explain the relationship between the abundance of these putative biomarkers and the four targeted quality traits. The consistency in direction of correlation of multiple subunits of individual protein complexes in the present study is an indicator of the robustness achieved with the large sample sizes under study in the present design. Two key energy metabolism related proteins (STBD1 and PHKA1) were identified across all quality traits and represent promising putative biomarkers for the explanation of

meat quality in large populations. The network and gene ontology analyses provided a robust validation of the critical role of muscle metabolism, especially calcium signalling, ATP synthesis and carbohydrate metabolism and the generation of precursor metabolites and energy, in the determination of the most important beef quality traits to consumers, i.e. beef texture and flavour. The novel candidate protein biomarkers need to be further evaluated and validated on a larger scale following the pipeline of biomarkers discovery using new populations of animals while considering the interacting factors at interplay [111].

Conflicts of interest

The authors declare no conflicts of interest.

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Table 1. Sensory evaluation score metrics for 14-day aged *Longissimus thoracis et lumborum* steaks from 34 Limousin-sired bulls

Sensory Attribute	Min[b]	Max[b]	Mean	SD[a]	CV(%)[a]
Tenderness	40.6	68.8	56.09	8.66	15.44
Chewiness	8.67	40.92	22.74	10.18	44.77
Stringiness	0.43	27.88	8.63	7.51	87.02
Flavour	25.08	52.06	39.28	6.33	16.12

[a] SD, Standard deviation; CV, Coefficient of Variation
[b] The scoring range of Tenderness, Chewiness, Stringiness and Flavour is 0–100

Tabl . The proteins are categorised manually into selected biological pathways based on their annotation in UniProt database (http://www.uniprot.org/).

Gene name	Full protein name	Tenderness	Chewiness	Stringiness	Flavour	Total [1]
	Muscle contraction and structure proteins **(n=22)** [2]					
CORO6	Coronin	-0.46**	0.45**		-0.51**	3
TTN	Titin	0.45**	-0.35*		0.55***	3
ACTN2	Alpha-actinin-2				-0.54***	1
ACTN3	Alpha-actinin-3				0.35*	1
CFL2	Cofilin-2	-0.38*				1
FHOD1	Formin homology 2 domain containing 1				0.34*	1
LUM	Lumican				0.36*	1
MYBPC2	Myosin binding protein C, fast type				0.47**	1
MYH1	Myosin-1				0.40*	1
MYH7	Myosin-7				-0.38*	1
MYL1	Myosin light chain 1/3, skeletal muscle isoform	-0.41*				1
MYL2	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform				-0.48**	1
MYL3	Myosin light chain 3	-0.37*				1
MYLK2	Myosin light chain kinase 2, skeletal/cardiac muscle				0.65***	1
MYLPF	Myosin regulatory light chain 2, skeletal muscle isoform	-0.38*				1
MYOZ3	Myozenin 3	-0.35*				1
PDLIM3	PDZ and LIM domain protein 3	-0.35*				1
SYNPO2	SYNPO2 protein				-0.36*	1
TNNI1	Troponin I1, slow skeletal type	-0.37*				1
TNNT1	Troponin T, slow skeletal muscle	-0.42*				1
TPM3	Tropomyosin alpha-3 chain	-0.42*				1
WDR1	WD repeat-containing protein 1	-0.36*				1
	Energy metabolism **(n=24)**					
PHKA1	Phosphorylase b kinase regulatory subunit alpha, skeletal muscle isoform	0.42*	-0.35*	-0.35*	0.41*	4
STBD1	Starch-binding domain-containing protein 1	0.36*	-0.37*	-0.36*	0.44*	4
PHKB	Phosphorylase b kinase regulatory subunit beta		-0.41*	-0.38*	0.67***	3
ATP5F1C	ATP synthase subunit gamma, mitochondrial	-0.48**			-0.42*	2
MDH2	Malate dehydrogenase, mitochondrial	-0.35*			-0.38*	2
ALDOC	Fructose-bisphosphate aldolase				0.42*	1
AMPD1	AMP deaminase				0.34*	1
ATP2A1	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	0.38*				1
ATP5F1A	ATP synthase subunit alpha				-0.48**	1
ATP5F1B	ATP synthase subunit beta				-0.37*	1
ATP5MF	ATP synthase subunit f, mitochondrial				-0.38*	1

CKM						1
DLAT	Acetyltransferase component of pyruvate dehydrogenase complex				-0.39*	1
ENO2	Enolase 2				0.34*	1
GOT2	Aspartate aminotransferase, mitochondrial				-0.48**	1
HADHB	Trifunctional enzyme subunit beta, mitochondrial				-0.49**	1
NDUFV1	NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial				-0.36*	1
PDHA1	Pyruvate dehydrogenase E1 component subunit alpha				-0.42*	1
PGM1	Phosphoglucomutase-1				0.38*	1
PHKG1	Phosphorylase kinase catalytic subunit gamma 1				0.54***	1
PREP	Prolyl endopeptidase				0.37*	1
SLC25A3	Phosphate carrier protein, mitochondrial	0.38*				1
UQCRC1	Cytochrome b-c1 complex subunit 1, mitochondrial				-0.59***	1
UQCRC2	Cytochrome b-c1 complex subunit 2, mitochondrial				-0.34*	1
	***Heat shock proteins* (n=5)**					
HSPB6	Heat shock protein beta-6	-0.49**		0.41*	-0.35*	3
CRYAB	Alpha-crystallin B chain	-0.38*		0.34*		2
HSPD1	60 kDa heat shock protein, mitochondrial	0.40*	-0.34*			2
HSPA5	Endoplasmic reticulum chaperone BiP				-0.36*	1
HSPG2	Heparan sulfate proteoglycan 2	0.40*				1
	***Oxidative stress proteins* (n=3)**					
AKR1B1	Aldo-keto reductase family 1 member B1	-0.47**	0.35*			2
PRDX6	Peroxiredoxin-6	-0.48**		0.38*		2
PDIA3	Protein disulfide-isomerase				0.44**	1
	***Other pathways: regulation of cellular processes, binding, apoptosis and transport proteins* (n=31)**					
CACNA1S	Voltage-dependent L-type calcium channel subunit alpha	0.53**	-0.35*		0.40*	3
LGALS1	Galectin-1	-0.42*	0.38*	0.48**		3
SYPL2	Synaptophysin-like 2	0.37*	-0.41*		0.42*	3
APOBEC2	Probable C->U-editing enzyme APOBEC-2	-0.47**		0.34*		2
CYCS	Cytochrome c	-0.38*			-0.36*	2
EIF4A2	Eukaryotic initiation factor 4A-II	0.39*			0.40*	2
PTGR2	Prostaglandin reductase 2	-0.37*			-0.42*	2
RTN4	Reticulon	0.48**			0.39*	2
A2M	Alpha-2-macroglobulin				0.47**	1
AHSG	Alpha-2-HS-glycoprotein				0.55***	1
ANXA5	Annexin				0.43*	1
ANXA6	Annexin A6				-0.34*	1
CACNA2D1	Voltage-dependent calcium channel subunit alpha-2/delta-1				0.63***	1
CAMK2D	Calcium/calmodulin-dependent protein kinase type II subunit delta				0.42*	1

CAN					1
CLTC	Clathrin heavy chain			0.34*	1
CMBL	Carboxymethylenebutenolidase homolog		0.36*		1
EEF2	Elongation factor 2			0.39*	1
EIF5A	Eukaryotic translation initiation factor 5A-1	-0.37*			1
ESD	S-formylglutathione hydrolase	-0.40*			1
FBP2	Fructose-1,6-bisphosphatase isozyme 2			0.43*	1
MB	Myoglobin	-0.34*			1
NDRG2	Protein NDRG2			0.45**	1
NPEPPS	Aminopeptidase			0.45**	1
OGN	Mimecan	-0.44**			1
RYR1	Ryanodine receptor 1			0.35*	1
TPT1	Translationally-controlled tumor protein			-0.34*	1
TRIM72	Tripartite motif containing 72	0.38*			1
UBA1	Ubiquitin-like modifier-activating enzyme 1			0.40*	1
VDAC1	Voltage-dependent anion-selective channel protein 1			-0.43*	1
VDAC3	Voltage-dependent anion-selective channel protein 3			-0.48**	1

[1] Total: total number of sensory traits for which protein abundance showed significant correlations.

[2] Correlation significance: * P < 0.05; ** P < 0.01; *** P < 0.001.

Tabl

Dependent variable	R-squared [1]	S.E	Entered independent variable [2]	Partial R-squared	Regression coefficient	t-value	P-value
Tenderness	0.71***	0.13	CACNA2D1	0.43	+0.81	+6.31	0.000
		0.15	EIF5A	0.14	-0.64	-4.24	0.000
		0.10	STBD1	0.08	+0.27	+2.63	0.014
		0.14	WDR1	0.05	-0.33	-2.30	0.029
Chewiness	0.51***	0.17	**SOD1** [3]	0.32	+0.79	+4.80	0.000
		0.19	PHKA1	0.13	-0.57	-3.10	0.004
		0.18	ATP5F1C	0.06	+0.36	+1.95	0.060
Stringiness	0.58***	0.16	TPT1	0.18	+0.82	+5.04	0.000
		0.15	**SOD1**	0.14	0.63	+4.30	0.000
		0.17	TPM3	0.18	+0.60	+3.54	0.001
		0.16	HPX	0.08	-0.67	-4.28	0.000
Flavour	0.70***	0.11	PHKG1	0.3[illegible]	+0.35	+3.20	0.003
		0.10	CORO6	0.2[illegible]	-0.59	-5.75	0.000
		0.11	ATP5F1A	0.16	-0.44	-3.99	0.000

[1] Significances of the regression equation models: *** P < 0.001.

[2] Variables (protein gene names) retained in the regression equations.

[3] Proteins in bold (SOD1) are common to different quality traits.

Tabl L) for meat quality traits.

Gene Name	UniProt ID	Chromosome	QTL linked to QTL database
CKMT2*	Q3ZBP1	Chr.7	Shear force
MYOZ3	F1N0W6		
ANXA6	P79134		
VDAC1	P45879		
OGN	P19879	Chr.8	
FBP2	Q2KJJ9		
MYL3	P85100	Chr.22	
UQCRC1	P31800		
SLC25A3	P12234	Chr.5	
MB	P02192		
PGM1	Q08DP0	Chr.3	
RTN4	Q1RMR8	Chr.11	
VDAC3	Q9MZ13	Chr.27	
CMBL	F1N2I5	Chr.20	
MYLK2	A4IFM7	Chr.13	
MYL2	Q3SZE5	Chr.17	
PREP	Q9XTA2	Chr.9	Tenderness score
PTGR2	Q32L99	Chr.10	
ACTN3	Q0III9	Chr.29	
NDUFV1	P25708		
MDH2	Q32LG3	Chr.25	Meat flavor score
APOBEC2	Q3SYR3	Chr.23	Marbling score
SLC25A3	P12234	Chr.5	Fat thickness at the 12th rib
MB	P02192		
MDH2	Q32LG3	Chr.25	
PHKG1	Q29RI2		
ATP2A1	Q0VCY0		
MYLPF	Q0P571		
CYCS	P62894	Chr.4	Intramuscular fat
MYLK2	A4IFM7	Chr.13	
AHSG	P12763	Chr.1	
EIF4A2	Q3SZ65		
ACTN3	Q0III9	Chr.29	Lignoceric acid content
NDUFV1	P25708		
PGM1	Q08DP0	Chr.3	
TPM3	Q5KR47		
HADHB	O46629	Chr.11	
EIF5A	Q6EWQ7	Chr.19	Myristic acid content
NPEPPS	E1BP91		
ALDOC	Q3ZBY4		
MYH1	Q9BE40		
CLTC	P49951		
CORO6	A6QLZ8		

* The proteins were from the 85 proteins significantly correlated with beef texture and flavour (**Table 1**) and further analysed by ProteQTL tool included in ProteINSIDE (http://www.proteinside.org/)

Tabl

tenderness by using the ProteINSIDE (http://www.proteinside.org/) website service.

GO term	Function	Gene Name	GO frequency within the dataset (%)	GO frequency within the genome (%)	P-Values
		Biological Process (BP)			
GO:0006936	muscle contraction	CACNA1S TPM3 CRYAB CKMT2 TNNT1 TNNI1 MYL1 TTN	21.62	3.76	4.12E-14
GO:0045214	sarcomere organization	TTN WDR1 TNNT1	8.11	7.89	1.94E-06
GO:0060048	cardiac muscle contraction	MYL3 TNNI1 TTN	8.11	6.67	2.95E-06
GO:1905907	negative regulation of amyloid fibril formation	CRYAB HSPG2	5.41	100	4.49E-06
GO:0006942	regulation of striated muscle contraction	ATP2A1 TNNI1 MYL3	8.11	3.7	1.36E-05
GO:0030042	actin filament depolymerization	WDR1 CFL2	5.41	25	2.76E-05
GO:0030836	positive regulation of actin filament depolymerization	CFL2 WDR1	5.41	15.38	5.89E-05
GO:0050821	protein stabilization	HSPD1 RTN4 CRYAB	8.11	1.97	6.54E-05
GO:000	skeletal muscle contraction	TNNT1 TNNI1	5.41	7.41	0.000

3009					182
GO:0008637	apoptotic mitochondrial changes	ATP2A1 HSPD1	5.41	3.51	0.000060
GO:0070509	calcium ion import	ATP2A1 CACNA1S	5.41	3.17	0.000695
GO:1902082	positive regulation of calcium ion import into sarcoplasmic reticulum	ATP2A1	2.7	100	0.002298
GO:2001213	negative regulation of vasculogenesis	RTN4	2.7	100	0.002298
GO:1900260	negative regulation of RNA-directed 5-3 RNA polymerase activity	EIF4A2	2.7	100	0.002298
GO:1990036	calcium ion import into sarcoplasmic reticulum	ATP2A1	2.7	100	0.002298
GO:0045766	positive regulation of angiogenesis	RTN4 HSPB6	5.41	1.57	0.002298
GO:1990809	endoplasmic reticulum tubular network membrane organization	RTN4	2.7	100	0.002298
GO:0031448	positive regulation of fast-twitch skeletal muscle fiber contraction	ATP2A1	2.7	100	0.002298
GO:0048291	isotype switching to IgG isotypes	HSPD1	2.7	100	0.002298

GO:1905580	positive regulation of ERBB3 signaling pathway	RTN4	2.7	100	0.002298
GO:0046314	phosphocreatine biosynthetic process	CKMT2	2.7	100	0.002298
GO:0002842	positive regulation of T cell mediated immune response to tumor cell	HSPD1	2.7	100	0.002298
GO:0090076	relaxation of skeletal muscle	ATP2A1	2.7	50	0.002825
GO:0051659	maintenance of mitochondrion location	ATP2A1	2.7	50	0.002825
GO:0046370	fructose biosynthetic process	AKR1B1	2.7	50	0.002825
GO:0034165	positive regulation of toll-like receptor 9 signaling pathway	RTN4	2.7	50	0.002825
GO:1905653	positive regulation of artery morphogenesis	RTN4	2.7	50	0.002825
GO:0061723	glycophagy	STBD1	2.7	50	0.002825
GO:0032470	positive regulation of endoplasmic reticulum calcium ion concentration	ATP2A1	2.7	50	0.002825
GO:003	slow-twitch skeletal muscle fiber contraction	TNNT1	2.7	50	0.002

Molecular Function (MF)					
GO:0005515	protein binding	OGN TNNI1 AKR1B1 CKMT2 PDLIM3 CRYAB ESD CFL2 STBD1 EIF5A SLC25A3 PTGR2 MYLPF TPM3 LGALS1 EIF4A2 TTN RTN4 CORO6 PHKA1 SYPL2 PRDX6 CACNA1S ATP5F1C ATP2A1 MYOZ3 CYCS TNNT1 HSPG2 HSPD1	81.08	0.47	1.35E-24
GO:0005101	actin filament binding	CORO6 TTN CFL2 TPM3 WDR1	13.51	3.57	9.96E-09
GO:0008307	structural constituent of muscle	TTN MYL3 MYLPF MYL1	10.81	8.7	1.88E-08
GO:0003723	RNA binding	RTN4 EIF5A HSPD1 MDH2 APOBEC2 EIF4A2 LGALS1 ATP5F1C	21.62	0.49	1.49E-07
GO:0030021	extracellular matrix structural constituent conferring compression resistance	OGN HSPG2	5.41	33.33	1.89E-05
GO:0031433	telethonin binding	TTN MYOZ3	5.41	28.57	2.31E-05
GO:0051082	unfolded protein binding	HSPB6 HSPD1 CRYAB	8.11	2.63	3.05E-05
GO:0051371	muscle alpha-actinin binding	TTN PDLIM3	5.41	11.11	9.83E-05
GO:0042802	identical protein binding	PRDX6 ESD APOBEC2 CRYAB TTN	13.51	0.35	0.00159
GO:0042803	protein homodimerization activity	ATP2A1 RTN4 HSPB6 CRYAB	10.81	0.53	0.00022

3					9
GO:0001540	amyloid-beta binding	HSPG2 CRYAB	5.41	5.56	0.000028
GO:0031625	ubiquitin protein ligase binding	PRDX6 RTN4 HSPD1	8.11	1.02	0.000324
GO:0016887	ATPase	ATP5F1C EIF4A2 HSPD1	8.11	0.76	0.000686
GO:0051087	chaperone binding	HSPB6 HSPD1	5.41	2.2	0.013533
GO:0018738	S-formylglutathione hydrolase activity	ESD	2.7	100	0.022988
GO:0043795	glyceraldehyde oxidoreductase activity	AKR1B1	2.7	100	0.022988
GO:0032038	myosin II heavy chain binding	MYL3	2.7	50	0.028225
GO:0047522	15-oxoprostaglandin 13-oxidase activity	PTGR2	2.7	33.33	0.033437
GO:0034186	apolipoprotein A-I binding	HSPD1	2.7	25	0.035555
GO:0031014	troponin T binding	TNNT1	2.7	25	0.035555
GO:	glycogen binding	STBD1	2.7	25	0.0

2001069					0.03555
GO:0030060	L-malate dehydrogenase activity	MDH2	2.7	25	0.03555
GO:0005315	inorganic phosphate transmembrane transporter activity	SLC25A3	2.7	25	0.03555
GO:0051373	FATZ binding	MYOZ3	2.7	20	0.038
GO:0047184	1-acylglycerophosphocholine O-acyltransferase activity	PRDX6	2.7	20	0.038
GO:0004689	phosphorylase kinase activity	PHKA1	2.7	20	0.038
GO:0004111	creatine kinase activity	CKMT2	2.7	20	0.038
GO:0047499	calcium-independent phospholipase A2 activity	PRDX6	2.7	16.67	0.04312
GO:0001758	retinal dehydrogenase activity	AKR1B1	2.7	14.29	0.04633
GO:0004032	alditol:NADP+ 1-oxidoreductase activity	AKR1B1	2.7	14.29	0.04633

Table 6. Top 30 significant Gene Ontology (GO) terms computed from the list of the 59 candidate protein biomarkers that correlated with beef flavour by using the ProteINSIDE (http://www.proteinside.org/) website service.

GO term	Function	Gene Name	GO frequency within the dataset (%)	GO frequency within the genome (%)	P-Values
Biological Process (BP)					
GO:0006119	oxidative phosphorylation	ATP5F1C UQCRC1 UQCRC2	5.17	25	4.34E-07
GO:0042776	mitochondrial ATP synthesis coupled proton transport	ATP5F1C ATP5F1B ATP5F1A	5.17	14.29	1.57E-06
GO:0002026	regulation of the force of heart contraction	MYL2 CAMK2D MYH7	5.17	10.71	2.97E-06
GO:0009060	aerobic respiration	UQCRC2 UQCRC1 MDH2	5.17	9.68	3.64E-06
GO:0006000	glycolytic process	PGM1 ENO2 ALDOC	5.17	7.69	6.22E

0096					-06
GO:0006754	ATP biosynthetic process	ATP5F1B ATP5F1A ATP5F1C	5.17	7.5	6.24E-06
GO:0021762	substantia nigra development	SYPL2 NDRG2 HSPA5	5.17	7.14	6.94E-06
GO:0060048	cardiac muscle contraction	TTN MYH7 MYLK2	5.17	6.67	7.53E-06
GO:0006816	calcium ion transport	CACNA1S CACNA2D1 RYR1 TPT1	6.9	2.12	7.53E-06
GO:0014728	regulation of the force of skeletal muscle contraction	ACTN3 MYH7	3.45	100	7.98E-06
GO:0006	muscle contraction	CACNA1S MYH7 TTN RYR1	6.9	1.88	1.08E-

9 3 6 G O :0 0 0 6 9 4 1	striated muscle contraction	MYH7 TTN MYLK2	5.17	3.7	0 5 3 . 2 5 E - 0 5
G O :1 9 0 2 5 1 4	regulation of calcium ion transmembrane transport via high voltage-gated calcium channel	CAMK2D CACNA2D1	3.45	28.57	3 . 7 6 E - 0 5
G O :0 0 3 0 3 8 8	fructose 1,6-bisphosphate metabolic process	FBP2 ALDOC	3.45	28.57	3 . 7 6 E - 0 5
G O :0 0 0 6 0 8 6	acetyl-CoA biosynthetic process from pyruvate	PDHA1 DLAT	3.45	28.57	3 . 7 6 E - 0 5
G O :0 0 7 1 3 1 3	cellular response to caffeine	RYR1 CACNA1S	3.45	25	4 . 5 1 E - 0 5
G O :0 0 3 4 9	protein folding in endoplasmic reticulum	PDIA3 HSPA5	3.45	14.29	0 . 0 0 0 1 1

GO:0055003	cardiac myofibril assembly	TTN MYL2	3.45	13.33	1.00001199
GO:0006000	fructose metabolic process	ALDOC FBP2	3.45	13.33	0.00001199
GO:0048041	focal adhesion assembly	ACTN3 ACTN2	3.45	8.33	0.00002499
GO:0015986	ATP synthesis coupled proton transport	ATP5F1C ATP5F1A	3.45	7.14	0.00003133
GO:0043536	positive regulation of blood vessel endothelial cell migration	ATP5F1A ATP5F1B	3.45	6.9	0.00003336
GO:0086091	regulation of heart rate by cardiac conduction	CACNA2D1 CAMK2D	3.45	5.56	0.00004844

1 GO:0045214	sarcomere organization	TTN ACTN2	3.45	5.26	0.000515
GO:0006094	gluconeogenesis	PGM1 FBP2	3.45	4.55	0.000664
GO:0006091	generation of precursor metabolites and energy	PHKB ATP5F1B PHKA1	5.17	1.07	0.000686
GO:0055010	ventricular cardiac muscle tissue morphogenesis	MYH7 MYL2	3.45	4.26	0.000716
GO:0005977	glycogen metabolic process	PHKB PHKA1	3.45	3.77	0.000841
GO:0043066	negative regulation of apoptotic process	VDAC1 TPT1 ANXA5 HSPA5	6.9	0.49	0.000844

GO:0071277	cellular response to calcium ion	RYR1 CAMK2D	3.45	3.7	0.000844
		Molecular Function (MF)			
GO:0005515	protein binding	UBA1 MYH1 FHOD1 CLTC PITRM1 LUM ENO2 HSPA5 UQCRC1 PHKA1 PTGR2 CAMK2D ATP5F1B TPT1 ALDOC ATP5F1A PDHA1 CACNA1S CAND2 ATP5F1C A2M EEF2 FBP2 HADHB SYPL2 TTN MYH7 MYL2 MYLK2 NDRG2 UQCRC2 VDAC1 COPO6 DLAT STBD1 ACTN2 ANXA6 PGM1 NDUFV1 AMPD1 MYBPC2 RTN4 SYNPO2 ANXA5 EIF4A2 CYCS ACTN3 PDIA3 PHKB	84.48	0.76	3.43E-41
GO:0003723	RNA binding	MDH2 GOT2 RTN4 CLTC ATP5F1C TPT1 UBA1 HADHB PDIA3 EIF4A2 ATP5F1A EEF2	20.69	0.74	4.97E-11
GO:0008307	structural constituent of muscle	TTN MYBPC2 ACTN3 MYL2 ACTN2	8.62	10.87	3.6E-10
GO:0042802	identical protein binding	ACTN3 DLAT FHOD1 ACTN2 CAMK2D PDIA3 TTN ANXA6 AMPD1 FBP2	17.24	0.7	3.69E-09
GO:0005509	calcium ion binding	TTN ANXA6 RYR1 MYL2 HSPA5 ANXA5 TPT1	12.07	1.02	1.49E-07

GO:0046933	proton-transporting ATP synthase activity, rotational mechanism	ATP5F1B ATP5F1A ATP5F1C	5.17	23.08	5.03E-07
GO:0044325	transmembrane transporter binding	VDAC1 ACTN2 ACTN3 CAMK2D	6.9	3.64	1.57E-06
GO:0051015	actin filament binding	FHOD1 ANXA6 CORO6 TTN	6.9	2.86	3.34E-06
GO:0005245	voltage-gated calcium channel activity	CACNA2D1 CACNA1S RYR1	5.17	7.5	6.24E-06
GO:0005516	calmodulin binding	TTN RYR1 MYLK2 CAMK2D	6.9	2.11	7.53E-06
GO:0043532	angiostatin binding	ATP5F1B ATP5F1A	3.45	50	1.89E-05
G	titin binding	CAMK2D ACTN2	3.45	18.18	7

GO:0031432					.66E-05
GO:0008308	voltage-gated anion channel activity	VDAC1 VDAC3	3.45	11.11	0.00015
GO:0051371	muscle alpha-actinin binding	TTN SYNPO2	3.45	11.11	0.00015
GO:0042288	MHC class I protein binding	ATP5F1A ATP5F1B	3.45	11.11	0.00015
GO:0019901	protein kinase binding	VDAC1 CLTC EEF2 TTN	6.9	0.72	0.00025
GO:0004683	calmodulin-dependent protein kinase activity	MYLK2 CAMK2D	3.45	7.14	0.00031
GO	cholesterol binding	ANXA6 VDAC1	3.45	4.55	0.

:0015485					0.000664
GO:0005544	calcium-dependent phospholipid binding	ANXA5 ANXA6	3.45	4.26	0.000716
GO:0031625	ubiquitin protein ligase binding	UQCRC1 HSPA5 RTN4	5.17	1.02	0.000751
GO:0045296	cadherin binding	EEF2 RTN4 HSPA5	5.17	1.02	0.000751
GO:0043621	protein self-association	FHOD1 TTN	3.45	3.7	0.000844
GO:0048306	calcium-dependent protein binding	ANXA6 A2M	3.45	3.39	0.000957
GO:0	ATPase	EIF4A2 ATP5F1C HSPA5	5.17	0.76	0.0

0168887					0.01503
GO:0051087	chaperone binding	HSPA5 HSPB6	3.45	2.2	0.00213
GO:0047742	dihydrolipoyllysine-residue acetyltransferase activity	DLAT	1.72	100	0.00277 4
GO:0051082	unfolded protein binding	HSPB6 HSPA5	3.45	1.75	0.00277 4
GO:0005262	calcium channel activity	RYR1 ANXA6	3.45	1.83	0.00277 4
GO:0005178	integrin binding	ACTN2 ACTN3	3.45	1.83	0.00277 4
GO:00	protease binding	TTN A2M	3.45	1.61	0.00

0 2 0 2 0	3 2 2 9

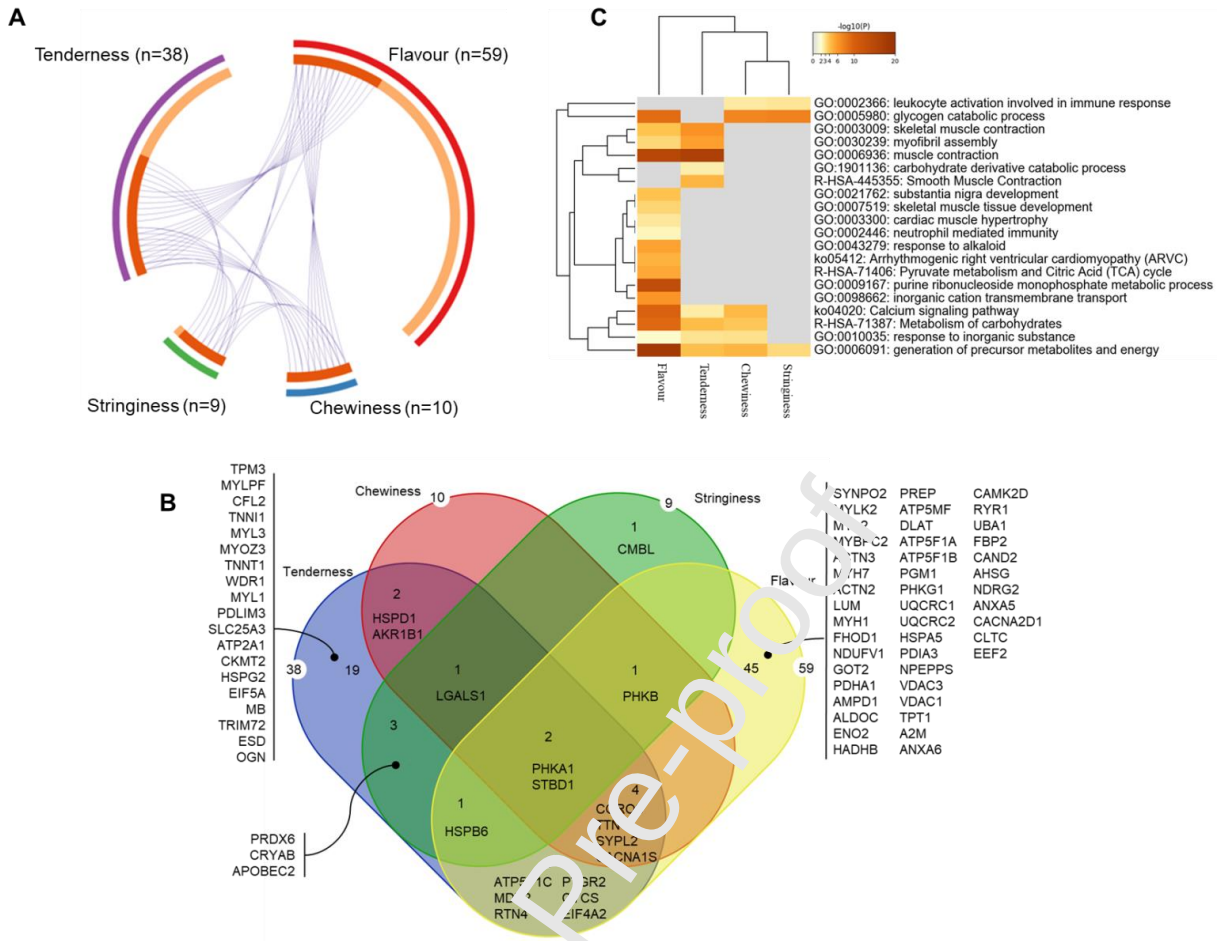


Figure 1. Shared significant trait relationships and pathway and process enrichment analysis for 85 proteins significantly correlated with sensory traits. (a) Circos plot highlighting the shared significant proteins across sensory traits. (b) Venn diagram detailing the proteins significantly correlated with each quality trait and those presenting overlap (c) Hierarchical Heatmap clustering indicating the Top 20 representative enriched GO terms (one per cluster) among the 85 proteins, coloured by p-values using Metascape® (https://metascape.org/). The most statistically significant term within a cluster is chosen to represent the cluster, while the darker the brown the more significant with the grey colour indicating no significant association was found.

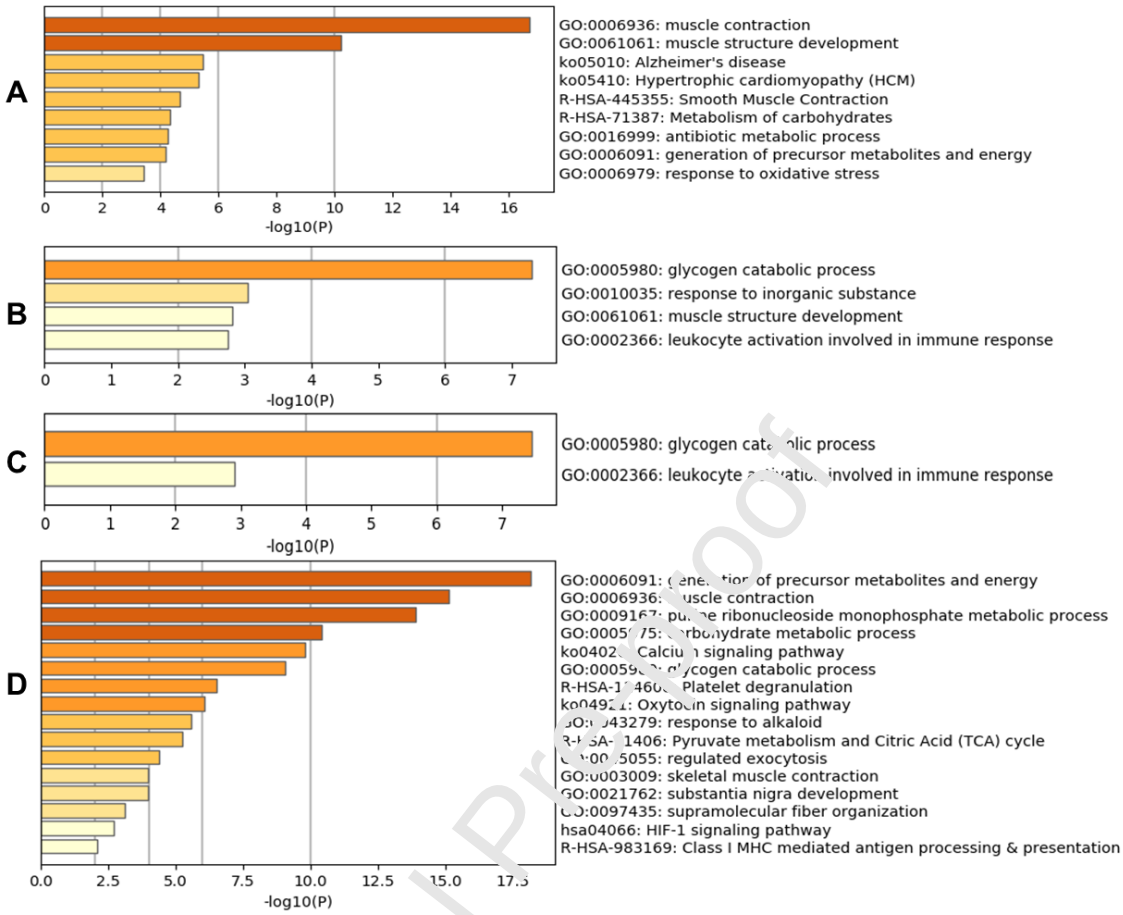


Figure 2. Bar graph and Hierarchical heatmap of enriched Gene Ontology terms significantly associated with the proteins correlated with individual texture traits and flavour (a) tenderness, (b) chewiness, (c) stringiness and (d) flavour. These are coloured by p-values, with darkest brown being most significant and the grey colour indicating lack of significant association.

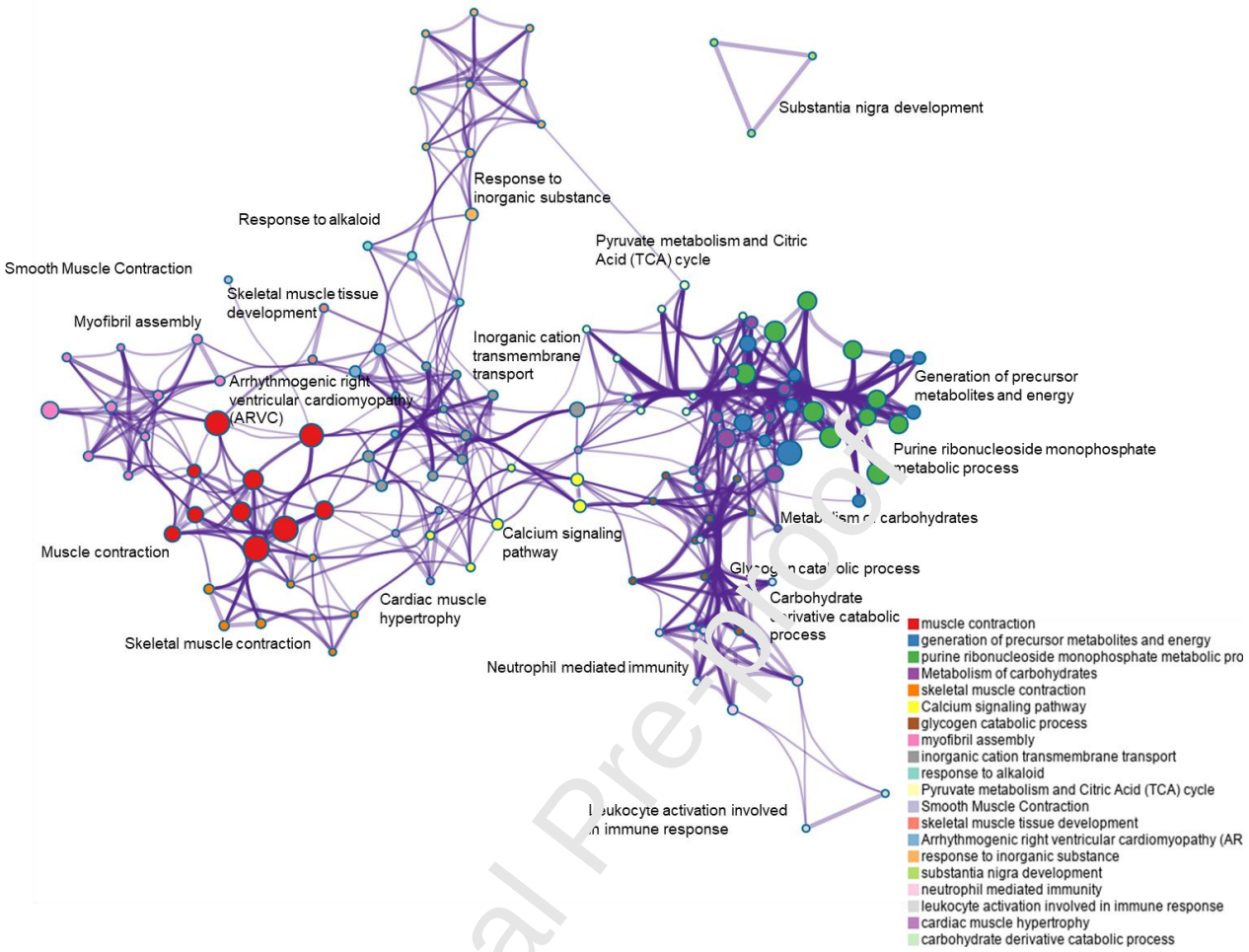


Figure 3. Functional enrichment network plot with nodes representing top 20 enriched terms based on the 85 shortlisted protein biomarkers using Metascape® (https://metascape.org/). The nodes are coloured by cluster ID, where nodes that share the same cluster ID are typically close to each other, and the size of node is proportional to the quantity of the proteins (gene names) included in that specific term. There are less than 15 terms per cluster and no more than 250 terms in total.

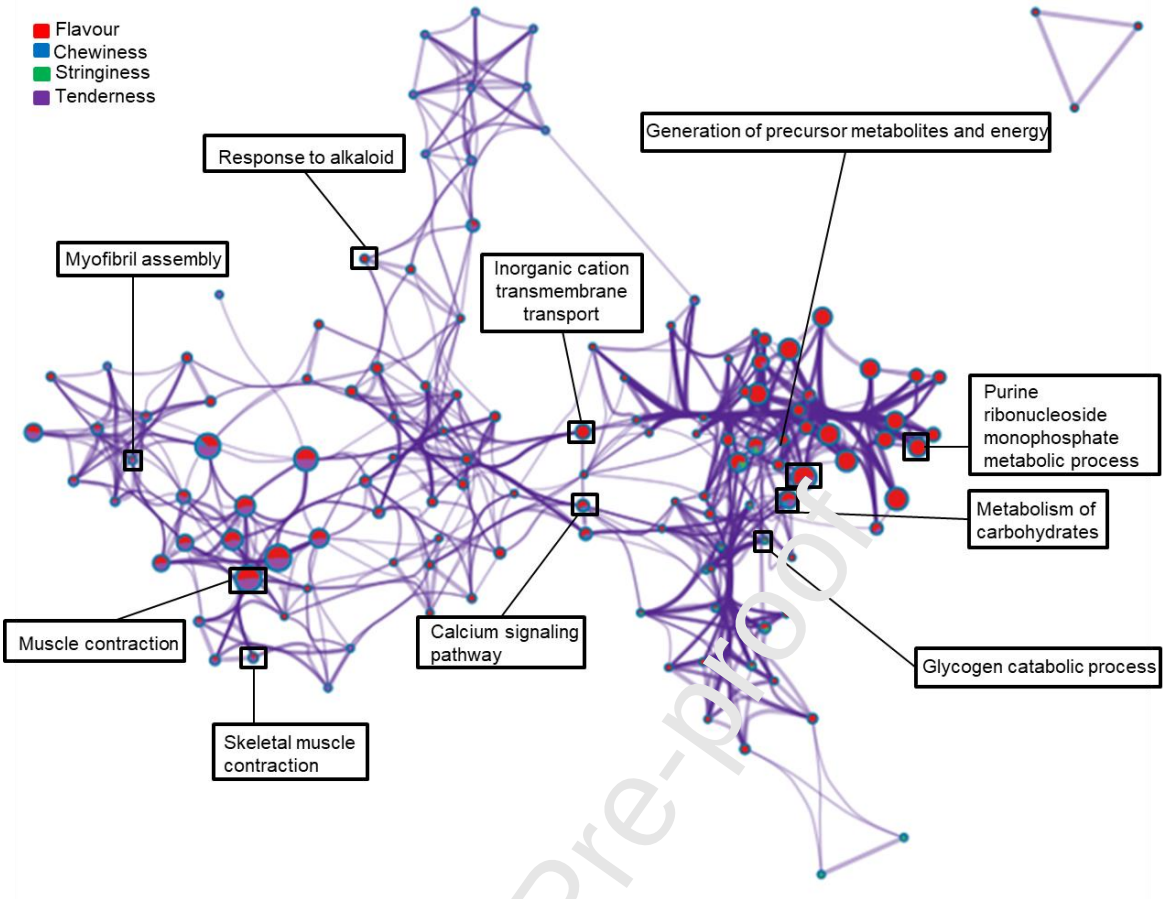
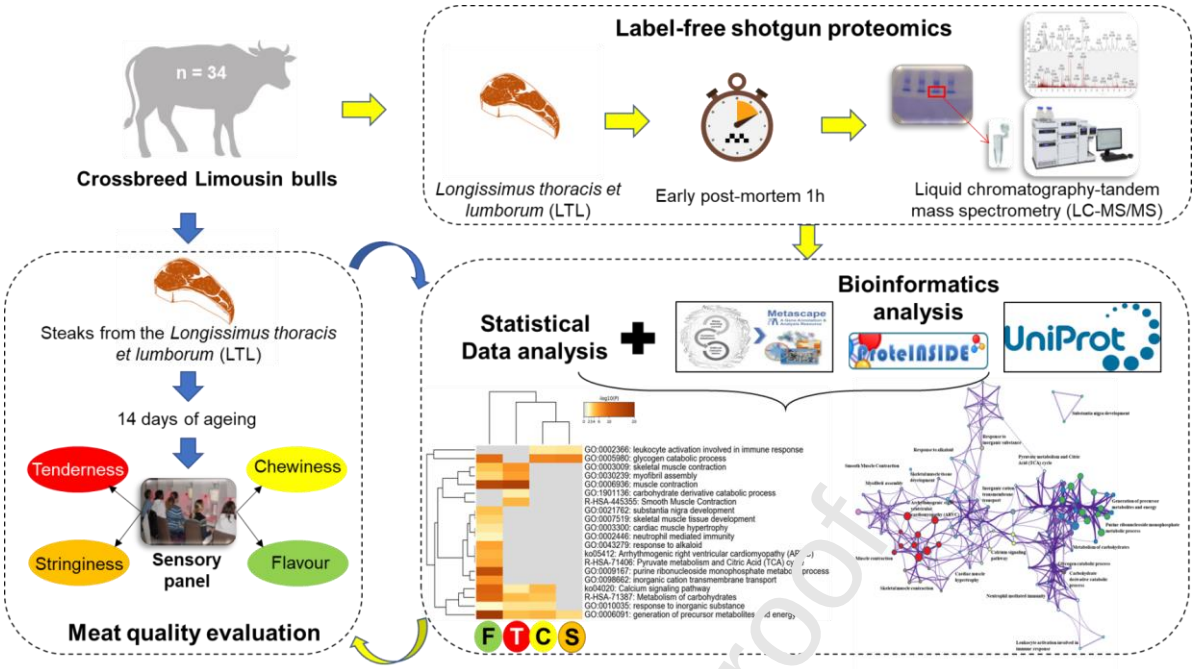


Figure 4. Functional enrichment network plot with nodes represented as pie charts based on the 85 shortlisted protein biomarkers using Metascape® (https://metascape.org/). The nodes are color-coded based on the identities of the proteins (gene names) lists (Red: Flavour; Blue: Chewiness; Green: Stringiness; Purple: Tenderness). The size of a slice is proportional to the percentage of proteins under the term that originated from the corresponding protein list. Network with boxes indicating the annotation of selected top 10 enriched GO terms.

Conflicts of interest

The authors declare no conflicts of interest.



Highlights

- Label-free proteomics to study multiple beef eating quality traits
- 85 proteins were significantly correlated beef tenderness, chewiness, stringiness and flavour
- Generation of precursor metabolites and energy was associated with beef quality determination irrespective of quality attribute
- PHKA1 and STBD1 with all the quality traits and proposed as putative biomarkers
- The underlying mechanisms regulating multiple beef eating quality traits were described