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Shotgun proteomics for the preliminary identification of biomarkers of beef sensory tenderness, juiciness and chewiness from plasma and muscle of young Limousin-sired bulls

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Abstract

Label free shotgun proteomics was used to analyse plasma and *Longissimus* muscle biopsies of Limousin-sired bulls, classified as 5 high-quality and 5 low-quality meat based on sensory texture traits (tenderness, juiciness and chewiness). A total of 31 putative protein biomarkers (16 in plasma and 15 in muscle) differed significantly in abundance between the two quality groups. The proteins were associated with muscle structure, energy metabolism, heat shock proteins, oxidative stress and proteolysis related pathways. Among them, B2M, AHSG, APOA4 and HP-20 (plasma), PFKM, MYH2, PTER, GSTM1 and MYPN (muscle) were good predictors of the three texture quality traits. Further, significant correlations were identified for FETUB, SERPINA7, ASL, TREH, HP, HP-25, AZGP1, APCS and SYT15, which are novel biomarkers from plasma that warrant further evaluation. This study is a significant step forward in elucidating proteomic profiles in bovine bio-fluids and muscle tissue, which may ultimately provide opportunities to processors for early assessment of beef sensory quality.

Keywords: Cattle; Meat tenderness; Protein biomarkers; Muscle; Plasma; Bio-fluids; Omics.

1. Introduction

Among the palatability traits associated with red meat, texture traits are major contributors to the consumer's overall perception of their beef-eating experience (O'Quinn et al., 2018). Texture comprises a group of related traits, which can be examined in more or less detail but usually including, initial bite tenderness, juiciness and chewiness. Juiciness is the amount of liquid released from the meat after biting with the molars, while chewiness is the energy required when people chew solid food to the point that it is ready to swallow (Kasapis et al., 2017); tenderness has long been considered the primary attribute concerning consumers and it has an important influence on repeat buying decisions (Miller et al., 2001). For the beef industry, it is an ongoing challenge to produce high-value table cuts of consistent quality that meet consumers' expectations (McCarthy et al., 2017, Gagaoua et al., 2019a), and there is a need to develop efficient and non-invasive tools to identify animals/carcasses with desired quality attributes before or soon after slaughter (Ouali et al., 2013). With this ambition, during the last two decades, high-throughput molecular techniques collectively known as proteomic approaches, have been applied in meat science (Di Luca et al., 2016; Gagaoua et al., 2020a; Picard & Gagaoua, 2020a; Munekata et al., 2020). Proteomics investigations on beef tenderization allow the simultaneous study of a large number of muscle proteins to characterize the changes occurring in the *post mortem* period from meat of divergent quality, leading to the identification of putative protein markers (Gagaoua et al., 2021a).

In addition to studies on muscle tissue, bio-fluids such as blood or plasma, are of growing interest in the search for biomarkers with proteomic approaches (Boudon et al., 2020). Blood can be collected *in vivo* or *peri mortem* and contains a wide variety of proteins. It fulfils the practicality recommendations to implement routine biomarker profiling. Differential abundances of plasma proteins can distinguish the physiological or pathological status of mammals. In the field of meat science, few studies are available, but all demonstrate there is potential in using on-line accessible bovine bio-fluids to identify alternative biomarkers of meat tenderness (Grubbs et al., 2016; Boudon et al., 2020).

In the present study, label-free quantitative shotgun proteomics (LC-MS/MS) was applied to identify candidate biomarkers of meat quality, which are differentially abundant in two divergent

groups of high *versus* low texture meat quality using plasma and muscle samples from the same young Limousin-sired bulls.

2. Materials and Methods

2.1. Blood sampling and plasma preparation

Twenty-three Limousin-sired crossbred bulls were finished in controlled conditions at Irish Cattle Breeders Federation Progeny Test Centre, transported together to a commercial EU-licensed abattoir, where they were slaughtered in a single group at an average age of 22 months and weight of 650 kg under standard conditions. Blood was collected from the jugular vein flow of each animal immediately after sticking in a sterile universal container and subsequently transferred to 6 mL EDTA-coated tubes to prevent blood clotting. After inversion of the tube 8 – 10 times, blood samples were centrifuged at 3000 × g for 10 min at room temperature to collect the plasma. Plasma samples were decanted into sterile 2 mL Eppendorf tubes, frozen on dry ice before storage at −80 °C until further analysis.

2.2. Animals and muscle tissue sampling

. At 1 h *post-mortem, Longissimus thoracis* et *lumborum* (LTL) muscle biopsies were collected from the 10th rib of each carcass on the right side. The LTL samples were stabilised using RNAlater® (Zhu et al., 2019), stored overnight at 4 °C before the RNAlater® was removed and the muscle transferred to a freezer at -80 °C until protein extraction. Carcasses were chilled in a common chill room, deboned at 48 h *post mortem* and the LTL aged for 14 days, blast frozen and stored at - 20 °C prior to sensory analysis.

2.3. Meat quality (texture) evaluation by trained sensory panellists

The protocol used for sensory beef assessment was modified from AMSA guidelines (AMSA, 2015). Briefly, 14 days aged muscle samples (2.54 cm thick) were defrosted in refrigerated conditions overnight, grilled in an electric cooker to a core temperature of 70 °C. After grilling, each steak was cut into 20 mm cubes that were immediately served to 8 trained panellists. The steaks were scored based on a scale of 1 to 10 for tenderness, juiciness and chewiness, with a score of 1 representing extremely tough, dry and less chewy and a score of 10 representing extremely tender, juicy and chewy. From the 23 animals, 5 animals with high quality (low

chewiness, high tenderness and juiciness) and 5 with low quality (chewy, tough, dry) texture were selected for the proteomic analyses (**Table 1**).

2.4. Protein extraction and quantification

2.4.1. Plasma proteins

To obtain a comprehensive blood protein profile, serum albumin was first reduced following the protocol of Pierce™ Albumin Depletion Kit (Thermo Fisher Scientific, Wilmington, DE, USA). To accurately deplete albumin from the plasma, the slurry was subjected to the procedure described by the manufacturer. Before loading the samples into the kit, 200 µL plasma samples were first desalted using a desalting column containing 25 mM Tris, 25 mM NaCl at pH 7.5. After desalting overnight at 4°C, a wide-bore micropipette tip was used to transfer 400 µL of the resin slurry into a spin column that was then placed in a 1.5 mL collection tube. These were centrifuged at 12,000 × g for 1 minute to remove excess liquid, the flow-through was discarded and the spin column was placed back into the same collection tube. Around 200 µL of binding/wash buffer containing 25 mM Tris, 75 mM NaCl (pH 7.5) was added to the spin column and centrifuged at 12,000 × g for 1 minute, the flow-through was discarded and the spin column placed into a fresh collection tube. Then, 25 µL of desalted plasma sample was loaded and incubated for 1-2 minutes at room temperature. Afterwards, the tubes were centrifuged at 12,000 × g for 1 minute to allow albumin binding to the column. The resin column was washed by adding a 25% volume of Binding/Wash Buffer to release proteins. The flow-through was retained after centrifuged at 12,000 × g for 1 minute and finally stored at -80°C.

2.4.2. Muscle proteins

Prior to protein extraction, frozen muscle tissue samples (80 mg) were first homogenized in 2 mL of extraction buffer containing 8.3 M urea, 2 M thiourea, 1% Dithiothreitol (DTT), 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 2% Immobilized pH gradient (IPG) buffer pH 3-10 using a T 25 digital ULTRA-TURRAX® (Picard & Gagaoua, 2020a). Subsequently, the protein homogenates were incubated with shaking for 30 min on wet-ice, followed by a 30 min centrifugation at 10000 × g. The supernatant was then transferred into Eppendorf tubes and stored at -80 °C until protein quantification.

2.4.3. Protein quantification using the Bradford method

Protein concentrations were determined using a spectrophotometer (UV-1700, Pharmaspec, SHIMADZU), using the dye-binding method of Bradford (1976). Bovine serum albumin was used as a standard (Bio-Rad Protein Assay kit, Bio-Rad, France).

2.5. Shotgun proteomics: LC-MS/MS, protein identification and label-free quantitation

The protein extracts were concentrated on 1D stacking gel of SDS-PAGE using commercial Mini-PROTEAN® TGX™ precast gels of 8.6 × 6.7 × 0.1 cm and 12% polyacrylamide (Bio-Rad Laboratories, Deeside, UK). First, the muscle protein extract was diluted with water and mixed with 1:1 Laemmli sample buffer (Bio-Rad Laboratories, Deeside, UK). Roughly, 20 µg proteins were loaded in each gel lane and the electrophoresis was run for about 15 min to concentrate the proteins. Subsequently, the gels were washed three times with pure water, stained with EZ Blue Gel staining reagent (Sigma, USA) with gentle shaking for 2 h and then washed with distilled.

The washed gels were used to excise the protein bands of each sample with a sterile disposable scalpel. The protein bands were carefully transferred into Eppendorf tubes and first destained by 200 µL of 25 mM ammonium bicarbonate (Sigma, USA), 5% acetonitrile for 30 min (Picard et al., 2016) and in a second time by two washing with 200 µL of 25 mM ammonium bicarbonate, 50% acetonitrile for 30 min each. Then, disulfide bonds were reduced with 200 µL of 10 mM dithiothreitol (Sigma-Aldrich) in 50 mM ammonium bicarbonate buffer for 30 min at 56 °C. The alkylation of proteins was carried out with 200 µL of 55 mM iodoacetamide (Sigma-Aldrich) in 50 mM ammonium bicarbonate buffer for 30 min in darkness. Finally, bands were dehydrated with 100% acetonitrile for 10 min and the liquid was discarded. Subsequently, the dried protein bands were stored at -80 °C until LC-MS/MS analysis. The hydrolysis of the bands was carried out with 48 µl of a 25mM ammonium bicarbonate buffer – 12.5 ng/µl trypsin solution (Promega) per band for 5 h in an oven at 37 °C. A volume of 30 µL buffer was added periodically during hydrolysis so that the bands were always covered with liquid. The extraction of the peptides was carried out under ultrasonication for 15 min with 38.4 µl of 99.95% acetonitrile – 0.05 % trifluoroacetic acid. Then, the supernatant was transferred into 500 µL Eppendorf tubes and dry concentrated using a Speedvac for 2 h. The volume was adjusted exactly to 20 µL with a solution of isotopologic peptides (50 pmol/µL) that is diluted 18 times in a 0.05 % TFA solution (internal quality control). After passing through the ultrasonic

bath (10 min), the entire supernatant was transferred to an HPLC vial prior to LC-MS/MS analysis.

For the separation, peptides mixtures were analysed by nano-LC-MS/MS (Thermo Fisher Scientific) using an Ultimate 3000 system coupled to a QExactive HF-X mass spectrometer (MS) with a nanoelectrospray ion source. 1 µL of hydrolyzate was first preconcentrated and desalted at a flow rate of 30 µl/min on a C18 pre-column 5 cm length × 100 µm (Acclaim PepMap 100 C18, 5µm, 100A nanoViper) equilibrated with Trifluoroacetic Acid 0.05% in water to remove contaminants that could potentially disrupt the efficiency of the mass spectrometry analysis. After 6 min, the concentration column was switched online with a nanodebit analytical C18 column (Acclaim PepMap 100 - 75 µm inner diameter × 25 cm length; C18 - 3 µm -100Å - SN 10711310) operating at 400 nL/min equilibrated with 96 % solvent A (99.9 % H_2O, 0.1 % formic acid). The peptides will then be separated according to their hydrophobicity, thanks to a gradient of solvent B, a solution of acetonitrile (ACN / FA-99.9 / 0.1) of 4 to 25% in 50 minutes (The mass spectrometer operates in data dependent mode : the parent ion is selected in the orbitrap cell (FTMS) at a resolution of 120,000 and each MS analysis is succeeded by 18 MS / MS with analysis of the MSMS fragments at a resolution of 15,000.

At the end of the LC-MS/MS analysis, raw data processing, MS/MS ion search was performed with Mascot v2.5.1 (http://www.matrixscience.com) against bos taurus database (i.e. ref_bos_taurus, 23970 sequences). The following parameters were used during the request: precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da, a maximum of two missed cleavage sites of trypsin, carbamidomethylation (C), oxidation (M) and deamidation (NQ) set as variable modifications. Protein identification was validated when at least two peptides originating from one protein showed statistically significant identity above Mascot scores with a False Dicovery Rate of 1%. Ions score is -10 log(P), where P is the probability that the observed match is a random event. For plasma proteome, the Mascot score was 37 with a False Discovery Rate (FDR) of 1% and the adjusted p-value was 0.0088. For muscle, the Mascot score was 36 with and at an FRD of 1% the adjusted p-value was 0.0098.

For label-free protein quantitation analysis, LC-Progenesis was used with the same identification parameters described above with the phenotypic data among the high and low quality beef texture. All unique validated peptides of an identified protein were included and the

total cumulative abundance was calculated by summing the abundances of all peptides allocated to the respective protein. Statistical analysis was performed using the "between subject design" and *p*-values were calculated by a repeated measures analysis of variance using the normalized abundances across all runs. LC-Progenesis analysis yielded 546 unique gene name proteins for muscle and 188 proteins for plasma.

2.6. Statistical analyses

The statistical analyses were performed using a range of software tools, mainly XLSTAT 2018.2 (AddinSoft, Paris, France) as well as the online tools NormalyzerDE and MetaOmGraph. Raw data means were scrutinized for data entry errors, and any missing data and outliers. Log2 transformation and mean normalization gave the best performance in reducing variability among replicate samples and was applied to protein abundance in both plasma and muscle datasets.

A one-way ANOVA was used for the comparison of protein abundances between the high-quality meat *versus* low-quality on each proteome source separately. Differences in protein abundances between the high and low groups for each beef sensory quality trait were considered significant at $P < 0.05$ and tendencies towards significance were defined at $P < 0.1$. For the separation of the least-squares means comparisons, the Tukey test was used. Subsequently, all the differentially abundant proteins in muscle or plasma were projected using principal component analysis (PCA) with the corresponding meat quality variables of each group.

Spearman's correlation coefficients were computed between the differential proteins within each proteome source and the texture traits. Correlation values were considered significant at $P < 0.05$ and tendencies at $P < 0.1$. Furthermore, correlations among the differential proteins from muscle or plasma were used to build biological correlation networks following the procedure by Gagaoua et al. (2015a). Only correlations significant at $P < 0.05$ were used.

To identify the suitability of the identified differentially abundant proteins to explain variation in the three sensory texture traits, Partial Least Squares (PLS) regressions on standardized data were conducted (Gagaoua et al., 2020b) for each meat quality trait within muscle and plasma. This generated explanatory models using the optimal number of components in each case. By including all the proteins in one model, this approach allowed identification of the proteins that had a strong association with the trait thanks to the Variable Importance in Projection (VIP)

criteria. The VIP filter was applied at both VIP > 1.0 and > 0.8 for variable selection (Gagaoua et al., 2020b).

2.7. Bioinformatics analyses

Protein-protein interactions among the differential proteins were analyzed separately for muscle and plasma using the STRING web service database (https://string-db.org/). In addition, the differential proteins among the two quality groups and for both sources (plasma and muscle) were compared for the enriched Gene Ontology (GO) terms using the web tool Metascape® (https://metascape.org/) as described by Gagaoua et al. (2021a). Furthermore, the web-based ProteINSIDE tool (http://www.proteinside.org/) was used to assign GO terms for the categories Biological Process (BP), Molecular Function (MF) and Cellular Component (CC) at Benjamini–Hochberg P <0.05 against human orthologs.

3. Results

Shotgun proteomics applied in this study allowed the identification of a total of 188 proteins in plasma and 546 in muscle (**Fig. 1**). These proteins were identified at an FDR of 1%. 37 proteins were common (**Figure 1**), and the GO of these proteins was computed (**Table S1**).

3.1. Putative protein biomarkers of meat texture traits identified from plasma

3.1.1. Differential proteins in plasma

From the plasma proteins, 16 belonging to different biological pathways, including 6 protease inhibitors, were significantly different in abundance between the high and low texture quality. These were identified with a log2 fold change ranging from -2.48 to 0.97 (**Table 2**). Among the 16 proteins, 9 were significantly different ($P < 0.05$): beta-2-microglobulin (B2M), alpha-2-HS-glycoprotein (AHSG), fetuin-B (FETUB), protein HP-20 homolog (HP-20), protein HP-25 homolog 1 (HP-25), serum amyloid P-component (APCS), apolipoprotein A-IV (APOA4), heat shock-related 70 kDa protein 2 (HSPA2), zinc-alpha-2-glycoprotein (AZGP1). The remaining 7 proteins showed a tendency ($P < 0.1$) towards significance: haptoglobin (HP), serpin A3-6 (SERPINA3-6), serpin A3-7 (SERPINA3-7), serpin peptidase inhibitor, clade A, member 7 (SERPINA7), uncharacterized protein (SYT15) that seemed to be synaptotagmin-15-like isoform X1, argininosuccinate lyase (ASL) and trehalase (TREH) (**Table 2**).

The 16 proteins were correlated with the three sensory texture quality traits (**Table 2**). Six proteins (B2M, SERPINA3-7, HP-20, HP-25, APOA4 and TREH) were common for tenderness, juiciness and chewiness scores. The first five proteins were negatively correlated with tenderness and juiciness and positively correlated with chewiness. The inverse correlation was found for the TREH, *i.e.* negative with chewiness and positive for tenderness and juiciness. FETUB (positive) and SYT15 (negative) were correlated with tenderness and juiciness, while SERPINA3-6 and APCS were correlated with juiciness (negative) and chewiness (positive), respectively. The other proteins were related specifically to one quality trait: HP (negative), AHSG and ASL (both positive) with juiciness; SERPINA7 was negatively correlated with tenderness and, HSPA2 was positive with chewiness scores. No correlation was found for AZGP1. From the correlation analyses, Beta-2-microglobulin (B2M) was strongly and significantly correlated with the three texture traits as exemplified in **Fig. 2**. To better present the associations between the 16 significant plasma proteins and the three beef texture attributes, a principal component (PC) analysis was performed (**Fig. 3**). The first two PC explained 73.7 % of the variability and separated well the high and low quality texture steaks. The first PC accounted for most of the variation (59.8 %) that was positively correlated with AHSG, FETUB, TREH, ASL and juiciness and tenderness scores, while negatively correlated with all the other proteins that were projected with chewiness namely SERPINA3-6, SERPINA3-7, SERPINA7, HSPA2, AZGP1, SYT15, B2M, APOA4, APCS, HP, HP-20 and HP-25. The second PC explained a weak variation of around 13.9 %. The main variables in the second PC were SERPINA3-6, SERPINA3-7, SERPINA7, HSPA2 on the positive side and APCS, HP, HP-20 and HP-25 on the negative side of PC.

3.1.2. Partial least squares to explain the variability of the texture traits by the 16 plasma proteins

Following variable importance in projection (**Fig. 4**), six proteins had a VIP of more than 1.0 for tenderness: B2M, APOA4, HP-20, AHSG, HP-25, HSPA2; 7 proteins had a VIP of more than 1.0 for juiciness: B2M, HP-20, HP-25, APOA4, TREH, AHSG, APCS; and 7 proteins reached this threshold for chewiness: B2M, AHSG, HP-20, APOA4, HSPA2, SERPINA3-6, SERPINA3-7 (**Fig. 4A-C**). Among them, 4 proteins were common to all three quality traits: B2M, HP-20, APOA4 and AHSG (**Fig. 4D**). HSPA2 that is the heat shock-related 70 kDa protein 2 was

common for tenderness and chewiness. HP-25, which is protein HP-25 homolog 1, was common for tenderness and juiciness (**Fig. 4D**). Four more proteins were found to be specific for juiciness (TREH and APCS) and chewiness (SERPINA3-6 and SERPINA3-7) as highlighted in the Venn diagram (**Fig. 4D**).

3.1.3. Protein-protein interactions and correlation networks on the putative plasma biomarkers

Protein-Protein interactions were assessed using the bioinformatics STRING database (https://string-db.org/) by the 16 differentially abundant proteins in the plasma (**Fig. 5A**). Furthermore, to visualize significant associations among the proteins in the dataset, another network based on the Spearman's correlation coefficients ($P < 0.05$) was constructed (**Fig. 5B; Supplementary Table S2.**). In this correlation network, the solid and dash lines represent the positive and negative correlations, respectively.

The network in **Fig. 5B** demonstrates that B2M interacts with the highest number of proteins (9 interactions) and relates the two distinct hubs together via HSPA2. APOA4, HP-20 and AHSG were all correlated significantly 8 times and contribute strongly to the correlation network. Together, HSPA2, APOA4, HP-20 and AHSG were all robustly retained in the PLS regression models with VIP > 1.0 (**Fig. 4D**) and were further common for the three sensory texture traits. From the above list of four proteins, AHSG was the only one that was negatively correlated with all the three proteins. Associations between AHSG and FETUB, AHSG and APCS, SERPINA3-7 and SERPINA3-6 were identified in both networks (**Fig. 5A and 5B**).

3.2. Putative protein biomarkers of meat texture traits identified from Longissimus thoracis muscle

3.2.1. Differential proteins in Longissimus thoracis muscle

From the muscle, 15 proteins were identified as differentially abundant between the high and low texture quality steaks with fold changes ranging from -0.47 to 1.51 (**Table 3**). Among them, the abundances of 8 proteins were significantly different ($P < 0.05$), namely myosin-2 (MYH2), myopalladin (MYPN), kelch-like protein 41 (KLHL41), bleomycin hydrolase (BLMH), ATP-dependent 6-phosphofructokinase (PFKM), starch-binding domain-containing protein 1 (STBD1), glutathione S-transferase Mu 1 (GSTM1) and lysine-tRNA ligase (KARS1). Seven other proteins showed tendencies towards significance ($P < 0.1$): myosin-1 (MYH1), tubulin

alpha-8 chain (TUBA8), proteasome subunit beta type-2 (PSMB2), 26S proteasome non-ATPase regulatory subunit 1 (PSMD1), protein-arginine deiminase type-2 (PADI2), phosphotriesterase-related protein (PTER) and translationally-controlled tumour protein (TPT1). Of these 15 proteins, 5 were from the 'cytoskeletal structure' and 4 were involved in 'proteolysis' pathways followed by 4 proteins belonging to 'metabolism' and 'hydrolases' pathways (**Table 3**).

The 15 proteins were correlated with the 3 beef texture quality traits, while chewiness is negatively correlated with the tenderness and juiciness (**Table 3**). It was observed for the proteins differentially abundant in muscle that 6 proteins (MYH2, MYPN, KLHL41, BLMH, PFKM and PTER) were common and significantly ($P < 0.05$) correlated with tenderness, juiciness and chewiness scores (**Table 3**). MYH2, MYPN and KLHL4 belong to the cytoskeletal pathway. MYH2 (myosin-2) was negatively correlated with tenderness and juiciness, but positively with chewiness. For the other 5 proteins (MYPN, KLHL41, BLMH, PFKM and PTER), the inverse pattern was observed as they were all positively correlated with tenderness and juiciness and negatively with chewiness. MYH1 and STBD1 were correlated with tenderness (positive) and chewiness (negative), while GSTM1 and KARS1 were correlated with juiciness (positive) and chewiness (negative). PSMB2 and PADI2 were specific for juiciness and chewiness respectively, and they were both negative. Finally, no significant correlation was observed for three proteins, being TUBA8, PSMD1 and TPT1. From these correlation analyses, ATP-dependent 6-phosphofructokinase (PFKM) was strongly and significantly correlated with the three texture traits (**Fig. 6**).

A principal component (PC) analysis (PCA) was performed to examine the associations between the 15 *Longissimus thoracis* muscle proteins and the 3 texture attributes (**Fig. 7**). The first two PC explained 67.6 % of the variability allowing the separation of the high and low quality steaks from *Longissimus thoracis* muscle. The first PC accounted for most of the variation explaining alone 53.3 % and was positively correlated with MYH1, KLHH41, TRT1, TUBA8, STBD1, PFKM, PSMD1, MYPN, GSTM1, PTER, KARS1, PADI2, BLMH and juiciness and tenderness scores, while negatively correlated with PSMB2, MYH2 and chewiness. The second PC explained 14.3 % of the variability. The main proteins positively correlated with this axis were PSMB2 and MYH1, while BLMH was negatively correlated.

3.2.2. Partial least squares to explain the variability of the texture traits by the 15 muscle proteins

PLS-R was used to rank the most important proteins based on the VIP score for each sensory quality trait (**Fig. 4**). Accordingly, 7 proteins (PFKM, MYH2, PTER, GSTM1, MYPN, MYH1 and STBD1) had VIP scores > 1.0 for tenderness; 8 proteins (PFKM, MYH2, PTER, GSTM1, MYPN, KLHL41, PSMB2 and KARS1) for juiciness; and 7 proteins for chewiness PFKM, MYH2, PTER, GSTM1, MYPN, STBD1 and PADI2 (**Fig. 8A-C**). Among them, 5 proteins were common for the 3 quality traits: PFKM, MYH2, PTER, GSTM1 and MYPN (**Fig. 8D**). STBD1, a protein involved in metabolism and autophagy, was common for tenderness and chewiness. Furthermore, MYH1 was as expected specific to tenderness, PADI2 was for chewiness, and KLHL41, PSMB2 and KARS1 were all specific for juiciness (**Fig. 8D**).

3.2.3. Protein-protein interactions and correlation networks on the putative muscle biomarkers

The network highlighted that the proteins with the highest number of interactions were MYH2 and KARS1 with 5 interactions each. MYPN and PFKM had 4 interactions and STBD1 and GSTM1 had 3 interactions. MYH2 was the only protein that negatively correlated with 5 other proteins and MYPN was the only one that positively correlated with 4 other proteins. These correlations between the differential proteins in *Longissimus thoracis* muscle are in agreement with the results of the PLS regressions (**Fig. 8**).

3.3. Enriched Gene Ontology terms and comparison between the differential proteins from plasma and muscle

The significant GO terms employing a heat map were compared between the proteins identified in plasma and muscle (**Fig. 10**)**.** This analysis allowed visualization of the common biological pathways shared between the two sources of putative biomarkers of beef texture traits. Four GO terms were common for plasma and muscle, being immune system processes, metabolic processes, response to stimuli and multicellular organismal processes. The other pathways, such as negative or positive regulation of biological processes, locomotion and signalling were significant and specific to plasma; while negative regulation of biological processes, biological regulation, localization and cellular processes were specific to muscle.

4. Discussion

This study aimed to use both plasma and muscle tissue collected from the same young Limousin-sired crossbred bulls, finished and slaughtered together under the same conditions, of two divergent beef texture quality groups to identify putative biomarkers that would allow us to (i) understand the mechanisms behind the variation of beef texture quality traits and (ii) propose preliminary explanatory models of each trait using PLS models. Tenderness, juiciness and chewiness were divergent between the two texture groups with respective average scores of 7.39, 6.65 and 2.58 in the high-quality group compared to those of the low-quality group being 6.60, 5.96 and 3.30. This study is the first to investigate the relationships among the three texture traits from the same animals and the respective proteomes of two different protein sources (plasma and muscle) analysed using label-free shotgun proteomics. The underlying mechanisms behind the differential proteins for plasma and then muscle are discussed in the following sections.

4.1. Plasma putative biomarkers belonging to protease inhibitor pathway

Among the 7 putative protein biomarkers belonging to this pathway, only SERPINA3-6 and SERPINA3-7, which are members of the A3 clade of the serpins (serine peptidase inhibitors) superfamily (Gagaoua et al. 2015b) were identified in previous studies to be related to variation in beef quality, while five other proteins (AHSG, FETUB, SERPINA7, ASL and TREH) were identified for the first time in this study on cattle.

SERPINA3-6 and SERPINA3-7 were previously shown to be negatively related, as in this study, to beef tenderness, but in studies of muscle tissue (Boudon et al., 2020; Gagaoua et al., 2015b). SERPINA3, also known as α1-antichymotrypsin, is a member of the serpins superfamily. This is the largest family of protease inhibitors comprising around 3,000 members among which some show an inhibitory function as well as various intracellular and extracellular processes such as blood coagulation, fibrinolysis, apoptosis regulation and cell migration (Gagaoua et al., 2015b). In cattle, the SERPINA3 subfamily comprises natural caspase inhibitors and was suggested as a new target for apoptosis inhibition *in vivo* and *post mortem* muscle (Gagaoua et al., 2015b). This supports the negative association with tenderness identified in this study. Their increased abundance at a systemic level in the plasma of animals producing tougher meat, and strongly association with the traits studied here are noteworthy given their known ability to inhibit caspases. This may retard the onset of apoptosis, an essential process in the conversion of muscle to meat and texture determination (Ouali et al. 2013). An earlier study

reported a significant correlation between meat toughness and serpins, and proposed their ability to explain a large component of the tenderness variation (Zamora et al., 2005). The SERPINA3 gene was recently suggested to affect bovine muscle development, beef quality and yield (Yang et al., 2020). Although not yet reported in bovine muscle, it is worth mentioning that SERPINA3 was identified in pork as a positive biomarker of drip loss (Te Pas et al., 2013) and SERPINA7 was shown to also be involved in lipid biosynthesis (Ma et al., 2013). It could be deduced from the plasma data presented here that serpins can regulate directly or indirectly the development of beef texture mainly as secreted proteins. Our evidence, alongside the literature, suggests strongly that further targeted studies are warranted to fully elucidate the role of serpin members in the determination of beef quality.

Trehalase (TREH) is an enzyme that catalyses conversion of trehalose to glucose. The present study shows TREH for the first time as a putative biomarker of beef texture since it is not identified in the most recent study on a beef tenderness biomarker atlas (Gagaoua et al., 2021a). However, TREH was previously shown to be up-regulated in muscle of feed efficient pigs and positively related to muscle characteristics, related to meat quality, *i.e.* pork tenderness (Horodyska et al., 2018). Energy conversion of carbohydrates has an important influence on water-holding capacity and meat tenderness (Gagaoua et al., 2021a). This is in line with the trend observed in this study on cattle, where TREH was more abundant in tender and juicy beef. The growing evidence in cross-species studies for TREH suggests it may be worth further study. It was shown in this study to be important for more than one texture trait and also plays a role in production efficiency, and therefore sustainability of animal production.

AHSG was positively related with juiciness but also related to the other texture traits in the PLS models. It is also known as fetuin-A, which, in humans, is encoded by the AHSG gene and is known to be a multifunctional plasma carrier protein of approximately 60 kDa (Ketteler et al., 2003). Fetuins have roles in transport in the circulation, playing a similar role to bovine serum albumin. They belonged to the cystatin superfamily involved with the transport of calcium and free fatty acids (Karmilin et al., 2019). The importance of cystatins as potent predictors of meat tenderness was highlighted in a previous study (Gagaoua et al., 2015b). Here, FETUB, was found to be a positive biomarker of tenderness and juiciness. This study is the first to identify these two proteins as plasma biomarkers of beef texture traits. A previous study suggested that

the AHSG gene may be a useful marker for the fatty acid profile in sheep meat (Munyaneza et al., 2019). The AHSG gene was positively associated with different fatty acids including saturated and unsaturated fatty acids in five different sheep breeds. In the context of the present study, we suggest that it is through the regulation of circulating proteases that both protein inhibitors could have roles in muscle to meat conversion.

4.2. Plasma putative biomarkers belonging to immunity & metabolism pathways

The 5 putative protein biomarkers (B2M, HP, HP-20, HP-25, AZGP1) belonging to this pathway were for the first time revealed in this study to be associated with beef quality. The high number of proteins identified in this pathway draws attention to the importance of stress at slaughter plays for meat quality outcomes.

HP, also known as haptoglobin, is a major acute-phase protein (APP) that exists in the serum of cattle, in line with the identification of SERPINA3 members above that are also APPs (Réhault et al., 1999). HP is a stress marker proposed to monitor the welfare and production quality of animals (Coutinho et al., 2017). In line with our findings on negative correlation with juiciness, the HP level was also reported in pork to be negatively correlated with drip loss and cooking loss (Čobanović et al., 2020). The HP-20 and HP-25 belong to the liver-derived hibernation protein (HP) complex. This plays a role in hibernation but the HP genes are also conserved in non-hibernating mammals such as cows and pigs, and probably regulate physiological functions (Seldin et al., 2014). Furthermore, B2M and HP-20 were negatively related with beef tenderness and juiciness and positively related with chewiness. B2M is a 12 kDa secreted protein that has crucial roles in a broad range of biological processes, notably immune modulation (Bash et al., 2010).

4.3. Plasma putative biomarkers belonging to binding, heat shock & transport pathways

HSPA2 was reported in several earlier muscle studies in bulls, however, APCS, SYT15 and APOA4 are for the first time here associated with meat quality traits.

In this study, HSPA2 showed a lower abundance in plasma of animals with more tender meat in agreement with previous knowledge on muscle (Gagaoua et al., 2021a). It is widely accepted that HSPA2 has essential roles in *post mortem* skeletal muscle because of its function in hindering the onset of apoptosis to prevent cells from damage as well as other stressful

conditions (Ouali et al., 2013; Gagaoua et al., 2018; 2019b). In plasma, other members of the heat shock protein family (HSPs) were identified in heifers to be related to tenderness (Boudon et al., 2020). HSPA2 may modulate the activity of initiator caspases and protect the integrity of myofibrillar proteins by inhibition of proteolytic activity (Gagaoua et al., 2021a; Picard & Gagaoua, 2020a). HSPs are synthesized to protect the organism itself in response to stressful conditions, such as those to which animals are challenged pre- and during slaughter; high temperature, lack of food, or oxidative stress. Identification of HSPA2 is in line with the two pathways described above since inflammation is known to interact with proteins of this family, especially serpins as found in the correlation network (**Fig. 5B**). HSPs have consistently been reported to show different abundance between tender and tough beef evaluated using both instrumental and sensory tenderness methods (Picard & Gagaoua, 2020a; Gagaoua et al., 2021a).

SYT15 is a member of the synaptotagmin (Syt) family of membrane trafficking proteins. The Syt family are transmembrane proteins that act as calcium regulators of exocytosis. It is highlighted that SYT4 is found as a paralog of Syts that is negatively correlated with beef tenderness (Gonçalves et al., 2018), which seems to agree with the identification of SYT15, with low abundance in this study in tender beef. Moreover, SYT4, a paralog of synaptotagmin 11 (SYT11), is a calcium sensor that plays a role in the regulation of the synaptic transmission (Glavan et al., 2009). This ability to modulate calcium ions could be linked with beef tenderization during meat ageing.

APOA4 is a member of the Apolipoprotein family, which is related to lipid transporter activity and shares characteristics with members of the apoptotic Bcl-2 family (Uzureau et al., 2016). Apolipoproteins have been associated with pork tenderness, furthermore, APOA5 and APOC3 genes were associated with several technological meat quality traits including cooked weight percentage, drip loss, colour and tenderness of pork (Hui et al., 2013). In another study, APOL3 transcript had higher expression in tough beef of *Bos indicus* cattle (Gonçalves et al., 2018), which is also in line with the trends of APOA4 in the present study. Therefore, this may suggest that an up-regulation of lipid transportation and metabolism pathways are associated with low tenderness scores.

4.4. Muscle putative biomarkers belonging to cytoskeleton and the myofibrillar pathway

The identification of a high number of myofibrillar proteins to be related to beef texture agrees with a recent integromics study which found this pathway to be the foremost molecular signature driving tenderness determination (Gagaoua et al., 2021a). Myosin heavy chain isoforms have previously been shown to be robust biomarkers of beef tenderness regardless of breed, gender or muscle type (Picard & Gagaoua, 2020a). The identification of MYH1 (fibres MyHC-IIX, fast glycolytic) is in accordance with the theory that muscles which contain more type-II fibres are more susceptible to rapid *post mortem* glycolysis, contributing to more tender meat (Ruusunen & Puolanne, 2004). Besides, the identification of a positive association between MYH1 and tenderness and a negative association of this trait with MYH2 (fibres MyHC-IIA, fast oxido-glycolytic) is in line with the literature on their important role in beef texture (Gagaoua et al., 2019b; Picard & Gagaoua, 2020b). During *post mortem* storage of meat, significant proteolytic changes occur in myosin proteins including MYH1 and MYH2 as well as cytoskeletal muscle proteins (Gagaoua et al., 2021a,b), such as tubulin, which lead to their degradation, hence playing a pivotal role in the final beef quality outcome. This supports a relationship between *post mortem* degradation of cytoskeleton proteins and beef tenderness. MYH1 and MYH2 can be considered robust biomarkers of beef tenderness as they have been identified multiple times in the literature as summarized in the integromics meta-analysis of Gagaoua et al. (2021a).

Tubulin is a highly conserved αβ dimeric protein binding with two moles of guanosine triphosphate that can assemble into microtubules (Valenstein & Roll-Mecak, 2016). TUBA8 was identified as positively correlated with tenderness in this study, which is consistent with the result of Zapata et al. (2009). In that investigation, meat toughness was negatively associated to α and β tubulins. The relationship could be explained by proteolytic breakdown of microtubules whose function is to maintain the shape of cells, leading to better tenderness. Also, it has recently been shown that microtubules are regulated by many microtubule-associated proteins, including tubulin, whose deletion is related to modification of sarcoplasmic reticulum organization and calcium release, resulting in possible muscle weakness (Sébastien et al., 2018).

MYPN has been identified for the first time as a putative biomarker of beef tenderness in this study. It is a constituent of the sarcomere which binds nebulin and nebulette to α-actinin at the Z line (Bang et al., 2001). MYPN is positively correlated with tenderness in our study, and it is

worth noting that earlier studies indicated that MYPN could be considered as a candidate gene for meat quality in pigs and rabbit (Wang et al., 2017).

Kelch-like protein 41, which is also known as KLHL41, had been reported as a positive beef tenderness biomarker in two previous studies, which agrees with our results (Gagaoua et al., 2021a). It is involved in skeletal muscle development and differentiation, which is important in myofibril assembly by promoting lateral fusion of adjacent thin fibrils into mature myofibrils (Gupta & Beggs, 2014).

4.5. Muscle putative biomarkers belonging to proteolysis pathway

The identification of proteins involved in proteolysis agrees with the previous section. Proteasome subunit beta type-2 (PSMB2) is a component of the 20S core proteasome complex which is involved in the proteolytic degradation of most intracellular proteins (Ouali et al., 2013) and identified to play a role in the changes that occur in beef muscle during aging (Gagaoua et al. 2021b). This complex plays numerous essential roles within the cell by associating with different regulatory molecules. It enables the ATP-dependent degradation of ubiquitinated proteins. PSMB2 was negatively related to tenderness. This contrasts with earlier findings of a positive relationship to beef tenderness in the *Longissimus thoracis* muscle of young bulls (Picard & Gagaoua, 2020a). PSMC2 and PSMD1 are two subunits of the proteasome 26S, and PSMC2 had been identified to be positively correlated with beef tenderness, which aligns with the effect on PSMD1 as shown in this study.

BLMH is categorized as a member of a proteolysis biological pathway. Although the normal physiological role of BLMH is not completely clear, it shows general aminopeptidase activity by catalyzing the process of inactivation of bleomycin and protecting normal and malignant cells from bleomycin toxicity. A recent genome-wide association analysis study in multibreed Angus-Brahman showed a significant association between the region hosting BLMH and shear force (Leal et al., 2019).

4.6. Muscle putative biomarkers associated with metabolism & binding proteins

During the early *post mortem* period, there is an anoxic situation caused by a sudden loss of blood, shifting the energy metabolism from aerobic and oxidative pathways toward the glycolytic pathway (Gagaoua et al. 2021a). Of seven putative biomarkers, GSTM1 is a member

of the glutathione S-transferase family involved in the metabolism of xenobiotic and catalyzing reactions between glutathione and a range of potentially toxic and carcinogenic compounds (Al-Husseini et al., 2014). A previous study showed that GSTM1 was highly expressed in steers phenotypically ranked high for residual feed intake in Angus (Al-Husseini et al., 2014). GSTM1 was for the first time identified as correlated with beef tenderness in our study and showed a positive relationship with beef tenderness and juiciness. STBD1 is a cargo receptor for glycogen, which delivers its cargo to an autophagic pathway, resulting in the transport of glycogen to lysosomes (Jiang et al., 2010). It was positively related to tenderness and juiciness in this study and has previously been identified as a novel candidate gene for porcine meat quality and carcass traits in *Longissimus thoracis* of Yorkshire pigs (Li et al., 2016).

PFKM showed a strong correlation with the three quality traits and ranked in the top 2 proteins in the PLS. PFKM, also known as ATP-dependent 6-phosphofructokinase, is one of the energy metabolism related enzymes involved in the phosphorylation process. PFKM catalyzes the first committed step of glycolysis and was found to be positively associated with deoxymyoglobin, while negatively with oxygen consumption in beef based on mitochondrial proteomics (Wu et al., 2020). It could be speculated that the higher expression of PFKM means a faster glycolysis process, which can contribute to more tender meat. In addition, phosphotriesterase-related protein is related to the catabolic process and hydrolase activity and is predicted to be involved in epithelial cell differentiation. It is important to note that this protein is shown here to have significantly elevated expression in tender and juicy meat.

5. Conclusion

Differences in proteomic profiles across the meat quality groups investigated in this study point towards the importance of increased oxidation metabolism and apoptosis, proteolysis of structure proteins, decreased glycolytic metabolism in higher quality meat. The results expand our knowledge of the plasma and muscle proteomes of a commercially important muscle in Limousin-sired crossbred bulls. As a next step, the putative biomarkers identified in this study should be further evaluated and validated in a larger group of animals, with a view to refining explanatory biomarkers panels for the prediction of beef quality.

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Conflicts of Interest

The authors declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material

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Table 1. Sensory quality scores of the texture attributes evaluated by trained panellists on the meat steaks from young Limousin bulls (n = 23) and selected animals (n = 5 per group).

	Quality traits[1]	**Mean**	**SD**	**Min**	**Max**
n = 23 All animals	Tenderness	6.99	0.30	6.44	7.57
	Juiciness	6.23	0.44	5.56	7.00
	Chewiness	2.91	0.32	2.43	3.56
n = 5 per group of the selected bulls	Tenderness (+)	7.39	0.11	7.29	7.57
	Juiciness (+)	6.65	0.15	6.43	6.78
	Chewiness (-)	2.58	0.13	2.43	2.78
	Tenderness (-)	6.60	0.10	6.44	6.70
	Juiciness (-)	5.96	0.19	5.78	6.20
	Chewiness (+)	3.30	0.20	3.10	3.56

[1] A structured scale from 1 to 10 was used to score the attributes.

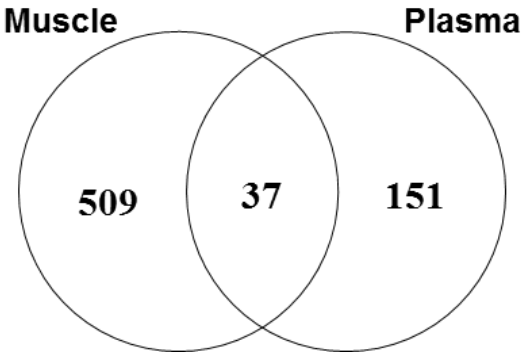

Fig. 1. Venn diagram highlighting the number of common proteins identified by LC-MS/MS in *Longissimus thoracis et lumborum* muscle and plasma from the 10 animals with divergent meat texture traits.

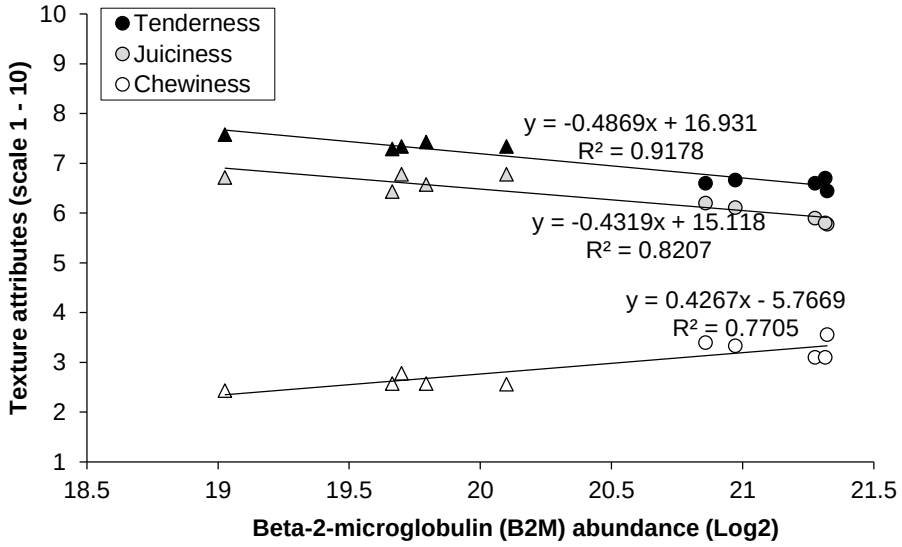

Fig. 2. Example of significant correlation between the abundance of Beta-2-microglobulin (B2M) identified in plasma and the three texture attributes. The high quality samples are shown by triangles (Δ) and low quality by circles (○). The R-squared values of each correlation are given.

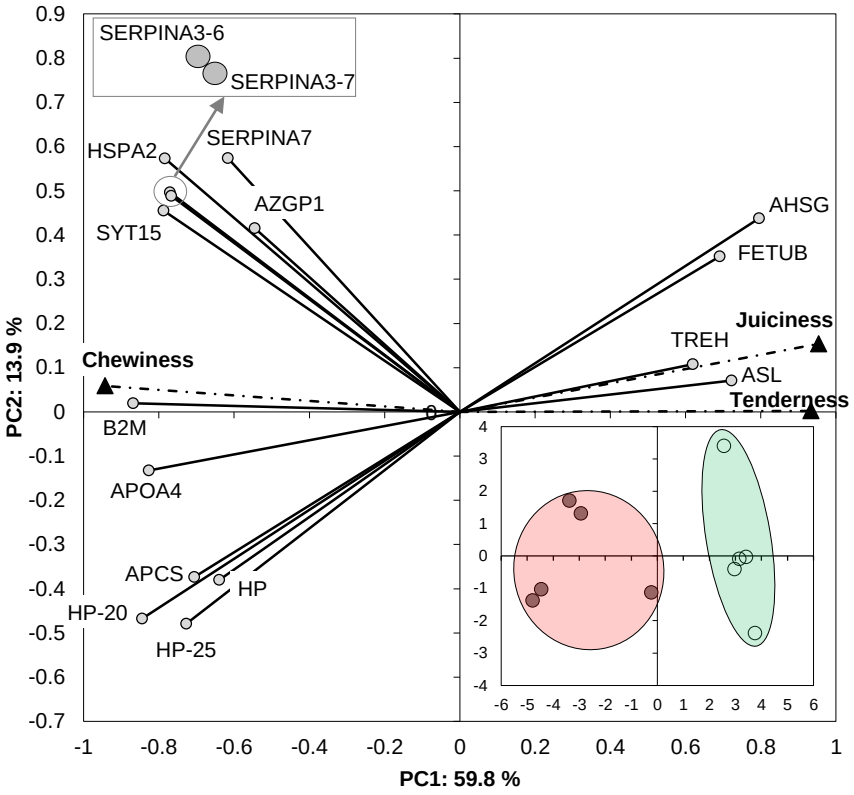

Fig. 3. Projection on a principal component analysis (PCA) of the 16 plasma proteins (using their gene names) differentially abundant between the texture groups (e.g., tender and tough samples) (full information in **Table 2**). The three texture attributes (tenderness, juiciness and chewiness) were shown with the proteins and highlighted by black triangles. An insert, bi-plot, at the bottom right of the PCA is given to show the extent of separation and distribution of the 10 individuals (n = 5 per group). An overall Kaiser-Meyer-Olkin measure of sampling adequacy of 0.59 was computed.

Table 2. List of the plasma proteins (n = 16) identified as being significantly different among the two texture groups and their correlations with tenderness, juiciness and chewiness attributes evaluated by trained panellists.

Uniprot ID	Gene Name	Full protein name	Biological pathway	Differences		Spearman correlations[1]		
				Fold change (Log2)	*P*-value	Tenderness	Juiciness	Chewiness
P01888	B2M	Beta-2-microglobulin	Immunity	-1.46	0.000	-0.81**	-0.83**	0.79*
Q2TBU0	HP	Haptoglobin	Immunity	-2.48	0.064		-0.75*	
P12763	AHSG	Alpha-2-HS-glycoprotein	Protease inhibitor	0.39	0.017		0.77*	
Q58D62	FETUB	Fetuin-B	Protease inhibitor	1.35	0.044	0.73*	0.70*	
A2I7N2	SERPINA3-6	Serpin A3-6	Protease inhibitor	-1.24	0.055		-0.64^{t}	0.64^{t}
A2I7N3	SERPINA3-7	Serpin A3-7	Protease inhibitor	-2.02	0.063	-0.62^{t}	-0.57^{t}	0.70*
Q3SYR0	SERPINA7	Serpin peptidase inhibitor, clade A, member 7	Protease inhibitor	-0.73	0.096	-0.62^{t}		
Q2KIT0	-[3]	Protein HP-20 homolog	Hibernation	-0.88	0.004	-0.82**	-0.92***	0.73*
Q2KIX7	-[4]	Protein HP-25 homolog 1	Hibernation	-0.57	0.021	-0.80**	-0.92***	0.65*
Q3T004	APCS	Serum amyloid P-component	Metal (Calcium)-binding	-1.62	0.040		-0.78*	0.63^{t}
E1BCH0	SYT15[2]	Uncharacterized protein	Calcium ion binding	-1.59	0.066	-0.60^{t}	-0.58^{t}	
F1N3Q7	APOA4	Apolipoprotein A-IV	Lipid transport	-0.46	0.005	-0.80**	-0.85**	0.74*
P34933	HSPA2	Heat shock-related 70 kDa protein 2	Stress response	-1.02	0.026			0.60^{t}
Q3ZCH5	AZGP1	Zinc-alpha-2-glycoprotein	Antigen processing	-0.72	0.044			
Q3SZJ0	ASL	Argininosuccinate lyase	Amino-acid biosynthesis	0.97	0.062		0.62^{t}	
E1B8N4	TREH	Trehalase	Glycosidase	0.02	0.094	0.66*	0.78*	-0.79*

[1] Significance of the correlations: *t*: tendency ($P < 0.1$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$;

[2] This protein should be a synaptotagmin-15-like isoform X1.

[3] The gene name of this protein was designated in the manuscript as HP-20.

[4] The gene name of this protein was designated in the manuscript as HP-25.

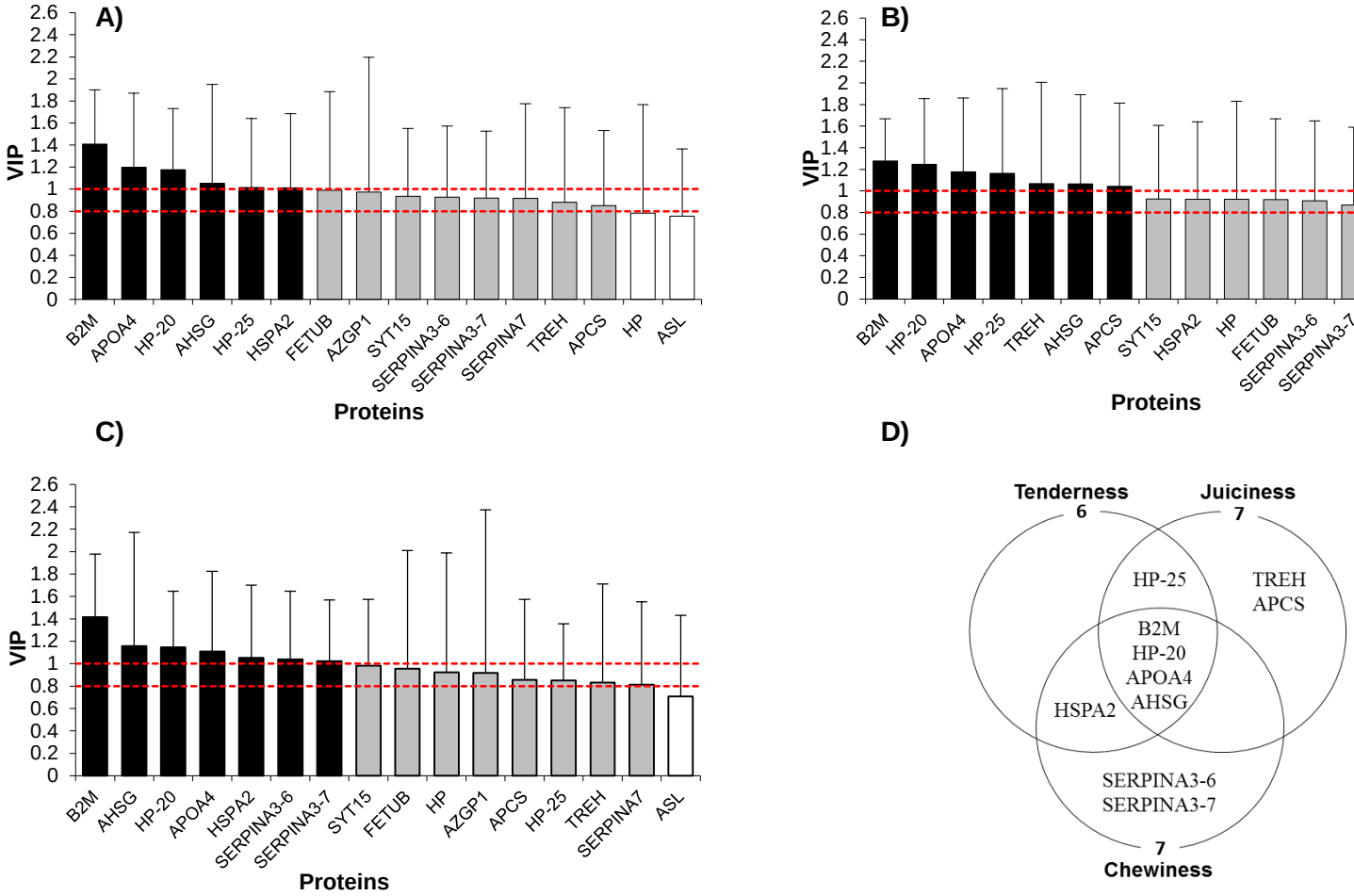

Fig. 4. Partial Least Squares (PLS) regressions between **A)** tenderness; **B)** juiciness and **C)** chewiness with the 16 putative plasma protein biomarkers. The dashed red lines highlight the cut-off by VIP >1.0 (black histograms), VIP > 0.8 (grey histograms) and VIP < 0.8 (white histograms). **D)** Venn diagram highlighting the overlap in the retained proteins from the three PLS regressions to explain the variability in the sensory texture attributes based on VIP > 1.0 only. Four proteins with VIP > 1.0 were common for the three quality traits, these being B2M, HP-20, APOA4 and AHSG. Full information on the proteins presented by their gene names are given in **Table 2**.

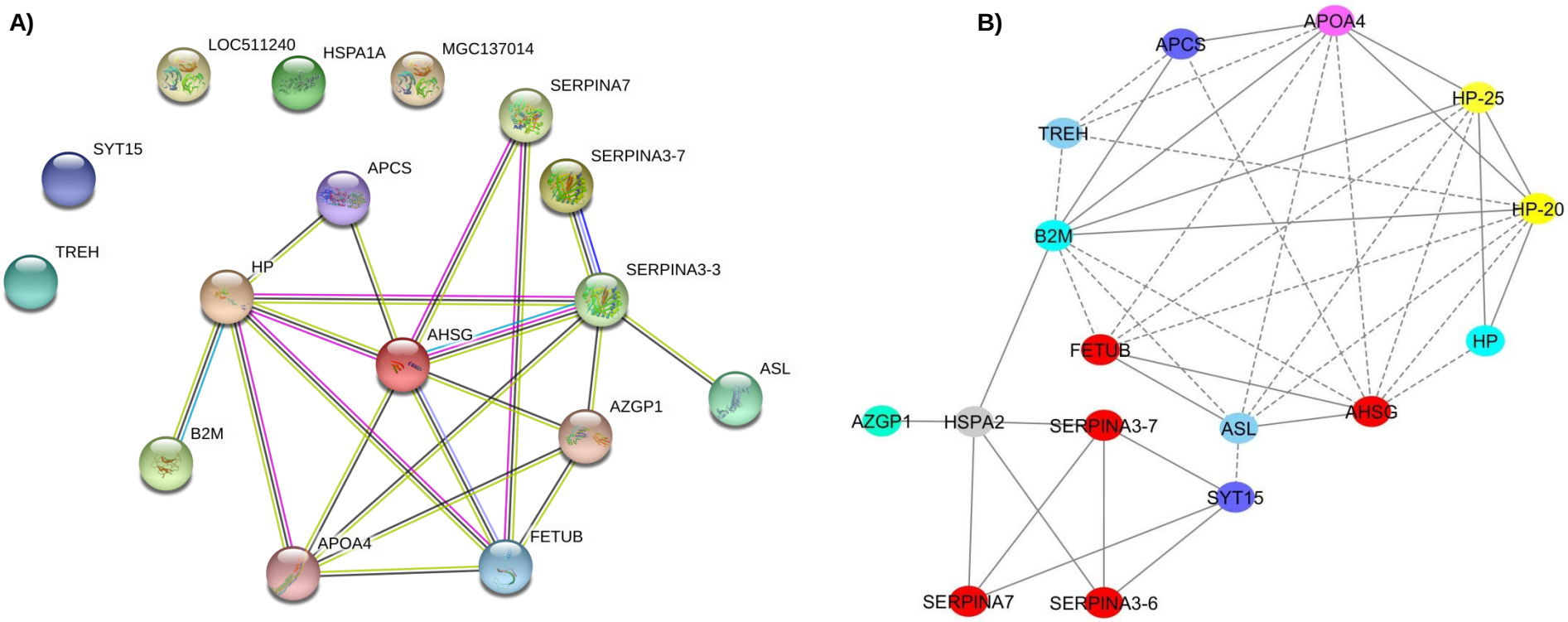

Fig. 5. Protein – Protein interactions networks among the 16 differentially abundant proteins in the plasma. **A)** A protein network built using the STRING database (https://string-db.org/). **B)** A protein correlation network built using the significant ($P < 0.05$) correlations among the 16 proteins. The solid and dash lines in this network represent positive and negative correlations, respectively. The proteins are colored following the biological pathway at which they belong: ● immunity; ● protease inhibitor; ● hibernation; ● calcium binding; ● lipid transport; ● stress response; ● enzymes & hydrolysis and ● antigen processing.

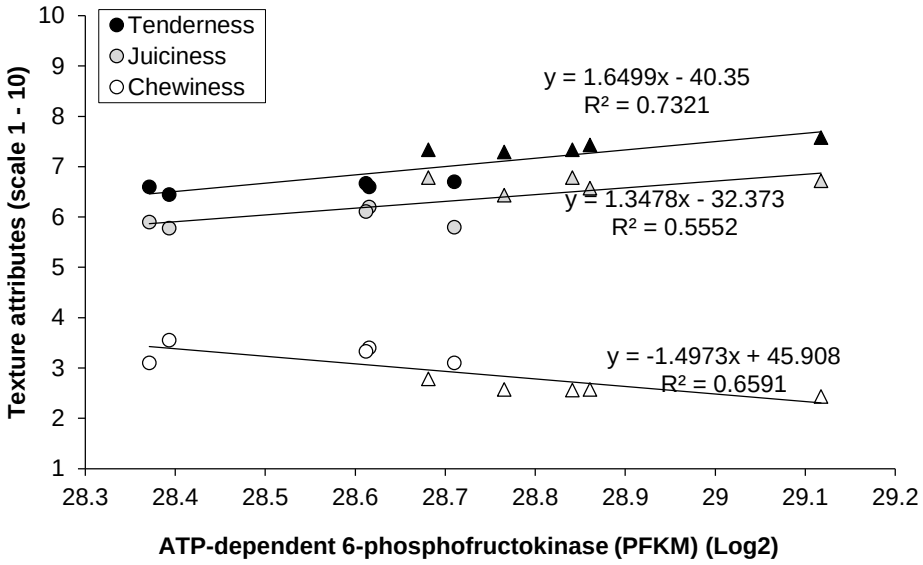

Fig. 6. Example of correlation between the abundance of ATP-dependent 6-phosphofructokinase (PFKM) identified in muscle and the three texture attributes. The high quality samples are shown by triangles (Δ) and low quality by circles (○). The R-squares of each correlation are given.

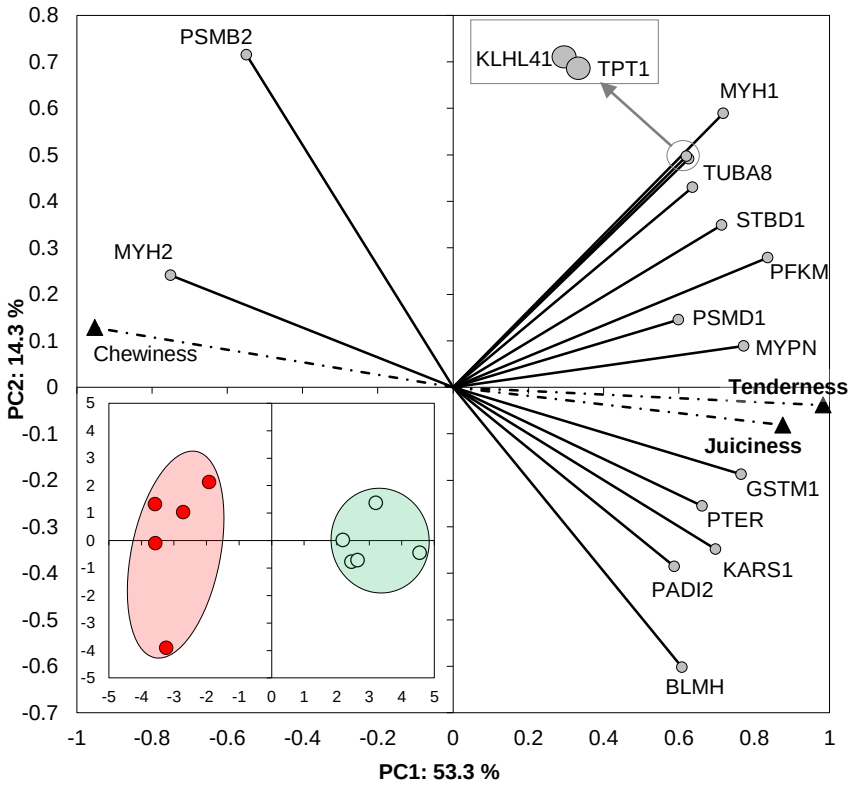

Fig. 7. Projection on a principal component analysis (PCA) of the 15 muscle proteins (using their gene names) differentially abundant between the texture groups (e.g., tender and tough samples) (full details on the proteins are in **Table 3**). The three texture attributes (tenderness, juiciness and chewiness) were also projected with the proteins and highlighted by black triangles. An insert at the bottom left of the PCA is given to show the separation and distribution of the 10 individuals (n = 5 per group) used in this trial. An overall Kaiser-Meyer-Olkin measure of sampling adequacy of 0.64 was computed.

Table 3. List of the muscle proteins (n = 15) identified as being significantly different among the two texture groups and their correlations with tenderness, juiciness and chewiness attributes evaluated by trained panellists.

Uniprot ID	Gene Name	Full protein name	Biological pathway	Differences		Spearman correlations[1]		
				Fold change (Log2)	*P*-value	Tenderness	Juiciness	Chewiness
Q9BE40	MYH1	Myosin-1	Cytoskeleton	0.47	0.059	0.75*		-0.72*
Q9BE41	MYH2	Myosin-2	Cytoskeleton	-0.47	0.014	-0.76*	-0.74*	0.92***
Q2HJB8	TUBA8	Tubulin alpha-8 chain	Cytoskeleton	0.32	0.074			
F1N0L9	MYPN	Myopalladin	Cytoskeleton	0.37	0.021	0.60^{t}	0.64^{t}	-0.66*
E1BAJ4	KLHL41	Kelch-like protein 41	Cytoskeleton	0.23	0.035	0.57^{t}	0.57^{t}	-0.57^{t}
E1BL29	BLMH	Bleomycin hydrolase	Proteolysis	0.32	0.041	0.57^{t}	0.58^{t}	-0.57^{t}
Q5E9K0	PSMB2	Proteasome subunit beta type-2	Proteolysis	-0.46	0.089		-0.58^{t}	
A7MBA2	PSMD1	26S proteasome non-ATPase regulatory subunit 1	Proteolysis	0.33	0.095			
Q0IIG5	PFKM	ATP-dependent 6-phosphofructokinase, muscle type	Metabolism/Glycolysis	0.31	0.014	0.91***	0.67*	-0.85**
E1BAJ4	STBD1	Starch-binding domain-containing protein 1	Metabolism/Autophagy	0.59	0.026	0.71*		-0.65*
F1N048	PADI2	Protein-arginine deiminase type-2	Hydrolase	1.51	0.052			-0.58^{t}
A6QLJ8	PTER	Phosphotriesterase-related protein	Hydrolase	0.54	0.058	0.80**	0.68*	-0.80**
Q9N0V4	GSTM1	Glutathione S-transferase Mu 1	Glutathione metabolic process	0.45	0.019		0.57^{t}	-0.75*
F1MMK8	KARS1	Lysine--tRNA ligase	Protein biosynthesis	0.44	0.021		0.56^{t}	-0.64^{t}
Q5E984	TPT1	Translationally-controlled tumor protein	Calcium ion binding	0.41	0.083			

[1] Significance of the correlations: *t*: tendency ($P < 0.1$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

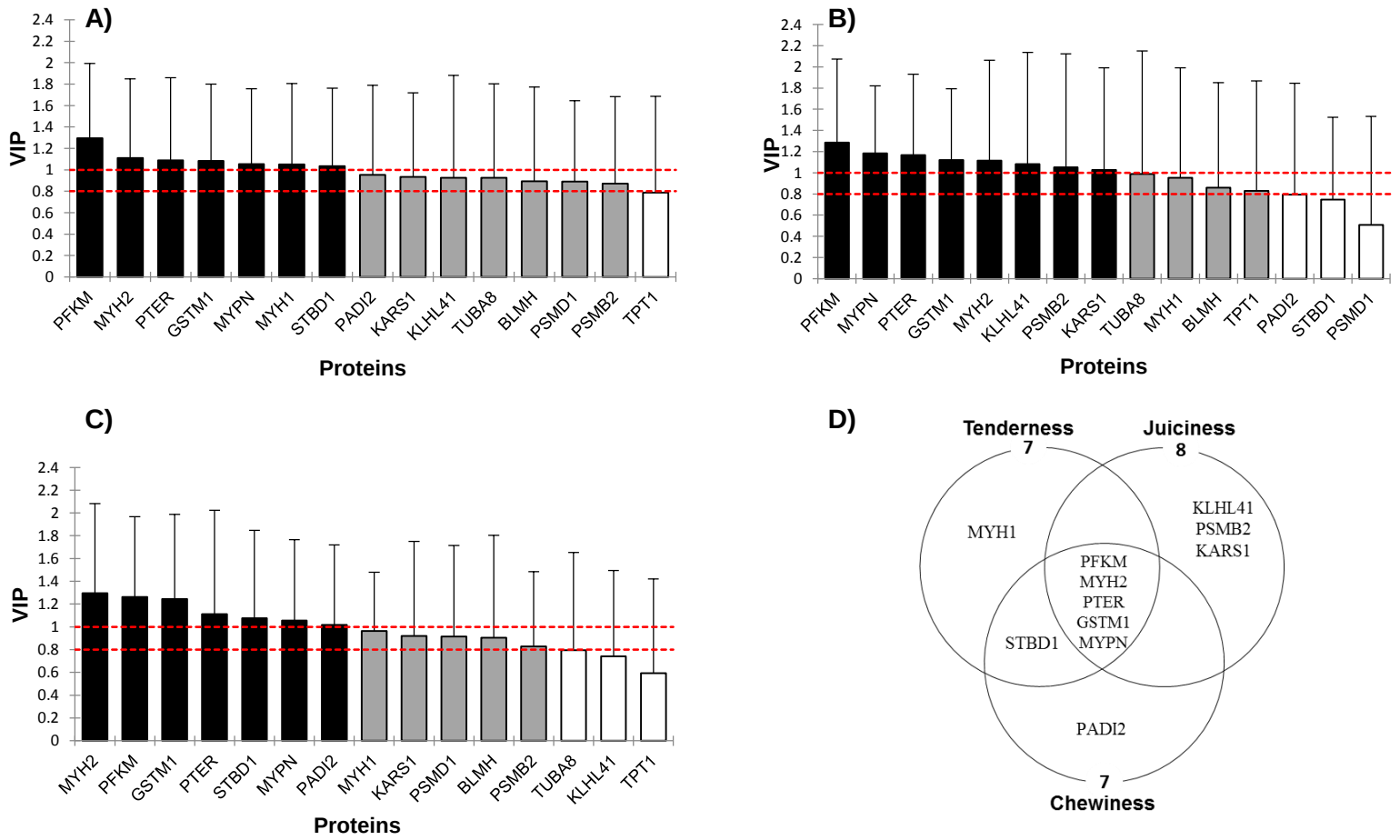

Fig. 8. Partial Least Squares (PLS) regressions between **A)** tenderness; **B)** juiciness and **C)** chewiness with the 15 putative muscle protein biomarkers. The dashed red lines highlight the cut-off by VIP >1.0 (black histograms), VIP > 0.8 (grey histograms) and VIP < 0.8 (white histograms). **D)** Venn diagram highlighting the overlap in the retained proteins from the three PLS regressions to explain the variability in the sensory texture attributes based on VIP > 1.0 only. Five proteins with VIP > 1.0 were common for the three quality traits, these being PFKM, MYH2, PTER, GSTM1 and MYPN. Full information on the proteins presented by their gene names are given in **Table 3**.

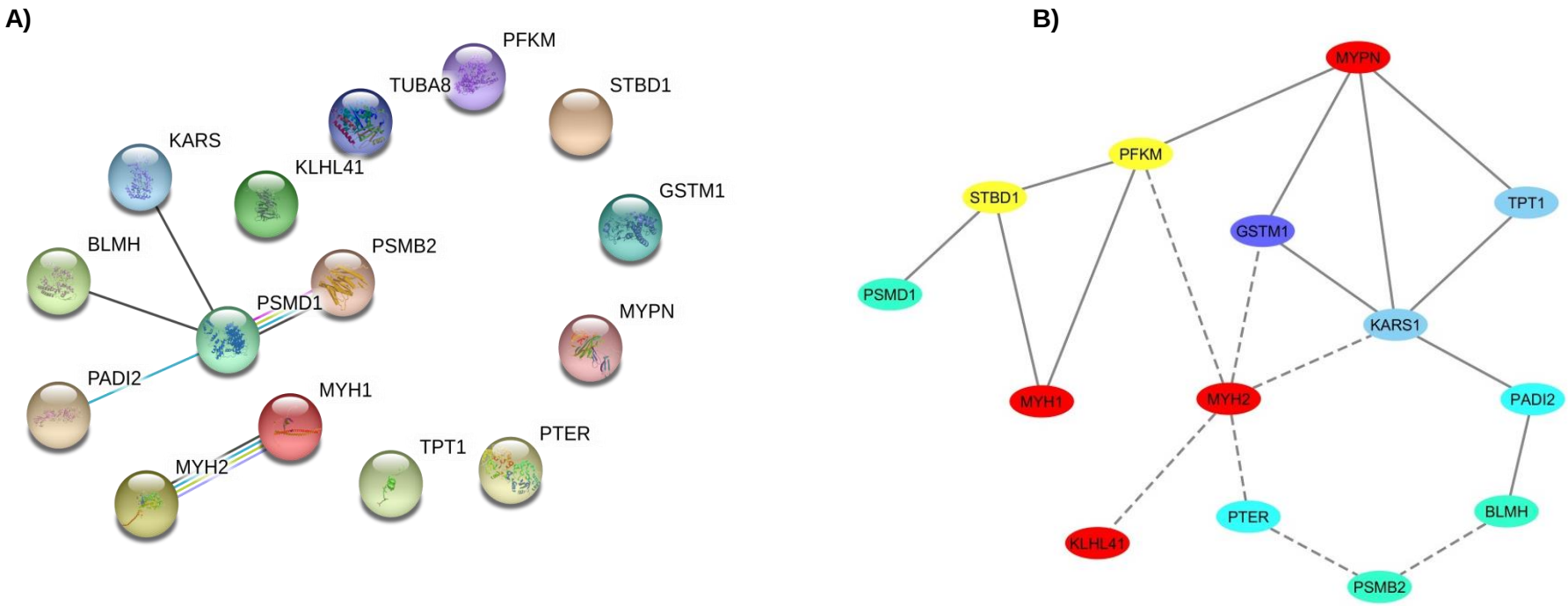

Fig. 9. Protein – Protein interactions networks among the 15 differentially abundant proteins in the *Longissimus thoracis* muscle. **A)** A protein network built using the STRING database (https://string-db.org/). **B)** A protein correlation network built using the significant ($P < 0.05$) correlations among the 15 proteins. The solid and dash lines in this network represent the positive and negative correlations, respectively. The proteins are colored following the biological pathway at which they belong: ● cytoskeletal; ● proteolysis. ● metabolism; ● hydrolases; ● glutathione metabolic process; and ● protein biosynthesis and calcium binding.

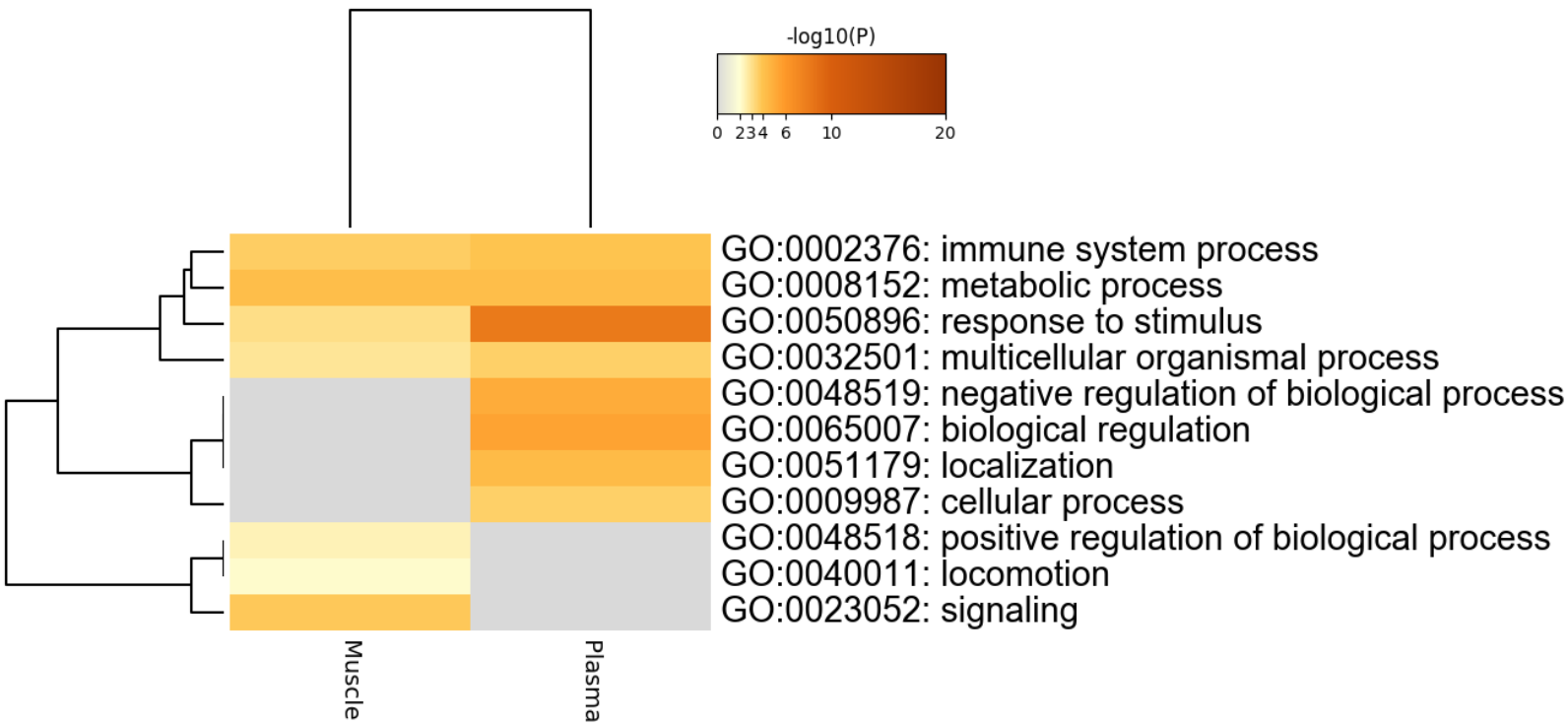

Fig. 10. Heat map showing the significant enriched Gene Ontology (GO) terms comparing based on the proteins identified to be significantly different among the two texture groups from both plasma and muscle. A discrete colour scale by *P*-values to represent statistical significance was used. Grey boxes indicate a lack of significance. Four GO terms were common for plasma and muscle, being immune system process, metabolic process, response to stimulus and multicellular organismal process.