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Effects of mixing disbudded and horned young bulls during rearing on the post-mortem *Longissimus thoracis* muscle proteome

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ABSTRACT

Eighty-one young Swiss cross-bred bulls, half of which were disbudded at 7 weeks of age, were reared in 6 rearing groups. Using a balanced design, these groups contained only horned, or only disbudded bulls (unmixed groups), or both (mixed groups), with only half of the bulls disbudded. They were slaughtered using two protocols, one with limited, the other with supplementary stress. *Longissimus thoracis* (LT) muscle samples were collected 48 h after slaughter and subjected to individual shotgun proteomic analysis. Results show that horn status and slaughter conditions influenced only 16 (6.2 %) and 8 (3.1 %) proteins, respectively, which is not more than expected by random error. By contrast, rearing conditions influenced 40 (15.5 %) of the identified proteins, which is significantly more than expected by random error. Eighteen of these differentially abundant proteins were involved in energy metabolism, and 9 in muscle structure and contraction. Overall, these proteins indicated greater glycolytic capacity and greater proportions of fast twitch fibres in the LT of bulls in mixed groups. These results are coherent with the lower physical activity and different physiological stress reactivity of these same mixed groups of bulls as reported in previous companion studies.

1. Introduction

Rearing practices influence muscle physiology with multiple consequences for meat quality traits. For example, feeding regime can influence the developmental pathways involved in meat quality determination as well as the intrinsic properties of muscles, translating into variation in toughness, colour and fat content of resultant meat (Oddy et al., 2001; Soulat et al., 2016). Some rearing practices in beef production systems involve the removal of horns to increase stocking density and to reduce the risk of injuries to handlers and other animals by horned animals (Schoiswohl et al., 2020). Horns are used by animals in defence reactions to intimidate, or inflict damage on other animals within the herd (Knierim et al., 2015). The size of horns conveys an *a priori* dominance status. Cattle can differentiate horn size classes, creating or reinforcing a given social structure within a herd, which allows maintaining the social dominance status of an individual (Goonewardene et al., 1999). Cattle with horns tend to keep greater inter-individual distances and exhibit fewer physical interactions than cattle without horns (Knierim et al., 2015). Compared to horned individuals, disbudded cattle tended to express more agonistic behaviour, leading to more stable social relationships in horned groups under suitable management conditions (Knierim et al., 2015; Waiblinger et al., 2000). The absence or presence of horns can influence the animal's behaviour and physiology (Knierim et al., 2015; Reiche et al., 2019; Reiche et al., 2020). The composition of the group regarding horn status may also influence behaviour. Rearing groups containing both horned and disbudded bulls were less active and less reactive compared to groups containing only horned, or only disbudded bulls (Reiche et al., 2020). The absence or presence of horns influenced further meat quality. Particularly, within the non-mixed groups, disbudded bulls had

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significantly tenderer meat and longer sarcomeres than horned bulls (Reiche et al., 2019).

Meat quality is also influenced by slaughter conditions, due to the effects of stress (Ferguson & Warner, 2008; Sierra et al., 2021). Stress is described as a negative emotional state of an animal in the situation of a real or imagined threat (Terlouw et al., 2021). For cattle, disruption of social groups, handling practices, transportation, loading, unloading, unfamiliar conditions and mixing animals are important pre-slaughter stress factors (Ferguson & Warner, 2008; Terlouw et al., 2021). Stress causes behavioural and physiological reactions that modify ante-mortem metabolism of the animal before slaughter, which in turn influences post-mortem biochemical processes involved in the conversion of muscle into meat (Reiche et al., 2019; Terlouw et al., 2021). Lastly, rearing and slaughter conditions may influence meat quality interactively, as rearing conditions may influence responsiveness to stress (Díaz et al., 2020; Gagaoua et al., 2019). Greater stress reactivity increases the stress reactions during the pre-slaughter period, and hence, the effect of a given stress on meat quality (Díaz et al., 2020; Lensink et al., 2000; Terlouw et al., 2021).

The mechanisms and pathways underlying the transformation of muscle into meat are only partly known. To increase our understanding, high throughput techniques such as omics can be very useful (Gagaoua et al., 2024). Proteomics is the large-scale analysis of proteins including their identification, localization and interactions along with the quantitative and qualitative analyses of the variation in proteins (Gagaoua et al., 2024). They have provided insights in the muscle physiology and structure, and metabolic responses during the *post-mortem* period and their relationship with meat quality (Gagaoua et al., 2021). Several studies have shown that the muscle proteome varies according to the rearing and slaughter context (Díaz et al., 2020; Mouzo et al., 2020; Sierra et al., 2021). The present study evaluated the effects of horn status (horned versus disbudded bulls), rearing conditions (mixing, versus nonmixing, of bulls with different horn status) and slaughter conditions (higher versus lower pre-slaughter stress levels) on the post-mortem *Longissimus thoracis* muscle proteome of young bulls using shotgun proteomics to evaluate proteins and the pathways underlying the conversion of muscle to meat.

2. Materials and methods

The experiment respected the Swiss laws of animal protection and was authorized by the cantonal veterinary office of Fribourg, Switzerland (No. 2015_21_FR).

2.1. Animals and rearing conditions

The study was conducted on eighty-one crossbreed bulls (Limousin × Swiss dairy breeds) using a balanced design with two replicates (replicate 1: $n = 40$; replicate 2: $n = 41$). Behavioural and physiological results of the animals of this study have been published earlier (Reiche et al., 2019; Reiche et al., 2020). Calves were purchased at an average age of five weeks with a one-week delay between the two replicates. Half of the animals ($n = 42$) were disbudded at the age of seven weeks using a hot iron under both local and systemic analgesia as previously described (Reiche et al., 2019). The calves of each replicate were subsequently allocated to one of three rearing groups with 13 or 14 calves per pen, balanced for body weight: unmixed with horns ($n = 27$), unmixed without horns (n = 27) and mixed (n = 27; 50 % with horns, 50 % without horns). Hence, the calves differed in terms of Horn Status (HS: with or without Horns) and Rearing Conditions (RC: mixed or unmixed). Each of the 6 rearing groups was kept in a different room where animals did not have visual or physical contact with other rearing groups. All pens were identical and situated in the same building. Animals were kept on deep litter and had access to an outdoor pen.

Starting from the arrival date of the calves until their weaning, the space allocated was 3.9 m^2/calf. During this period, calves were given a milk replacer, ad libitum hay and a commercial compound feed (UFA 117 Prevacox, UFA, Herzogenbuchsee, Switzerland). After weaning, calves were allocated 9.4 m^2/calf and fed a roughage based fattening diet composed of hay, corn silage and concentrates (corn and rape), formulated for an average daily gain of 1300 g/d according to the Swiss feeding recommendation (Agroscope, 2016).

2.2. Slaughter conditions and muscle sampling

All animals were slaughtered at the age of 13 months (live weight 548.2 ± 4.6 kg). Slaughter was carried out over several days, with controlled levels of slaughter stress. As changes in group composition, including removal of animals, generally represents a stress for the remaining animals, it was necessary to slaughter all animals of a pen on the same day (Bouissou, 1980; Bourguet et al., 2010). Hence, each slaughter day, the bulls of one entire pen were slaughtered under two slaughter conditions (SC), half using a supplementary stress protocol (Supplementary Stress: $n = 40$) and half using a limited stress protocol (Limited Stress: $n = 41$). On the slaughter day, bulls to be slaughtered were feed deprived from 06:00 AM onwards. Bulls slaughtered under limited stress and supplementary stress conditions were transported separately, each in a cattle truck of 11 m^2 to a commercial slaughterhouse situated 45 min away from the farm. The bulls of the Limited Stress condition were transported at 08:00 AM. After they arrived at the slaughterhouse, they waited for 5–10 min in the truck until unloading. Then they were guided into a quiet waiting pen of approximately 20 m^2, partially visually separated from the other waiting pens at the slaughterhouse. Two hours later, these bulls were guided into the slaughter corridor and slaughtered immediately. Slaughter involved introduction of the animal into the stunning box, followed by stunning with a captive bolt pistol, hoisting and bleeding. Further details can be found in Reiche et al. (2019).

Bulls of the Supplementary Stress condition left the farm 2 h later than the bulls of the Limited Stress condition. After arrival at the slaughterhouse, they waited for 30 min in the truck before unloading. The bulls were then unloaded directly into the slaughter corridor and were driven back and forth in the slaughter corridor for 30 min by two purposely noisy unfamiliar experimenters (shouting and hitting metal bars with a stick), following a predetermined protocol to provoke additional emotional stress and physical activity. Bulls of both slaughter conditions remained within their slaughter group and were not in contact with unfamiliar animals until stunning. Hence, each slaughter day corresponded to slaughtering all animals of one rearing group, with approximately 7 bulls in the Supplementary and 7 in the Limited Stress group. Hot carcasses were weighed within 30 min after slaughter and were then chilled at 2 °C. Carcass weights were 309.5 ± 2.7 kg. For further details, see Reiche et al. (2019).

Forty-eight hours *post-mortem*, 20 cm of the *Longissimus thoracis* (LT) muscle were excised with a sterile scalpel from the region between 10th and 12th ribs of the left side of the carcass and immediately frozen in liquid nitrogen and stored at −80 °C for various determinations (Reiche et al., 2019), including shotgun proteomics analysis, which took place 2 years after sample collection.

2.3. Shotgun proteomics analysis on the post-mortem muscle samples

2.3.1. Protein extraction

Frozen muscle tissue samples (150 mg) were first 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. (2004). To remove non-extracted cellular components, fat, insoluble proteins, the protein homogenates were incubated with shaking for 30 min at 4 °C followed by a 30 min centrifugation at 10,000 ×*g* as previously described (Lamri et al., 2023). The supernatant was then transferred into Eppendorf tubes for protein

quantification using the dye-binding protocol of Bradford, (1976).

2.3.2. One dimensional SDS-PAGE and protein bands preparation

For the preparation of the protein bands for shotgun proteomics analysis, the protein extracts were mixed (1:1) with Laemmli sample buffer (Bio-Rad Laboratories, Deeside, UK) as recently described (Lamri et al., 2023). Then, the extracts were concentrated in duplicates on 1D stacking gel of sodium dodecyl sulphate polyacrylamide gel electrophoresis (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). In each gel lane, 20 μg proteins were loaded and the electrophoresis was run at 4 watts for about 15 min to concentrate the proteins in the stacking gel (Zhu et al., 2021).

The gels were washed three times with Milli-Q water, stained with EZ Blue Gel staining reagent (Sigma, Saint Louis, MO, USA) with gentle shaking for 2 h then washed with Milli-Q water. The immobilised proteins in the 1D gel bands were discoloured/reduced-alkylated (Lamri et al., 2023). The protein bands were excised from the washed gels using a sterile scalpel and immediately placed into Eppendorf tubes. A treatment with 50 mM ammonium bicarbonate - 50 % ethanol during 20 min at room temperature was applied and after removal of this buffer, bands were dehydrated with 100 μL of 100 % ethanol. Reduction of the disulfide bonds was performed with 200 μL of 10 mM dithiothreitol (Sigma-Aldrich) in 50 mM ammonium bicarbonate buffer for 30 min at 56 °C and alkylation of proteins was carried out with 200 μL of 55 mM iodoacetamide (Sigma-Aldrich, Saint Louis, USA (MO)) in 50 mM ammonium bicarbonate buffer for 30 min in darkness. The destaining step was carried out using 200 μL of 25 mM ammonium bicarbonate (Sigma, Saint Louis, MO, USA) -5 % acetonitrile for 30 min before two washing steps using 200 μL of 25 mM ammonium bicarbonate 50 % acetonitrile for 30 min each. Finally, they were dehydrated with 100 % acetonitrile for 10 min, and the liquid was discarded. The immobilised proteins in the 1D gel bands were discoloured/reduced-alkylated as recently described (Lamri et al., 2023). The dried protein bands were stored at −20 °C until LC-MS/MS analysis.

Hydrolysis of the protein bands was carried out with 48 μL of a 25 mM ammonium bicarbonate buffer −12.5 ng/ μL trypsin solution (Promega) per band for 5 h in an oven at 37 °C. 30 μL buffer was added periodically during hydrolysis so that the bands did not dry out. The extraction of the peptides was carried out under ultrasound (25 min) with 48 μL acetonitrile and trifluoroacetic acid. The supernatant was transferred into 500 μL Eppendorf tubes and dehydrated using a Speedvac for 2 h. The volume was adjusted to exactly 20 μL with a solution of isotopologic peptides (50 pmol/μL) that was diluted 18 times in a 0.05 % Trifluoroacetic acid (TFA) solution. After passing through an ultrasonic bath (10 min), the entire supernatant was transferred to a High-Performance Liquid Chromatography (HPLC) vial before LC-MS/MS analysis.

2.3.3. LC-MS/MS analysis

For the separation, 6 μL of the hydrolysate was injected into a nano HPLC (Ultimate 3000, Dionex) which is coupled via a nano ESI source to a mass spectrometer of type Orbitrap Q Extractive HFX (Thermo Fisher Scientific) functioning in a data dependent mode. Briefly, 6 μL of hydrolysate was first pre-concentrated and desalted at a flow rate of 30 μL/min on a C18 pre-column 5 cm length × 100 m (Acclaim PepMap 100 C18, 5 μm, 100 Å 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 put in line with a nano debit analytical column operating at 400 nL/min. The peptides were then separated according to their hydrophobicity (column C18, length 25 cm, diameter 75 μm, SN 10711310), using a gradient of a solution of acetonitrile (ACN/FA-99.9/0.1) of 4 to 25 % for 50 min. For MS analysis, eluted peptides were electrosprayed in positive-ion mode at 1.6 kV through a Nano electrospray ion source heated to 250 °C. The mass spectrometer operated in data-dependent mode: the parent ion is selected in the orbitrap cell (FTMS) at a resolution of 120,000, and 18 MS/MS succeeds each MS analysis with analysis of the MS/MS fragments at a resolution of 15,000).

The raw files from the LC-MS/MS were aligned against the *Bos taurus* database (i.e.,ref_bos_taurus, 23,970 sequences) with Mascot V.2.5.1 (http://www.matrixscience.com, accessed on 07/11/2019). The precursor and fragment mass tolerance were set up at 10 ppm and 0.02 Da, respectively. The variable modifications included carbamidomethylation (C), oxidation (M) and deamidation (NQ). Protein identification could be verified when at least two peptides derived from one protein showed statistically significant identity. The Mascot score was 35 with a False Discovery Rate of 1 %, and the *P*-value was adjusted to a given threshold (0.007851).

2.4. Statistical analyses

The generated protein abundance dataset was subjected to a Log2 transformation and mean normalisation. The proteomics data were statistically analyzed using XLSTAT 2021.4 (AddinSoft, Paris, France). To study the influence of the three treatment factors on the protein abundances, the data were subjected to ANOVA. Slaughter day was confounded with rearing group and replicate because each slaughter day corresponded to slaughtering of one rearing group and animals of a given rearing group belonged to one of the two replicates. Therefore, initial analyses included only slaughter day as random factor using PROC MIXED in SAS (SAS System 9.0). As this random factor was not significant in any of the analyses, it was removed from the final analysis. Subsequently, although the effect of replicate was not a research question, it was included as fixed rather than random effect because there were only two levels. A random factor needs at least 5 or 6 levels. Hence, the ANOVA included replicate and three treatment factors: RC, SC, HS and their interactions (RC*SC, RC*HS, SC*HS, RC*SC*HS, as well as the 2 and 3-way interactions with replicate). Replicate was removed from the model if it had no significant effect. The two-tailed sample z-test for proportions without continuity correction was used (using an expected proportion of 0.05) to test for each treatment factor whether the percentage of proteins significantly influenced significantly exceeded 5 % of the total amount identified. The Heatmap function from the "ComplexHeatmap" library R Statistical Software (v4.1.2; R Core Team 2021) was used to create a heatmap of significant correlations.

2.5. Bioinformatics analyses

For the bioinformatics analyses, the differentially abundant proteins were analyzed for their interrelationships by building protein-protein interactions (PPI) networks using the STRING database (https://string-db.org/). We used the default settings, medium confidence of 0.4 and 4 criteria for linkage: co-occurrence, experimental evidence, existing databases, and text mining. Since the existing data resources for *Bos taurus* Gene Ontology (GO) are limited, we used human Uniprot IDs orthologous for this analysis, to benefit from the most complete annotations available (Gagaoua, Warner et al., 2021). Furthermore, Gene Ontology (GO) pathway and process enrichment analyses were conducted using the Metascape® webservice tool (https://metascape.org/; Zhou et al., 2019) to investigate the main enriched GO terms (Biological Processes) from the differentially abundant proteins influenced by the three treatment factors (RC, SC and Horns). Where relevant, Cytoscape (version 3.10.3) was used to map expression correlation between proteins influenced by treatments, to visualise their quantitative relationships as expressed by Pearson correlations. Finally, ingenuity pathway analysis on IPA system (version 42,012,434, Ingenuity Systems; Qiagen China Co., Ltd.) was used to identify canonical pathways, molecular function, regulator effects, upstream regulators and molecular networks associated with the treatment factors. For canonical pathway analysis, disease and function, the −log (*P*-value) >2 was taken as threshold, the

Z-score > 2 was defined as the threshold of significant activation, whilst Z-score < −2 was defined as the threshold of significant inhibition. For regulator effects and molecular networks, consistency scores were calculated, where a high consistency score indicates accurate results for the regulatory effects analysis. For upstream regulators, the P-value of overlap <0.05 was set as the threshold. The algorithm used for calculating the Z-scores and *P*-values of overlap has been described previously (Krämer et al., 2014).

3. Results

Two-hundred and fifty-eight proteins were quantified in this study.

3.1. Effect of rearing condition on the longissimus thoracis muscle proteome

RC significantly influenced the abundance of 40 proteins (z-test for proportions: $p < 0.0001$; Table 1). Twenty-three of these proteins had greater and 17 had lower abundances in mixed groups (Table 1). An interaction with replicate was found for 6 proteins ($P < 0.05$). The interaction was due to an absence of effect of RC in replicate 2 (ACTN1 ($P = 0.03$; Alpha-actinin-1) and PYGM ($P = 0.01$; Glycogen phosphorylase, muscle associated)), and to a larger effect in replicate 1 compared to replicate 2 for the other proteins (IMMT ($P = 0.04$; Inner Membrane Mitochondrial Protein), TUBB4A ($P = 0.05$; Tubulin beta-4A chain), NDUFS1 ($P = 0.001$; NADH: Ubiquinone Oxidoreductase Core Subunit S1), and PHB ($P = 0.01$; Prohibitin)).

Of the influenced proteins, 18 (45 %) were involved in energy metabolism, 9 (23 %) in muscle structure related pathways and 3 (7.5 %) in protein binding pathways (Fig. 1B). A GO terms enrichment analysis of the influenced proteins showed that the three most significant ontology terms relate to *energy derivation by oxidation of organic compounds* (GO:0015980), *glycogen metabolic processes* (GO:0005977) and the *tricarboxylic acid cycle* (GO:0006099) (Fig. 2**, C1**). Ten of the more abundant proteins were involved in energy metabolism related biological pathways, six of them were related to muscle structure biological pathways and two of them were related to protein binding biological pathways (Table 1).

The GO enrichment analysis comparing the pathways between proteins with different abundances between rearing groups revealed that "GO:0015980: *energy derivation by oxidation of organic compounds* was strongly influenced by RC. *Glycogen metabolic process* (GO:0005977) and *tricarboxylic acid cycle* related ontology terms (GO:0006099) were represented amongst proteins with greater abundances in mixed groups, while proteins involved in *mitochondrion organization* (GO:0007005) and *respiratory electron transport chain* (GO:0022904) had lower abundances in mixed groups (Figs. 2C**.2, 2C.3**).

The Protein-Protein Interaction (PPI) network (STRING database) of the proteins significantly influenced by RC showed a great number of interactions existing amongst those implicated in energy metabolism related biological pathways (Fig. 3A). The Cytoscape correlation network shows that the 40 RC-influenced proteins involved in the different functions as indicated in Table 1 showed also many quantitative relationships, within and between functions (Fig. 3B). The correlation heatmap of these proteins (Fig. 3C) shows various clusters of strong positive correlations, with amongst others a cluster containing proteins related to energy metabolism (NDUFS1, ATP5F1C (ATP synthase subunit gamma), ATP5H (ATP synthase subunit d)), a protein binding (PHB) and a miscellaneous protein (CHCHD3 (Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 3)) and another cluster containing proteins related to energy metabolism (ATP5B (ATP synthase subunit beta), IDH3A (Isocitrate dehydrogenase [NAD] subunit alpha), MT-CO2, HADHA), to calcium homeostasis (PHB) and a miscellaneous protein (IMMT) (Fig. 3C). Some smaller clusters of negative correlations were also found (Fig. 3C).

Upstream enrichment analysis using Ingenuity Pathway Analysis showed that three canonical pathways were found to be significantly activated in mixed groups. The detected signalling and metabolic pathways were associated with mitochondrial dysfunction, oxidative phosphorylation and the Tricarboxylic Acid (TCA) Cycle (Fig. 2C**.4**).

Based on the pattern of proteins with different abundances, upstream regulator analysis suggested that two master regulator proteins (TP53 (Tumor Protein p53, z-score of 1.817) and NR4A1 (Nuclear Receptor Subfamily 4 Group A Member 1, z-score of 2.414)) were putatively involved in regulation of muscle response to altered rearing condition. CMBL (Carboxymethylenebutenolidase homolog protein) and VIM (Vimentin) had both greater, and NDUFS1, lower abundances in the mixed groups. NDUFS1 is activated by both TP53 and NR4A1. Six other proteins, known to be inhibited by TP53, were up-regulated in the mixed groups: OGDH (Oxoglutarate Dehydrogenase), FH (fumarate hydratase) and SUCLA2 (Succinate-CoA Ligase ADP-Forming Subunit Beta) are involved in pathways related to energy metabolism, TMOD4 (Tropomodulin 4) is related to muscle structure, AHCY (Adenosylhomocysteinase) is related to protein binding and NPEPPS (Aminopeptidase Puromycin Sensitive) is related to proteolysis. Two of these, SUCLA2 and OGDH, are inhibited by TP53 but activated by NR4A1. Three more proteins, known to be activated by NR4A1, were influenced: PYGM was up-regulated in and IDH3A and ATP5B were down-regulated in the mixed groups. All these proteins are involved in energy metabolism related pathways which were activated.

3.2. Effect of horn status on the longissimus thoracis muscle proteome

HS significantly influenced sixteen proteins, which is not significantly different from 5 % of the 258 proteins (z-test for proportions: $P = 0.38$; Table 1). Of these proteins, 7 (43 %) were involved in energy metabolism related biological pathways, 3 (18 %) in oxidative stress related biological pathways and two (13 %) in muscle structure-related biological pathways (Fig. 1A). Eleven proteins were more abundant in the disbudded bulls (z-test: $P < 0.0001$). A GO enrichment analysis of the 16 proteins showed that the top ontology term was related to *generation of precursor metabolites and energy* (GO:0006091), followed by the ontology term related to *sulfur compound metabolic process* (GO:0006790) (Fig. 2A).

3.3. Effect of slaughter condition on the longissimus thoracis muscle proteome

SC significantly influenced 8 differentially abundant proteins, which is not significantly different from 5 % of the 258 proteins (z-test for proportions: $P = 0.16$; Table 1). The classification of the 8 proteins showed that only one was implicated in energy metabolism-related biological pathways, one was related to protein-binding biological pathways and one was implicated in oxidative stress related biological pathways (Fig. 1C). An interaction with replicate was found for FGB ($P = 0.01$), as the effect was only observed for replicate 2. A GO enrichment analysis of the ten differentially abundant proteins showed that the top ontology term was related to *regulation of body fluid levels* (GO:0050878) (Fig. 2B).

3.4. Overlap amongst the three treatment factors

Overall, 56 unique proteins were influenced by at least one of the three treatment factors. Twenty-one (38 %) of these proteins were linked to energy metabolism related biological pathways, eleven (20 %) involved in muscle structure related biological pathways and four (7 %) belong to protein-binding related biological pathways (**Fig. S1**). No protein was influenced by all three treatment factors (**Fig. S2A**), and five proteins were significantly influenced by both RC and HS, one by both SC and HS and two by RC and SC (**Fig. S2B**). The major GO term for the three protein lists was related to *energy derivation by oxidation of organic compounds* (**Fig. S2C**). The 11 enriched GO terms were used to create a

Table 1
LS means of differentially expressed proteins ($n = 56$) significantly influenced by Rearing Condition (RC), Slaughter Condition (SC) and Horn Status (HS) in the *Longissimus thoracis* muscle. Bold figures indicate greater contents compared to the counterpart treatment. Standard error of the means (SEM) is calculated across all animals. Where relevant, alternative abbreviations are between brackets (see Fig. 3A).

Uniprot ID	Gene Name	Full protein name	Horn Status (HS)		Rearing Condition (RC)		Slaughter Condition (SC)		SEM	P value[1]		
			Disbudded	Horned	Mixed	Unmixed	Limited Stress	Additional Stress		HS	RC	SC
Energy metabolism ($n = 21$)												
Q5E962	AKR1B1	Aldo-keto reductase family 1 member B1	**−4.87**	−5.50	−5.35	−5.02	−5.16	−5.21	0.09	***		
F1N206	DLD	Dihydrolipoyl dehydrogenase, mitochondrial	−2.42	**−2.19**	−2.38	−2.24	−2.36	−2.26	0.04	*		
Q29RI2	PHKG1	Phosphorylase b kinase gamma catalytic chain (skeletal muscle, heart)	−5.75	**−5.29**	−5.68	−5.36	−5.40	−5.64	0.09	**		
Q148N0	OGDH	2-oxoglutarate dehydrogenase complex component E1, mitochondrial	**0.29**	−0.06	**0.25**	−0.02	0.08	0.15	0.07	**	*	
A5PK73	ALDOB	Fructose-bisphosphate aldolase B	**0.22**	−0.08	**0.31**	−0.17	0.16	−0.03	0.07	*	***	
F1MGC0	SUCLA2	Succinate–CoA ligase [ADP-forming] subunit beta, mitochondrial	**−1.73**	−2.04	**−1.74**	−2.03	−1.86	−1.91	0.07	*	*	
P23004	UQCRC2	Cytochrome b-c1 complex subunit 2, mitochondrial	**2.62**	2.33	**2.62**	2.34	2.48	2.47	0.06	*	*	
F6QLM5	GYG1	glycogenin 1	−1.49	−1.38	**−1.21**	−1.66	−1.46	−1.41	0.06		***	
P68530	MT-CO2	Cytochrome c oxidase subunit 2	−1.69	−1.53	−1.75	**−1.47**	−1.74	**−1.48**	0.05		**	**
Q148D3	FH	Fumarate hydratase, mitochondrial	−1.20	−1.23	**−1.07**	−1.36	−1.22	−1.21	0.05		**	
F1MN74	IDH3A	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	−1.40	−1.31	−1.48	**−1.23**	−1.38	−1.33	0.04		**	
G3X778	PHKA1	Phosphorylase b kinase regulatory subunit alpha 1, skeletal muscle	−2.85	−3.00	−3.09	**−2.76**	−2.82	−3.03	0.06		**	
F1MHT1	AGL	Glycogen debranching enzyme	4.49	4.34	**4.51**	4.32	4.38	4.45	0.04		*	
P00829	ATP5B (ATP5F1B)	ATP synthase subunit beta. Mitochondrial	3.12	3.18	3.09	**3.21**	3.16	3.14	0.03		*	
P05631	ATP5F1C	ATP synthase subunit gamma. Mitochondrial	−3.97	−3.96	−4.22	**−3.71**	−4.08	−3.860	0.10		**	
P13620	ATP5H (ATP5PD)	ATP synthase subunit d, mitochondrial	−2.87	−3.02	−3.08	**−2.81**	−3.00	−2.88	0.05		*	
Q3SZ00	HADHA	Enoyl-CoA hydratase	−3.99	−3.88	−4.05	**−3.82**	−3.97	−3.90	0.05		*	
P15690	NDUFS1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	−1.45	−1.52	−1.60	**−1.38**	−1.52	−1.46	0.04		*	
F1MJ28	PYGM	Glycogen phosphorylase, muscle	6.82	6.76	**6.87**	6.71	6.79	6.78	0.04		*	
E1BAJ4	STBD1	Starch-binding domain-containing protein 1	0.95	1.01	**1.07**	0.90	0.92	1.04	0.04		*	
A5PJR4	ADSSL1 (ADSS1)	Adenylosuccinate synthetase isozyme 1	0.17	0.16	**0.24**	0.09	0.16	0.17	0.03		*	
Muscle structure ($n = 11$)												
F1N0W6	MYOZ3	Myozenin 3	−0.77	−0.49	−0.66	−0.60	−0.67	−0.60	0.06	*		
E1BF59	PLEC	Plectin	**1.73**	1.48	1.66	1.55	1.57	1.63	0.05	*		
E1BE25	FLNC[2]	Filamin C	3.61	3.55	**3.68**	3.48	3.61	3.55	0.03		***	
P81948	TUBB4A	Tubulin beta-4A chain	−6.20	−6.03	−6.48	**−5.75**	−6.14	−6.09	0.10		***	
G3X6W9	MYBPH[3]	Myosin binding protein H	−3.18	−2.95	−3.44	**−2.69**	−2.86	−3.27	0.13		**	
Q3B7N2	ACTN1	Alpha-actinin-1	−2.62	−2.75	−2.82	**−2.55**	−2.71	−2.66	0.06		*	
Q9BE41	MYH2	Myosin-2	0.19	0.10	**0.26**	0.03	0.11	0.18	0.05		*	
Q3SX40	PDLIM7	PDZ and LIM domain protein 7	2.94	2.89	**2.99**	2.84	2.90	2.93	0.03		*	
E1BPV6	SMTNL1[3]	Smoothelin like protein 1	−1.34	−1.33	**−1.20**	−1.47	−1.35	−1.33	0.06		*	
Q0VC48	TMOD4	Tropomodulin-4	0.62	0.63	**0.70**	0.55	0.59	0.65	0.03		*	
P48616	VIM[3]	Vimentin	−1.09	−1.05	**−0.97**	−1.16	−1.07	−1.06	0.05		*	
Protein binding (n = 4)												
Q3T165	PHB[2]	Prohibitin 1	−4.23	−4.39	−4.54	**−4.08**	−4.28	−4.34	0.07		***	
Q3MHL4	AHCY	Adenosylhomocysteinase	−1.80	−1.92	**−1.72**	−2.00	−1.90	−1.82	0.04		**	
P07033	ACYP2	Acylphosphatase-2	−2.50	−2.63	**−2.47**	−2.66	−2.60	−2.54	0.04		*	
A4IFK4	SYNPO2[3]	Synaptopodin-2	0.62	0.60	0.65	0.57	**0.67**	0.55[b]	0.03			*
Oxidative stress ($n = 3$)												
F1MUX6	GSTM3	Glutathione S-transferase Mu 3	**−3.61**	−4.32	−4.04	−3.90	−4.28	**−3.65**	0.14	*		*
E1BH17	GSTM1	Glutathione S-transferase Mu 1	**0.51**	0.33	0.48	0.36	0.42	0.42	0.04	*		
F1MNQ4	SOD1	Superoxide dismutase [Cu—Zn]	**−1.88**	−2.07	−1.92	−2.04	−2.00	−1.96	0.04	*		
Apoptosis (n = 3)												
P62935	PPIA	Peptidyl-prolyl cis-trans isomerase A	−3.87	**−3.63**	−3.73	−3.77	−3.83	−3.67	0.05	*		
P11116	LGALS1	Galectin-1	0.42	0.27	**0.51**	0.19	0.32	0.38	0.05		**	

(*continued on next page*)

Table 1 (*continued*)

Uniprot ID	Gene Name	Full protein name	Horn Status (HS)		Rearing Condition (RC)		Slaughter Condition (SC)		SEM	P value[1]		
			Disbudded	Horned	Mixed	Unmixed	Limited Stress	Additional Stress		HS	RC	SC
Q32PH8	EIF5A2	Eukaryotic translation initiation factor 5A-2	−2.67	−2.5	−2.70	**−2.46**	−2.55	−2.61	0.05		*	
Calcium homeostasis (n = 2)												
A6QNM9	SLC25A12	Electrogenic aspartate/glutamate antiporter SLC25A12, mitochondrial	−3.70	−3.66	−3.80	**−3.56**	−3.70	−3.66	0.06		*	
Q3SWX4	GBAS (NipSnap) [3]	Glioblastoma amplified sequence	−1.93	−1.88	−1.97	**−1.84**	−1.92	−1.89	0.03		*	
Proteolysis (n = 3)												
Q3T0Y5	PSMA2	Proteasome subunit alpha type-2	**1.64**	1.21	1.53	1.32	1.37	1.47	0.07	**		
E1BP91	NPEPPS	Aminopeptidase	−3.46	−3.56	**−3.37**	−3.64	−3.58	−3.44	0.05		**	
P62992	RPS27A	Ubiquitin-ribosomal protein eS31 fusion protein	0.92	0.83	**0.97**	0.79	0.88	0.88	0.04		*	
Miscellaneous (n = 9)												
Q3SZV7	HPX	Hemopexin	−3.07	**−2.80**	−3.00	−2.88	−2.88	−2.99	0.06	*		
F1N2I5	CMBL[2,3]	Carboxymethylenebutenolidase homolog	**−1.48**	−1.70	**−1.47**	−1.72	−1.61	−1.58	0.05	*	*	
Q5E9D3	CHCHD3	Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 3	−3.81	−3.87	−3.95	**−3.73**	−3.88	−3.80	0.04		*	
E1BBP7	H4C14	Histone H4	0.09	0.15	**0.20**	0.03	0.10	0.13	0.08		*	
F1MXX0	IMMT	Inner Membrane Mitochondrial Protein	−2.78	−2.74	−2.89	**−2.62**	−2.85	**−2.66**	0.04		***	*
F1MAV0	FGB[2]	Fibrinogen beta chain	−3.13	−3.15	−3.08	−3.20	**−2.91**	−3.37	0.08			**
F1MGU7	FGG	Fibrinogen gamma chain	−4.46	−4.52	−4.54	−4.44	**−4.26**	−4.72	0.08			**
A0A140T8A1	HSPB6	Heat shock protein beta-6	0.90	0.78	0.83	0.84	0.65	**1.02**	0.08			*
F1MCF8	IGLC3	Immunoglobulin lambda constant 3	−1.58	−1.39	−1.48	−1.49	**−1.27**	−1.70	0.09			*

[1] * p value ≤0.05, ** *p* value ≤0.01, *** p value ≤0.001, bold figures: higher protein abundance.
[2] protein showing in addition a 2-way interaction (See Table 2).
[3] protein showing in addition a 3-way interaction (See Table 3).

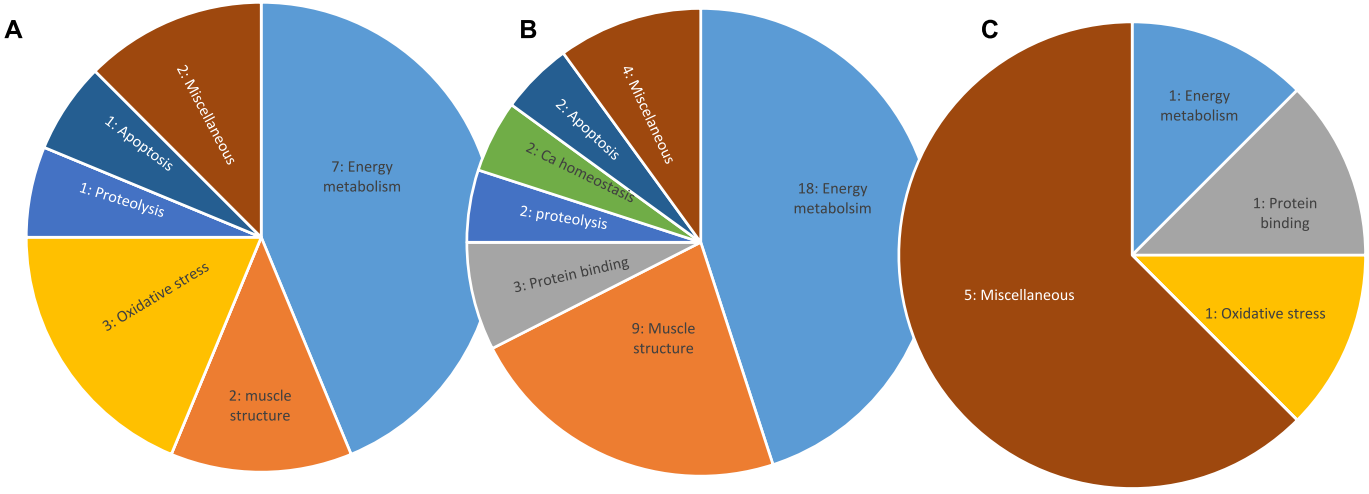

Fig. 1. Classification by family of the differentially expressed proteins significantly influenced by the 3 treatment factors (A. HS: Horn status B. RC: Rearing Condition, C. SC: Slaughter Condition) and their numbers in the *Longissimus thoracis* muscle of young bulls.

unique network (**Fig. S2D**).

The molecular pathways of the three protein lists (RC, SC and HS) did not reveal common enriched Ontology terms. RC and HS had common pathways including the *sulfur compound metabolic process* (GO:0006790), and *energy derivation by oxidation of organic compounds* (GO:0015980) (**Fig. S2E**).

3.5. Interactions amongst the treatment factors

Ten significant interactions were identified from the variance analysis: four second-degree interactions between RC and HS (Table 2) and six third-degree interactions amongst RC, SC and HS (Table 3). More specifically, in horned animals in mixed groups, PHB (Prohibitin) was less abundant compared to the other groups. Disbudded animals in mixed groups had greater abundances of FLNC (Filamin-C) and FGB (Fibrinogen beta chain) than disbudded animals in unmixed groups

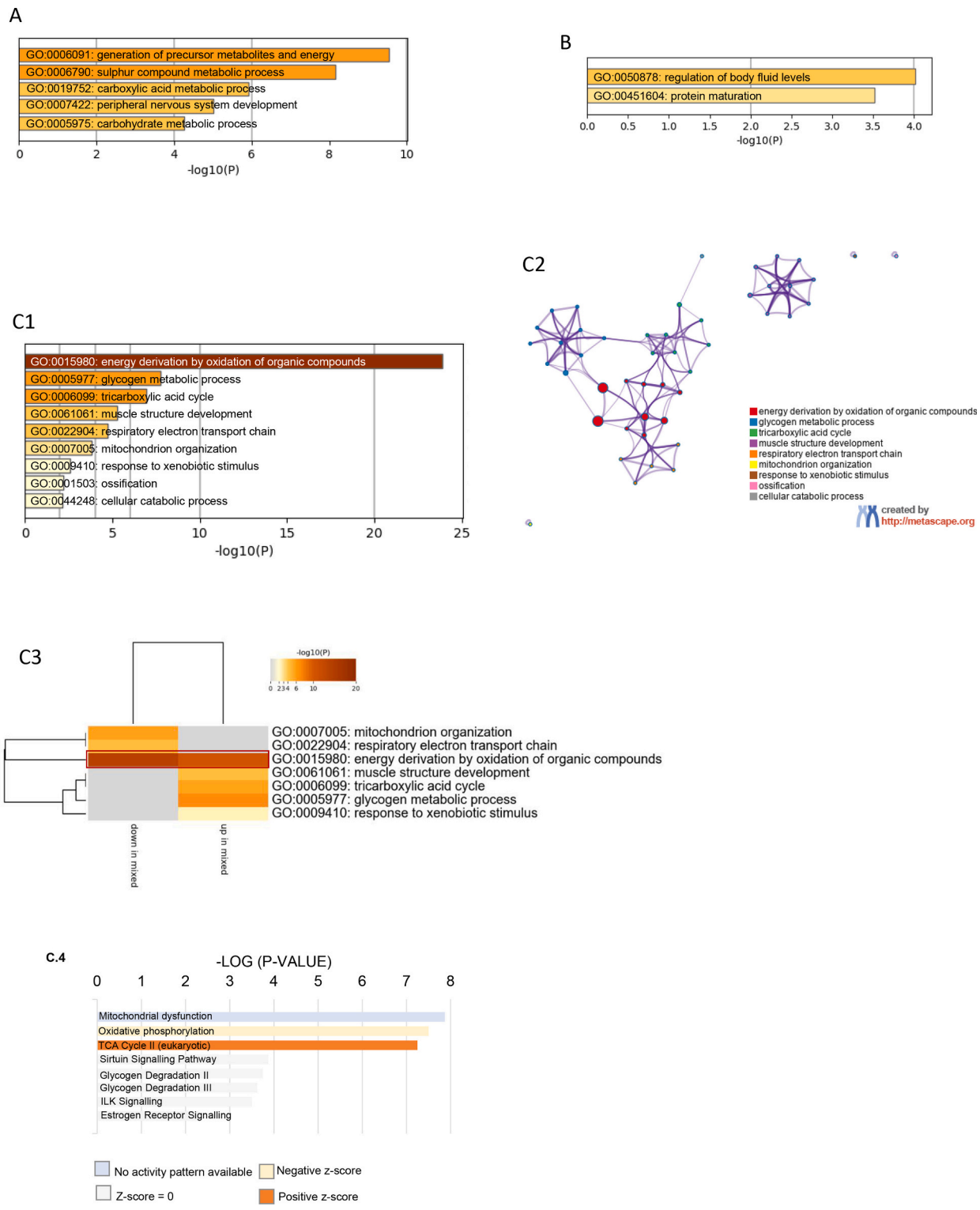

Fig. 2. Bioinformatics analysis (Biological Processes) using Metascape® (https://metascape.org/). of the proteins of which abundances were significantly influenced by the 3 treatment factors (RC: Rearing Condition, SC: Slaughter Condition and HS: Horns status) in the *Longissimus thoracis* muscle of young bulls.
A, B and C1: Gene ontology enrichment analysis): Heat maps showing the top 5 (**A**), 2 (**B**) and 9 (**C1**) significantly enriched ontology terms for the 16, 8 and 40 differentially expressed proteins influenced by **HS**, **SC** and **RC**, respectively.
C.2. Network of pathways and processes enrichment cluster of the 40 differentially expressed proteins significantly influenced by RC.
C.3. Gene ontology enrichment analysis: a heat map showing the top 7 significantly enriched ontology terms and the overlap between them for the 40 differentially expressed proteins influenced by RC. Terms are ranked by their *P*-value.
C.4. An Ingenuity Pathway Analysis of the 40 proteins significantly influenced by RC using the ingenuity pathways analysis system with a threshold of –Log (P-value) $>$ 2.67.

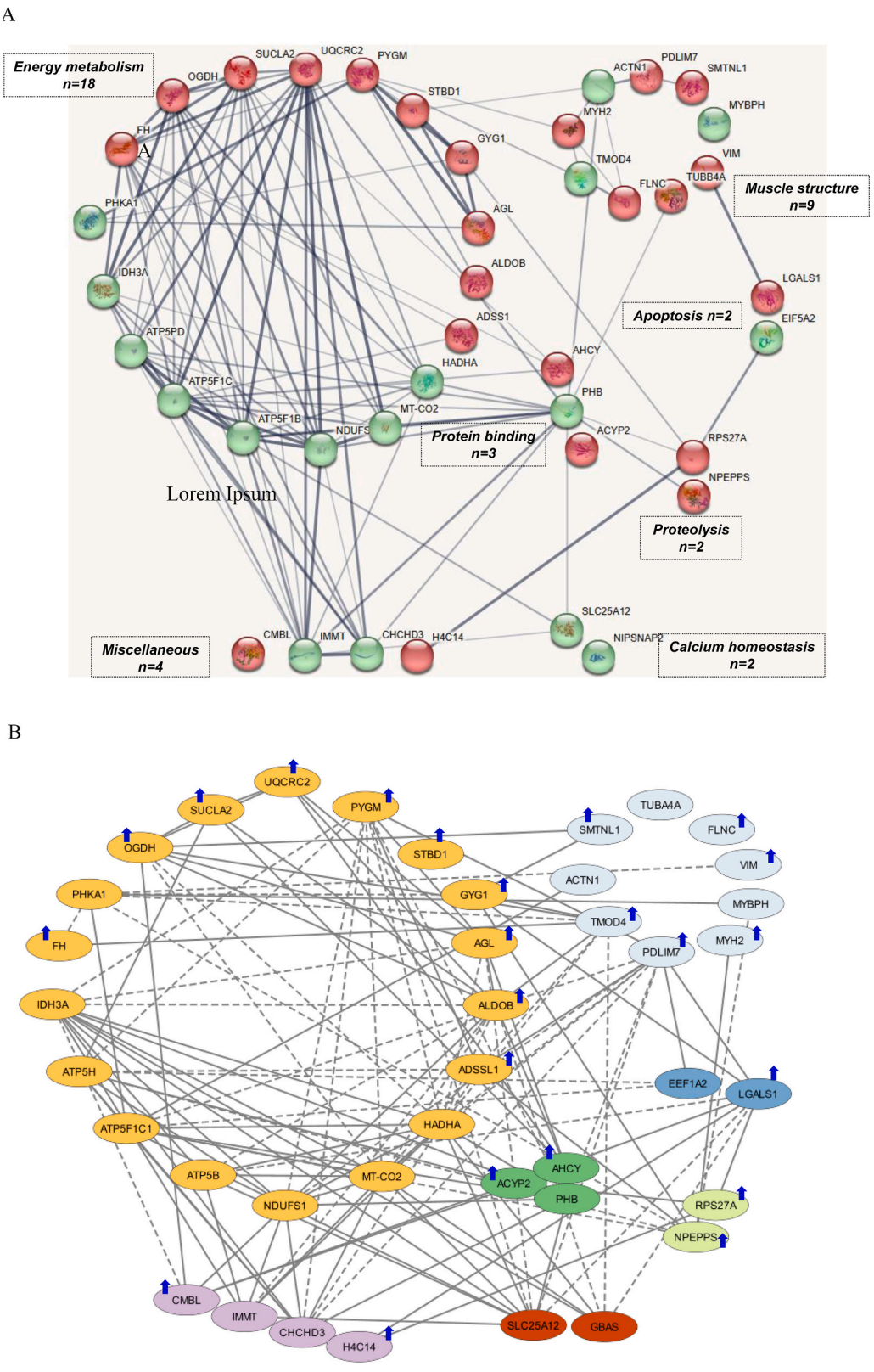

Fig. 3. A. Protein-protein interaction network (PPI) network linking the 40 proteins significantly influenced by Rearing Condition in the *Longissimus thoracis* muscle. The interaction map was generated from the web-based search STRING database (https://string-db.org/). Default settings of confidence of 0.4 and 4 criteria for linkage: Co-occurrence, experimental evidence, existing databases and text mining were used. Network statistics are: number of nodes: 40; number of edges: 112; average node degree: 5.6; average local clustering coefficient: 0.667; expected number of edges: 21 and PPI enrichment *P*-value: < 1.0e-16. Thicker edges indicate a greater degree of confidence prediction of the interaction based on existing data.
B. Protein-protein correlation network built as described by the method of Gagaoua et al. (2015) linking the 40 differentially expressed proteins significantly influenced by Rearing Condition in the *Longissimus thoracis* muscle. The interaction map was generated using Cytoscape 3.10.3. This map is a visual representation of significant Pearson correlations between abundances of proteins with *P*-values set at the <0.0002 level. The dashed lines represent the negative correlations between

the proteins and the continuous lines represent the positive correlations. The nodes representing the proteins were coloured and positioned according to their class (see Table 1 and Fig. 3A). The disconnected nodes represent the proteins with no correlations found. The blue arrows pointing up mark the proteins ($n = 23$) with higher abundances in mixed groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
C. Heatmap created in R using the Heatmap function from the "ComplexHeatmap" library. Hierarchical clustering is performed in the Heatmap() function; it is done by default. Coloured blocks indicate significant correlations. Proteins with no correlation at a *P*-value of <0.001 with other proteins were removed from the heatmap (STBD1 and FLNC).

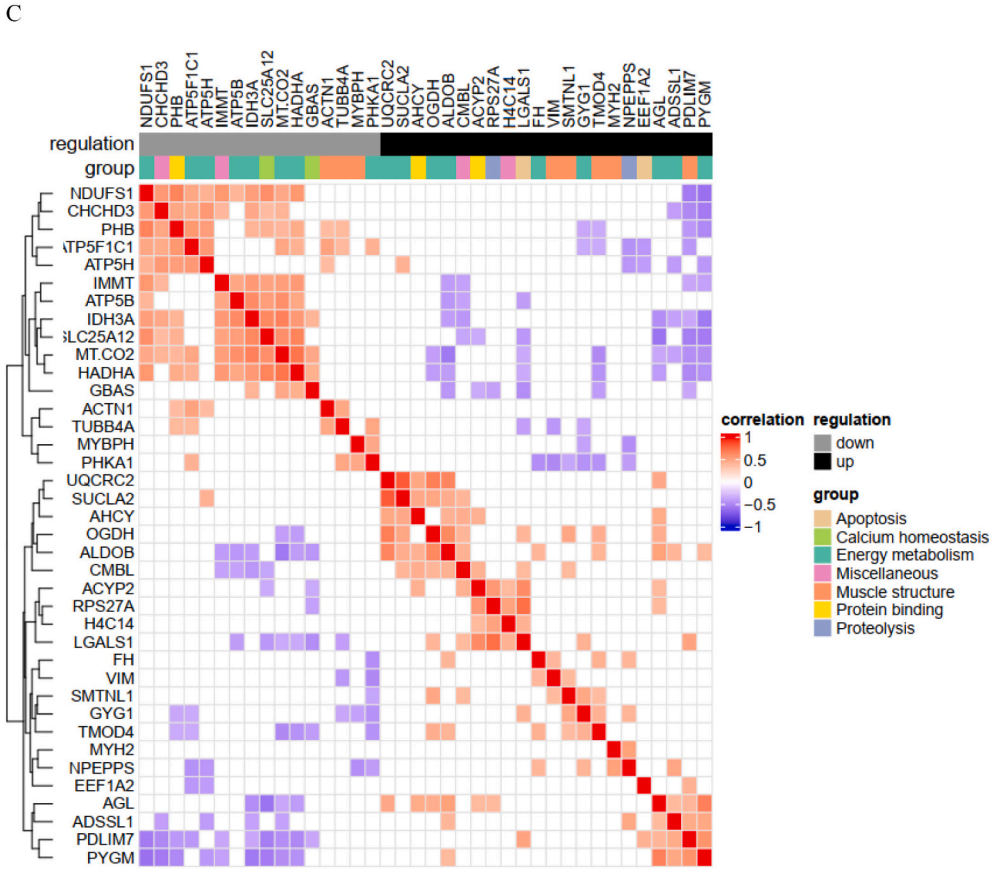

Fig. 3. (*continued*).

Table 2
LS means of proteins in the *Longissimus thoracis* muscle with statistically significant interactions between Rearing Condition (RC) and Horn Status (HS). Standard error of the means (SEM) is calculated across all animals.

Uniprot ID	Gene Name	Full protein name	Mixed* With Horns	Mixed* Without Horns	Unmixed*With Horns	Unmixed* Without Horns	SEM	RC*HS[1]
Q3T165	PHB	Prohibitin	−4.76^{b}	−4.33^{ab}	−4.03^{a}	−4.13^{a}	0.07	*
F1N2I5	CMBL	Carboxymethylenebutenolidase homolog	−1.69^{b}	−1.24^{a}	−1.71^{b}	−1.72^{b}	0.05	*
F1MAV0	FGB	Fibrinogen beta chain	−3.28^{ab}	−2.89^{a}	−3.01^{ab}	−3.38^{b}	0.08	*
E1BE25	FLNC	Filamin C	3.59^{ab}	3.77^{a}	3.51^{b}	3.44^{b}	0.03	*

[1] * p value ≤0.05.

(Table 2). Third-degree interactions involved amongst others CMBL, with greater abundance in disbudded animals within the mixed, limited stress groups, and GBAS (Glioblastoma amplified sequence or NipSnap), with lower abundance in disbudded animals within the mixed, limited stress (Table 3). In disbudded animals of the limited stress groups, SYNPO2 protein and SMTNL1 (Smoothelin like 1), had greater and lower abundances, respectively.

4. Discussion

If 20 out of 258 proteins are influenced by a given treatment factor, the probability that this is by chance is 4.3 % (two tailed z-test for proportions: $P = 0.043$). If 19 proteins are influenced by a treatment factor, the *P*-value is 0.08. Hence, if at least 20 proteins are influenced, this can be considered as having a statistical, that is, a biological meaning. The results were unexpected as only the rearing conditions influenced a significant number of the identified proteins. By contrast, the number of proteins influenced by horn status or slaughter stress were not significantly greater than 5 %, hence, no greater than expected by random fluctuations at a *P*-value of 0.05. The main part of the discussion will therefore focus on the observed effects of rearing conditions on the LT proteome.

4.1. Effect of rearing conditions on the LT muscle proteome

4.1.1. Energy metabolism was significantly affected by rearing conditions

The GO enrichment analysis evidenced a greater abundancy of proteins involved in glycolysis and an enrichment of terms related to energy

Table 3
LS means of proteins in the *Longissimus thoracis* muscle with statistically significant interactions amongst Rearing Condition (RC), Slaughter Condition (SC) and Horn Status (HS). Standard error of the means (SEM) is calculated across all animals.

Uniprot ID	Gene Name	Full protein name	Mixed* Limited Stress *With Horns	Mixed *Limited Stress *Without Horns	Mixed* Supplementary Stress *With Horns	Mixed* Supplementary Stress *Without Horns	Unmixed* Limited Stress* With Horns	Unmixed* Limited Stress* Without Horns	Unmixed* Supplementary Stress* With Horns	Unmixed* Supplementary Stress* Without Horns	SEM	RC*SC *HS[1]
F1N2I5	CMBL	Carboxymethylene -butenolidase homolog	-1.90^{c}	-1.10^{a}	-1.47^{abc}	-1.39^{ab}	-1.63^{bc}	-1.78^{bc}	-1.79^{bc}	-1.66^{bc}	0.05	*
Q3SWX4	GBAS	Glioblastoma amplified sequence	-1.82^{ab}	-2.14^{c}	-2.08^{bc}	-1.83^{ab}	-1.88^{ab}	-1.85^{ab}	-1.73^{a}	-1.91^{ab}	0.03	**
E1BPV6	SMTNL1	Smoothelin like 1	-1.50^{c}	-0.90^{a}	-1.04^{ab}	-1.36^{bc}	-1.37^{bc}	-1.61^{c}	-1.42^{bc}	-1.50^{c}	0.06	*
P48616	VIM	Vimentin	-1.27^{c}	-0.87^{ab}	-0.70^{a}	-1.05^{abc}	-1.00^{abc}	-1.13^{bc}	-1.22^{bc}	-1.28^{c}	0.05	*
G3X6W9	MYBPH	Myozin binding protein H	-2.58^{ab}	-3.87^{c}	-3.85^{c}	-3.44^{bc}	-2.63^{ab}	-2.36^{a}	-2.75^{ab}	-3.03^{abc}	0.13	*
A4IFK4	SYNPO2	SYNPO2 protein	0.68^{ab}	0.84^{a}	0.62^{ab}	0.46^{b}	0.64^{ab}	0.52^{b}	0.45^{b}	0.67^{ab}	0.03	*

[1] p value ≤0.05, ** p value ≤0.01.

metabolism and ATP metabolic processes. Amongst the proteins involved in glycogen metabolism, the only protein less abundant in mixed compared to unmixed groups, was phosphorylase b kinase regulatory subunit alpha (PHKA1), a cytosolic enzyme involved in glycogen biosynthesis. Mixed groups had greater abundance of the glycolytic enzyme fructose-biphosphate aldolase B (ALDOB), and of four other glycolysis-related cytosolic enzymes starch-binding domain-containing protein 1 (STBD1), glycogenin 1 (GYG1), glycogen debranching enzyme (AGL) and glycogen phosphorylase, muscle form (PYGM) involved in muscle glycogenolysis. ALDOB is an isoform usually found in the liver, but also in the muscle (Gagaoua et al., 2020), and involved in the carbohydrate degradation and glycolytic pathways. These five enzymes allow provision of energy to the muscle by breaking down glucose, fructose or glycogen. Hence, energy-generating glycolytic pathways were up-regulated in the mixed condition. Adenylosuccinate synthase 1 (ADSSL1) was also more abundant in mixed groups. ADDSL1 is muscle-specific and also involved in energy metabolism as it catalyses the first step in the conversion of inosine monophosphate (IMP) to adenosine monophosphate (AMP; Zhao et al., 2015).

Enzymes related to mitochondrial activity differed also according to rearing conditions. Two mitochondrial enzymes, isocitrate dehydrogenase [NAD] subunit alpha (IDH3A) and trifunctional enzyme subunit alpha (HADHA), were less abundant in the mixed groups. HADHA is a mitochondrial enzyme that catalyses three of the four final steps of the mitochondrial fatty acid beta-oxidation pathway ensuring the fatty acids breakdown into acetyl-CoA. HADHA was less abundant in type II than type I muscle fibres in an earlier study (Deshmukh et al., 2021). This finding is also coherent with a previous study reporting that an increase in pyruvate entry into the TCA cycle for oxidation inhibits fatty acid oxidation by limiting its transport into mitochondria (Wolfe et al., 1998). IDH3A is a catalytic subunit of the enzyme isocitrate dehydrogenase (ICDH), which catalyses the oxidation followed by the decarboxylation of isocitrate to α-ketoglutarate in the TCA cycle. IDH3 was more specifically found in the mitochondria of fast IIX and IIB fibres in mice (Schiaffino et al., 2015). In humans, IDH3A had greater abundance in fast IIX (glycolytic) compared to I and IIA fibres (Murgia et al., 2021). Although the lower abundance of IDH3A in animals of mixed groups contradicts their apparent greater glycolytic activity, it seems consistent with their lower levels of certain mitochondrial enzymes, discussed next.

Five mitochondrial enzymes within the mitochondrial respiratory chain, NDUFS1, MTCO2 (cytochrome *c* oxidase subunit 2), ATP5H, ATP5B and ATP5F1C, also had lower abundance in the mixed groups. These functionally related enzymes contribute to ATP production via oxidative phosphorylation in the inner mitochondrial membrane. The mitochondrial respiratory chain produces ATP utilizing the energy from electrons transferred from NADH and $FADH_2$. NDUFS1 is the core subunit of Complex I of this chain, using ubiquinone as an electron acceptor. MTCO2 is involved in electron transport in the inner mitochondrial membrane and part of Complex IV. Complex V, containing different ATP synthases, including ATP5H, ATP5B and ATP5F1C, is the last complex and synthesizes ATP from ADP and P_i. An earlier study found concurrent down-regulation of IDH3A, NDUF isoforms and ATP5F1A in cattle undergoing compensatory growth, and the involvement of a common upstream regulator (*PPARGC1A)* was hypothesized (Mullins et al., 2020). Effects on an upstream regulator may thus explain the lower abundance of IDH3A in the mixed groups described above. Ubiquinol-cytochrome *c* reductase core protein 2 (UQCRC2), which is part of complex III in the mitochondrion, was, in contrast, more abundant in the mixed groups.

Three other mitochondrial enzymes also showed greater abundances in mixed groups: 2-oxoglutarate dehydrogenase (E1) (OGDH), Succinyl-CoA ligase [ADP-forming] subunit beta (SUCLA2) and Fumarate hydratase (FH). OGDH is a component of the 2-oxoglutarate dehydrogenase complex (OGDHC) involved in the first step of the conversion of α-ketoglutarate into succinyl-CoA catalysed by the whole OGDHC (Nemeria et al., 2017). FH catalyses the oxidation of succinate to malate in the TCA cycle (Ajalla Aleixo et al., 2019). In human and rodent

muscle, OGDH and FH had greater abundances in type I compared to type II fibres (Murgia et al., 2021; Rakus et al., 2015). SUCLA2 catalyses the conversion of ADP and succinyl-CoA to succinate and ATP in the TCA cycle (Gagaoua et al., 2020). Previous studies reported a higher abundance of SUCLA2 in type I muscle fibres in rodents, but not in humans (Murgia et al., 2021; Rakus et al., 2015).

The *Longissimus thoracis* is a mixed oxido-glycolytic muscle; it contains slow oxidative type I, fast oxidoglycolytic type IIA and fast glycolytic type IIX muscle fibres with a higher proportion of the latter (Picard & Gagaoua, 2020). Slow-twitch muscle fibres (type I) contain less glycogen but more mitochondria than fast-twitch type II muscle fibres, which rely on glycolytic enzymes for a rapid generation of energy (Deshmukh et al., 2021; Ferguson & Gerrard, 2014; Gagaoua et al., 2020). The exact proportions of the different fibre types in the LT depends on breed and level and nature of exercise, amongst other influences (Picard & Gagaoua, 2020).

Overall, the findings suggest that the mixed groups have increased proportions of type II fibres, or an increased level of glycolytic enzyme activity, given the increased abundances of enzymes involved in the catabolism of glycogen. Increased abundance of STBD1, AGL and PYGM, which are specific to type II muscle fibres, suggesting greater glycolytic activity in mixed groups (Murgia et al., 2021; Yi et al., 2013). The greater abundance of ALDOB in the mixed groups is also consistent with their greater glycolytic activity. Earlier studies found that muscles with increased proportions of fast-twitch glycolytic muscle fibre contain higher levels of the skeletal muscle form of the ubiquitous glycolytic enzyme fructose-bisphosphate aldolase A (ALDOA) (Murgia et al., 2021; Gagaoua et al., 2020). PHKA1 was, in contrast, less abundant in the mixed groups, but an earlier study suggests that this enzyme may not be a good marker of fibre types (Murgia et al., 2021).

The greater abundances of STBD1, AGL and PYGM, and smaller abundances of mitochondrial IDH3A, HADHA and 5 mitochondrial respiratory chain related enzymes in the animals of the mixed groups suggest also greater glycolytic and lower oxidative activity of the LT muscle of these animals. However, their greater, rather than smaller abundances of OGDH, FH, SUCLA2, and UQCRC2, potentially indicative of greater mitochondrial activity, may seem contradictory to this interpretation. This discrepancy is possibly related to different enzyme contents of mitochondria of different fibre types (Leary et al., 2003). For example, exercise increased contents of enzymes involved in oxidative phosphorylation, but these were not the same enzymes in fast and slow fibres (Deshmukh et al., 2021). Overall, the increased expression of cytosolic glycolytic enzymes and lower abundance of various mitochondrial enzymes, with the exception of UQCRC2, are indicative of greater glycolytic activity in the LT of animals in mixed groups.

Mixed groups had, in addition, greater abundances of six structural proteins, Filamin-C (FLNC), Myosin-2 (MYH2), PDZ and LIM domain protein 7 (PDLIM7), smoothelin like 1 (SMTNL1), Tropomodulin 4 (TMOD4) and Vimentin (VIM) and three structural proteins, TUBB4A, Myosin-binding protein H (MYBPH) and ACTN1. These proteins are directly or indirectly related to muscle contraction and force generation. For example, SMTNL1 is believed to have multiple interactions with contractile regulators (Murali & MacDonald, 2018) and TMOD4 is involved in thin filament assembly during skeletal myofibrillogenesis (Nworu et al., 2015). FLNC is a muscle-specific filamin localized at the myotendinous junction, *Z*-disks and sarcolemma in skeletal muscle fibres. This protein is a muscle activity and mechanical stress biomarker playing an essential role in the maintenance and support of the structural integrity of skeletal muscles in response to mechanical stress (Fujita et al., 2012). VIM is an intermediate filament connecting Z-discs to adjacent myofibrils (Lazarides et al., 1982). MYH2, is a key protein involved in muscle contraction and required for cytoskeleton organization (Schiaffino, 2018; Wang et al., 2018). FLNC, MYH2, MYBPH, SMTNL and PDLIM7 are all known biomarkers for type IIA (fast oxido-glycolytic) muscle fibres (Castorena et al., 2015; Murgia et al., 2021; Picard & Gagaoua, 2020; Schiaffino, 2018; Wang et al., 2018). These results suggest that the greater glycolytic activity in the LT of mixed animals may be related to greater proportions of fast twitch muscle fibres.

Mixed groups had a greater abundance of galectin-1 (LGALS1), a type IIA muscle fibre specific protein in human muscles (Murgia et al., 2021), and a lower abundance of eukaryotic translation initiation factor 5 A-2 (EIF5A2). Both LGALS1 and EIF5A2 are involved in the regulation of apoptosis (Barba-Aliaga & Alepuz, 2022; Clement et al., 2003; He & Baum, 2004). During the early post-mortem period, apoptosis is known to influence the muscle structure and be favourable for the tenderisation process (Chen et al., 2020; Lamri et al., 2023). In addition to the above, 3 proteins involved in protein binding, 2 involved in calcium homeostasis, and 2 involved in proteolysis, and 4 miscellaneous proteins were influenced by rearing conditions, 5 of which had lower and 6 had greater abundances in mixed groups.

The increased abundance of specific structural proteins that are biomarkers of glycolytic fibre types, suggest longer-term structural changes in the properties of the LT muscle in mixed groups. Muscle fibre composition is highly genetically determined, but physical activity may induce structural and metabolic adaptations in the muscles. Fibre types may change from IIX ➔ IIA ➔ I and vice versa (Bogdanis, 2012; Deshmukh et al., 2021), including in cattle (Picard & Gagaoua, 2020). Endurance trained individuals have greater percentages of type I muscle fibres than sprint trained individuals (Allemeier et al., 1994; Bogdanis, 2012; Geng et al., 2010). Greater oxidative activity was also observed in the LT and other muscles of cattle that had greater physical activity (Gagaoua et al., 2017; Jurie et al., 2006). For instance, in steers receiving the same diet, walking had increased the activity of several oxidative enzymes in the *Semitendinosus* and *Rectus abdominis* (Jurie et al., 2006).

Longer-term structural changes would suggest an impact of different lifestyles. The mixed bulls had indeed different behavioural and physiological characteristics as described in earlier reports on these same animals (Reiche et al., 2019; Reiche et al., 2020). The mixed groups were less active, spending more time lying down, and less time walking compared to their unmixed counterparts (Reiche et al., 2020). The apparent greater glycolytic activity of the LT of bulls of the mixed groups may further be related to altered metabolic reactions to the stress and physical demands of the slaughter situation. During unloading for slaughter, which represents a strong physical effort, heart rates of mixed bulls were higher compared to their unmixed counterparts (Reiche et al., 2019). In contrast, in the slaughterhouse, when subjected to the Supplementary Stress conditions, certain stress indicators of the mixed bulls, amongst which adrenaline and noradrenaline levels, were lower or presented a delayed rise (Reiche et al., 2019). Adrenaline and noradrenaline levels and heart rate are controlled by the autonomic nervous system and involved in the stress response (Gordan et al., 2015). Possibly, the physiological responses to physical and psychological stress were different in the groups of mixed compared to unmixed bulls. The autonomic nervous system, specifically adrenaline, stimulates glycogen breakdown in the active muscle (Watt et al., 2001), and a modification in the functioning of this system could have an association with the different glycolytic profile of the LT of animals of mixed groups.

4.2. *Effect of horn status on the LT muscle proteome*

Horn status significantly influenced the abundance of sixteen proteins, seven of which are associated with the GO term *generation of precursor metabolites and energy*. Of these, only two, phosphorylase kinase, gamma 1 (PHKG1) and dihydrolipoyl dehydrogenase (DLD), had lower abundance in disbudded bulls. PHKG1 mediates the neural and hormonal regulation of glycogen breakdown by activating glycogen phosphorylase via its phosphorylation (Ma et al., 2014). DLD is a key factor in the cellular production of energy by converting pyruvate into acetyl-CoA (Duarte et al., 2021). The other five proteins of this GO term had greater abundances in disbudded bulls. Three of these proteins are

mitochondrial enzymes: OGDH and SUCLA2, enzymes of the TCA cycle, and UQCRC2 a component of the mitochondrial electron transport chain, possibly indicating greater mitochondrial activity in the LT of disbudded animals. The other two enzymes are cytosolic enzymes AKR1B1 (aldose reductase enzyme), involved in the reduction of aldehydes and ketones to alcohols, and ALDOB, catalysing different steps of glycolysis (Gagaoua et al., 2020; Murgia et al., 2021; Penning, 2015).

Glutathione S-transferase 3 (GSTM3), Glutathione S-transferase 1 (GSTM1) and Superoxide dismutase 1 (SOD1) had greater abundances for disbudded bulls. They are antioxidants; they scavenge reactive oxygen species, mainly produced by mitochondria (Deters & Hansen, 2020). The greater abundance of antioxidant proteins in LT muscles of bulls without horns is in keeping with the possible greater mitochondrial activity suggested above.

These results suggest that disbudding may influence the energy metabolism and muscle biology. Horn growth depends on the presence of various hormones involved in the use of energy, such as growth and thyroid hormones, prolactin, and testosterone (Bubenik, 1990) and while having horns may be an advantage with respect to social status, growing and maintaining horns is energetically costly (Bergeron et al., 2010). For instance, in a situation with limited energy resources, young cattle may use their energy resources towards gaining in body weight and away from horn growth (Cusson, 2020). Although results point to an effect of the presence of horns on energy metabolism, the number of proteins influenced by horn status was no greater than expected by chance and the observed trends would need further confirmation.

4.3. *Effect of slaughter conditions on the LT muscle proteome*

Our earlier study found that slaughter conditions influenced the metabolism and physiology of these bulls as well as post-mortem pH and temperature decline, proteolysis, water holding capacity and juiciness of the muscle and meat of these bulls (Reiche et al., 2019). The present study found however that slaughter conditions influenced very few proteins. The lack of difference in the proteome may potentially relate to the time of sampling post mortem. Proteome changes due to short-lasting or recent effects, are possibly not durably detectable post-mortem, as protein abundances evolve in the first 24 h of post-mortem storage (Jia et al., 2006; Lamri et al., 2023; Morzel et al., 2008). Certain proteins and fragments may disappear or be formed at different rates due to glycolytic activity, proteolysis, phosphorylation and apoptosis or other means of fragmentation of proteins (Gagaoua et al., 2021; Jia et al., 2006; Morzel et al., 2008). The large amounts of proteins influenced particularly by rearing conditions may indicate that the proteomic differences established *in vivo* by these processes were stable even after 48 h post-mortem, in contrast to potentially shorter lasting differences induced by slaughter conditions. Possibly, in order to fully evaluate the effects of stress on the muscle proteome, future proteomic studies should focus on the early post-mortem pre-rigor period.

5. Conclusion

The composition of the rearing group with respect to horn status, influenced the LT muscle post-mortem proteome. Particularly, being in a mixed, compared to an unmixed group induced a shift towards glycolytic metabolism of the LT, possibly induced by modifications in behaviour and physiological stress reactivity. These findings warrant further exploration using early post-mortem sampling times and combinations of various omics approaches, including for instance metabolomics to decipher the interconnected pathways related to energy metabolism. The combination of proteomics and metabolomics with machine learning methods may allow a better understanding of the mechanisms underlying the effects of pre-slaughter stress on meat quality. Such an approach would pave the way for better animal welfare and preserved meat quality through better practices at farm and slaughter levels.

CRediT authorship contribution statement

E.M. Claudia Terlouw: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rabiaa Ben Mbarek:** Writing – original draft, Investigation, Formal analysis, Data curation. **Ruth M. Hamill:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Conceptualization. **Anna-Maria Reiche:** Writing – review & editing, Validation, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Brigitte Picard:** Writing – review & editing, Validation, Supervision, Conceptualization. **Joseph Kerry:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Paolo Silacci:** Writing – review & editing, Validation, Resources, Investigation. **Anne Maria Mullen:** Writing – review & editing, Validation, Methodology, Investigation. **Didier Viala:** Data curation, Writing – review & editing. **Mohammed Gagaoua:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Consent

Informed consent was not necessary as the study did not involve human subjects.

Author statement

All listed authors have made substantial contributions to the conception, design, execution, or interpretation of the research and have approved the final manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meatsci.2025.109922.

Data availability

The data that has been used is confidential.

References

Agroscope. (2016). *Fütterungsempfehlungen für Wiederkäuer (Grünes Buch)*. Retrieved 16.01.2019 https://www.agroscope.admin.ch/agroscope/de/home/services/dienste/futtermittel/fuetterungsempfehlungen-wiederkaeuer.html.

Ajalla Aleixo, M. A., Rangel, V. L., Rustiguel, J. K., de Pádua, R. A., & Nonato, M. C. (2019). Structural, biochemical and biophysical characterization of recombinant human fumarate hydratase. *The FEBS Journal, 286*(10), 1925–1940.

Allemeier, C. A., Fry, A. C., Johnson, P., Hikida, R. S., Hagerman, F. C., & Staron, R. S. (1994). Effects of sprint cycle training on human skeletal muscle. *Journal of Applied Physiology, 77*(5), 2385–2390.

Barba-Aliaga, M., & Alepuz, P. (2022). Role of eIF5A in mitochondrial function. *International Journal of Molecular Sciences, 23*(3), 1284.

Bergeron, P., Grignolio, S., Apollonio, M., Shipley, B., & Festa-Bianchet, M. (2010). Secondary sexual characters signal fighting ability and determine social rank in Alpine ibex (Capra ibex). *Behavioral Ecology and Sociobiology, 64*, 1299–1307.

Bogdanis, G. C. (2012). Effects of physical activity and inactivity on muscle fatigue. *Frontiers in Physiology, 3*, 142.

Bouissou, M. F. (1980). Social relationships in domestic cattle under modern management techniques. *Italian Journal of Zoology, 47*(3–4), 343–353.

Bouley, J., Chambon, C., & Picard, B. (2004). Mapping of bovine skeletal muscle proteins using two-dimensional gel electrophoresis and mass spectrometry. *Proteomics, 4*(6), 1811–1824.

Bourguet, C., Deiss, V., Gobert, M., Durand, D., Boissy, A., & Terlouw, E. M. C. (2010). Characterising the emotional reactivity of cows to understand and predict their stress reactions to the slaughter procedure. *Applied Animal Behaviour Science, 125*(1–2), 9–21.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry, 72*(1–2), 248–254.

Bubenik, A. B. (1990). Epigenetical, morphological, physiological, and behavioral aspects of evolution of horns, pronghorns, and antlers. In *Horns, pronghorns, and antlers: Evolution, morphology, physiology, and social significance* (pp. 3–113). Springer.

Castorena, C. M., Arias, E. B., Sharma, N., Bogan, J. S., & Cartee, G. D. (2015). Fiber type effects on contraction-stimulated glucose uptake and GLUT4 abundance in single fibers from rat skeletal muscle. *American Journal of Physiology. Endocrinology and Metabolism, 308*(3), E223–E230.

Chen, C., Zhang, J., Guo, Z., Shi, X., Zhang, Y., Zhang, L., Yu, Q., & Han, L. (2020). Effect of oxidative stress on AIF-mediated apoptosis and bovine muscle tenderness during postmortem aging. *Journal of Food Science, 85*(1), 77–85.

Clement, P. M., Henderson, C. A., Jenkins, Z. A., Smit-McBride, Z., Wolff, E. C., Hershey, J. W., … Johansson, H. E. (2003). Identification and characterization of eukaryotic initiation factor 5A-2. *European Journal of Biochemistry, 270*(21), 4254–4263.

Cusson, P.-O. (2020). *Effet de la chasse aux trophées sur le comportement de prise de risque des béliers d'une population sauvage de mouflons d'Amérique (Ovis canadensis)*. Université de Sherbrooke.

Deshmukh, A. S., Steenberg, D. E., Hostrup, M., Birk, J. B., Larsen, J. K., Santos, A., … Murgia, M. (2021). Deep muscle-proteomic analysis of freeze-dried human muscle biopsies reveals fiber type-specific adaptations to exercise training. *Nature Communications, 12*(1), 304.

Deters, E. L., & Hansen, S. L. (2020). Invited review: Linking road transportation with oxidative stress in cattle and other species. *Applied Animal Science, 36*(2), 183–200.

Díaz, F., Díaz-Luis, A., Sierra, V., Diñeiro, Y., González, P., García-Torres, S., … Coto-Montes, A. (2020). What functional proteomic and biochemical analysis tell us about animal stress in beef? *Journal of Proteomics, 218*, Article 103722.

Duarte, I. F., Caio, J., Moedas, M. F., Rodrigues, L. A., Leandro, A. P., Rivera, I. A., & Silva, M. F. B. (2021). Dihydrolipoamide dehydrogenase, pyruvate oxidation, and acetylation-dependent mechanisms intersecting drug iatrogenesis. *Cellular and Molecular Life Sciences*, 1–18.

Ferguson, D. M., & Gerrard, D. E. (2014). Regulation of post-mortem glycolysis in ruminant muscle. *Animal Production Science, 54*(4), 464–481.

Ferguson, D. M., & Warner, R. D. (2008). Have we underestimated the impact of pre-slaughter stress on meat quality in ruminants? *Meat Science, 80*(1), 12–19.

Fujita, M., Mitsuhashi, H., Isogai, S., Nakata, T., Kawakami, A., Nonaka, I., … Kudo, A. (2012). Filamin C plays an essential role in the maintenance of the structural integrity of cardiac and skeletal muscles, revealed by the medaka mutant zacro. *Developmental Biology, 361*(1), 79–89.

Gagaoua, M., Franco, D., & Ramanathan, R. (2024). Meat Omics: Trends and applications of Omics strategies in meat research. *J Proteomics, 295*, 105090.

Gagaoua, M., Schilling, W., Zhang, X., & Suman, S. (2024). *Applications of proteomics in meat research. Encyclopedia of Meat Sciences* (pp. 513–527). Elsevier. https://doi.org/10.1016/B978-0-323-85125-1.00123-X.

Gagaoua, M., Hughes, J., Terlouw, E. M. C., Warner, R. D., Purslow, P. P., Lorenzo, J. M., & Picard, B. (2020). Proteomic biomarkers of beef colour. *Trends in Food Science & Technology, 101*, 234–252.

Gagaoua, M., Monteils, V., Couvreur, S., & Picard, B. (2017). Identification of biomarkers associated with the rearing practices, carcass characteristics, and beef quality: An integrative approach. *Journal of Agricultural and Food Chemistry, 65*(37), 8264–8278.

Gagaoua, M., Monteils, V., Couvreur, S., & Picard, B. (2019). Beef tenderness prediction by a combination of statistical methods: Chemometrics and supervised learning to manage integrative farm-to-meat continuum data. *Foods, 8*(7), 274.

Gagaoua, M., Terlouw, E. M. C., Boudjellal, A., & Picard, B. (2015). Coherent correlation networks among protein biomarkers of beef tenderness: What they reveal. *Journal of Proteomics, 128*, 365–374.

Gagaoua, M., Terlouw, E. M. C., Mullen, A. M., Franco, D., Warner, R. D., Lorenzo, J. M., … Troy, D. (2021). Molecular signatures of beef tenderness: Underlying mechanisms based on integromics of protein biomarkers from multi-platform proteomics studies. *Meat Science, 172*, Article 108311.

Gagaoua, M., Warner, R. D., Purslow, P., Ramanathan, R., Mullen, A. M., López-Pedrouso, M., … Terlouw, E. M. C. (2021). Dark-cutting beef: A brief review and an integromics meta-analysis at the proteome level to decipher the underlying pathways. *Meat Sci, 181*, 108611.

Geng, T., Li, P., Okutsu, M., Yin, X., Kwek, J., Zhang, M., & Yan, Z. (2010). PGC-1α plays a functional role in exercise-induced mitochondrial biogenesis and angiogenesis but not fiber-type transformation in mouse skeletal muscle. *American Journal of Physiology-Cell Physiology, 298*(3), C572–C579.

Goonewardene, L. A., Price, M. A., Liu, M. F., Berg, R. T., & Erichsen, C. M. (1999). A study of growth and carcass traits in dehorned and polled composite bulls. *Canadian Journal of Animal Science, 79*(3), 383–385.

Gordan, R., Gwathmey, J. K., & Xie, L.-H. (2015). Autonomic and endocrine control of cardiovascular function. *World Journal of Cardiology, 7*(4), 204.

He, J., & Baum, L. G. (2004). Presentation of galectin-1 by extracellular matrix triggers T cell death. *Journal of Biological Chemistry, 279*(6), 4705–4712.

Jia, X., Hollung, K., Therkildsen, M., Hildrum, K. I., & Bendixen, E. (2006). Proteome analysis of early post-mortem changes in two bovine muscle types: M. longissimus dorsi and M. semitendinosis. *Proteomics, 6*(3), 936–944.

Jurie, C., Ortigues-Marty, I., Picard, B., Micol, D., & Hocquette, J. F. (2006). The separate effects of the nature of diet and grazing mobility on metabolic potential of muscles from Charolais steers. *Livestock Science, 104*(1–2), 182–192.

Knierim, U., Irrgang, N., & Roth, B. A. (2015). To be or not to be horned—Consequences in cattle. *Livestock Science, 179*, 29–37.

Krämer, A., Green, J., Pollard, J., Jr., & Tugendreich, S. (2014). Causal analysis approaches in ingenuity pathway analysis. *Bioinformatics, 30*(4), 523–530.

Lamri, M., Della Malva, A., Djenane, D., Albenzio, M., & Gagaoua, M. (2023). First insights into the dynamic protein changes in goat semitendinosus muscle during the post-mortem period using high-throughput proteomics. *Meat Science, 202*, Article 109207.

Lamri, M., della Malva, A., Djenane, D., López-Pedrouso, M., Franco, D., Albenzio, M., … Gagaoua, M. (2023). Towards the discovery of goat meat quality biomarkers using label-free proteomics. *Journal of Proteomics, 278*, 104868.

Lazarides, E., Granger, B. L., Gard, D. L., O'Connor, C. M., Breckler, J., Price, M., & Danto, S. I. (1982). Desmin-and vimentin-containing filaments and their role in the assembly of the Z disk in muscle cells. *Cold Spring Harbor Symposia on Quantitative Biology, 46*, 351–378.

Leary, S. C., Lyons, C. N., Rosenberger, A. G., Ballantyne, J. S., Stillman, J., & Moyes, C. D. (2003). Fiber-type differences in muscle mitochondrial profiles. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 285*(4), R817–R826.

Lensink, B. J., Fernandez, X., Boivin, X., Pradel, P., Le Neindre, P., & Veissier, I. (2000). The impact of gentle contacts on ease of handling, welfare, and growth of calves and on quality of veal meat. *Journal of Animal Science, 78*(5), 1219–1226.

Ma, J., Yang, J., Zhou, L., Ren, J., Liu, X., Zhang, H., Yang, B., Zhang, Z., Ma, H., & Xie, X. (2014). A splice mutation in the PHKG1 gene causes high glycogen content and low meat quality in pig skeletal muscle. *PLoS Genetics, 10*(10), Article e1004710.

Morzel, M., Terlouw, C., Chambon, C., Micol, D., & Picard, B. (2008). Muscle proteome and meat eating qualities of Longissimus thoracis of "Blonde d'Aquitaine" young bulls: A central role of HSP27 isoforms. *Meat Science, 78*(3), 297–304.

Mouzo, D., Rodríguez-Vázquez, R., Lorenzo, J. M., Franco, D., Zapata, C., & López-Pedrouso, M. (2020). Proteomic application in predicting food quality relating to animal welfare. A review. *Trends in Food Science & Technology, 99*, 520–530.

Mullins, Y., Keogh, K., Kenny, D. A., Kelly, A., O'boyle, P., & Waters, S. M. (2020). Label-free quantitative proteomic analysis of M. Longissimus dorsi from cattle during dietary restriction and subsequent compensatory growth. *Scientific Reports, 10*(1), 2613.

Murali, M., & MacDonald, J. A. (2018). Smoothelins and the control of muscle contractility. *Advances in Pharmacology, 81*, 39–78.

Murgia, M., Nogara, L., Baraldo, M., Reggiani, C., Mann, M., & Schiaffino, S. (2021). Protein profile of fiber types in human skeletal muscle: A single-fiber proteomics study. *Skeletal Muscle, 11*, 1–19.

Nemeria, N. S., Gerfen, G., Guevara, E., Nareddy, P. R., Szostak, M., & Jordan, F. (2017). The human Krebs cycle 2-oxoglutarate dehydrogenase complex creates an additional source of superoxide/hydrogen peroxide from 2-oxoadipate as alternative substrate. *Free Radical Biology and Medicine, 108*, 644–654.

Nworu, C. U., Kraft, R., Schnurr, D. C., Gregorio, C. C., & Krieg, P. A. (2015). Leiomodin 3 and tropomodulin 4 have overlapping functions during skeletal myofibrillogenesis. *Journal of Cell Science, 128*(2), 239–250.

Oddy, V., Harper, G., Greenwood, P., & McDonagh, M. (2001). Nutritional and developmental effects on the intrinsic properties of muscles as they relate to the eating quality of beef. *Australian Journal of Experimental Agriculture, 41*(7), 921–942.

Penning, T. M. (2015). The aldo-keto reductases (AKRs): Overview. *Chemico-Biological Interactions, 234*, 236–246.

Picard, B., & Gagaoua, M. (2020). Muscle fiber properties in cattle and their relationships with meat qualities: An overview. *Journal of Agricultural and Food Chemistry, 68*(22), 6021–6039.

Rakus, D., Gizak, A., Deshmukh, A., & Wiśniewski, J. R. (2015). Absolute quantitative profiling of the key metabolic pathways in slow and fast skeletal muscle. *Journal of Proteome Research, 14*(3), 1400–1411.

Reiche, A.-M., Dohme-Meier, F., & Terlouw, E. C. (2020). Effects of horn status on behaviour in fattening cattle in the field and during reactivity tests. *Applied Animal Behaviour Science, 231*, Article 105081.

Reiche, A.-M., Oberson, J.-L., Silacci, P., Messadène-Chelali, J., Hess, H.-D., Dohme-Meier, F., Dufey, P.-A., & Terlouw, E. (2019). Pre-slaughter stress and horn status influence physiology and meat quality of young bulls. *Meat Science, 158*, Article 107892.

Schiaffino, S. (2018). Muscle fiber type diversity revealed by anti-myosin heavy chain antibodies. *The FEBS Journal, 285*(20), 3688–3694.

Schiaffino, S., Reggiani, C., Kostrominova, T. Y., Mann, M., & Murgia, M. (2015). Mitochondrial specialization revealed by single muscle fiber proteomics: Focus on the Krebs cycle. *Scandinavian Journal of Medicine & Science in Sports, 25*, 41–48.

Schoiswohl, J., Stanitznig, A., Waiblinger, S., Frahm, S., Krametter-Froetscher, R., & Wittek, T. (2020). Suppression of horn growth in cattle by clove oil and isoeugenol. *Journal of Veterinary Behavior, 36*, 1–3.

Sierra, V., González-Blanco, L., Diñeiro, Y., Díaz, F., García-Espina, M. J., Coto-Montes, A., … Oliván, M. (2021). New insights on the impact of cattle handling on post-mortem myofibrillar muscle proteome and meat tenderization. *Foods, 10*(12), 3115.

Soulat, J., Picard, B., Leger, S., & Monteils, V. (2016). Prediction of beef carcass and meat traits from rearing factors in young bulls and cull cows. *Journal of Animal Science, 94*(4), 1712–1726.

Terlouw, E. C., Picard, B., Deiss, V., Berri, C., Hocquette, J.-F., Lebret, B., … Gagaoua, M. (2021). Understanding the determination of meat quality using biochemical characteristics of the muscle: Stress at slaughter and other missing keys. *Foods, 10*(1), 84.

Waiblinger, S., Baars, T., & Menke, C. (2000). Understanding the cow-the central role of human-animal relationship in keeping horned dairy cows in loose housing. In *Proceedings of the 3rd workshop of the NAHWOA. Clermont-Ferrand, France*.

Wang, L., Geist, J., Grogan, A., Hu, L.-Y. R., & Kontrogianni-Konstantopoulos, A. (2018). Thick filament protein network, functions, and disease association. *Comprehensive Physiology, 8*(2), 631.

Watt, M. J., Howlett, K. F., Febbraio, M. A., Spriet, L. L., & Hargreaves, M. (2001). Adrenaline increases skeletal muscle glycogenolysis, pyruvate dehydrogenase activation and carbohydrate oxidation during moderate exercise in humans. *The Journal of Physiology, 534*(1), 269–278.

Wolfe, C. A., James, P. S., Mackie, A. R., Ladha, S., & Jones, R. (1998). Regionalized lipid diffusion in the plasma membrane of mammalian spermatozoa. *Biology of reproduction, 59*(6), 1506–1514.

Yi, H., Fredrickson, K. B., Das, S., Kishnani, P. S., & Sun, B. (2013). Stbd1 is highly elevated in skeletal muscle of Pompe disease mice but suppression of its expression does not affect lysosomal glycogen accumulation. *Molecular genetics and metabolism, 109*(3), 312–314.

Zhao, H., Chiaro, C. R., Zhang, L., Smith, P. B., Chan, C. Y., Pedley, A. M., … Benkovic, S. J. (2015). Quantitative analysis of purine nucleotides indicates that Purinosomes increase de novo purine biosynthesis*. *Journal of Biological Chemistry, 290*(11), 6705–6713.

Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A. H., Tanaseichuk, O., & Chanda, S. K. (2019). Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nature Communications, 10*(1), 1523.

Zhu, Y., Gagaoua, M., Mullen, A. M., Kelly, A. L., Sweeney, T., Cafferky, J., … Hamill, R. M. (2021). A proteomic study for the discovery of beef tenderness biomarkers and prediction of Warner–Bratzler shear force measured on longissimus thoracis muscles of young Limousin-sired bulls. *Foods, 10*(5), 952.