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## Dark-cutting beef: a brief review and an integromics meta-analysis at the proteome level to decipher the underlying pathways

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**Abstract**

Comprehensive characterization of the *post-mortem* muscle proteome defines a fundamental goal in meat proteomics. During the last decade, proteomic tools have been applied in the field of foodomics to help decipher factors underpinning meat quality variations and to enlighten us, through data-driven methods, on the underlying mechanisms leading to meat quality defects such as dark-cutting meat. In cattle, several proteomics studies have focused on the extent to which changes in the *post-mortem* muscle proteome relate to dark-cutting beef development. The present data-mining study firstly reviews proteomics studies which investigated dark-cutting beef, and secondly, gathers the protein biomarkers that differ between dark-cutting *versus* beef with normal-pH in a unique repertoire. A list of 139 proteins from eight eligible studies was curated and mined through bioinformatics for Gene Ontology annotations, molecular pathways enrichments, secretome analysis and biological pathways comparisons to normal beef color from a previous meta-analysis. The major biological pathways underpinning dark-cutting beef at the proteome level have been described and deeply discussed.

**Keywords:** OMICs; proteome; pH; DFD, meat color; cattle; muscle structure, TCA cycle; metabolism; mitochondria

## 1. Introduction

Recent developments in high-throughput omics techniques provide us with tools for efficient proteome profiling of muscle foods, increasing our understanding of muscle biology and biochemistry (Canto et al., 2015; Gagaoua, Monteils, Couvreur, & Picard, 2019; Gagaoua, Monteils, & Picard, 2018; Gagaoua, Troy, & Mullen, 2021; Munekata, Pateiro, López-Pedrouso, Gagaoua, & Lorenzo, 2021; Purslow, Gagaoua, & Warner, 2021) and allowing the discovery of meat quality biomarkers (Gagaoua, Bonnet, & Picard, 2020; Gagaoua, Terlouw, & Picard, 2017; López-Pedrouso, Lorenzo, Gagaoua, & Franco, 2020; Ouali et al., 2013; Picard & Gagaoua, 2020a; Picard, Gagaoua, & Hollung, 2017). A range of proteomics methods has been applied during the last decade to investigate important meat quality characteristics that influence eating experience and purchasing decisions. They were used (i) to study the dynamic changes in *post-mortem* muscle proteome and the modifications occurring in fresh and cooked meat products (Gagaoua, Troy, et al., 2021; Montowska & Pospiech, 2013; Tian et al., 2016); (ii) for the discovery and identification of protein biomarkers aiming to better understand variations in different meat quality traits (Gagaoua, Terlouw, Boudjellal, & Picard, 2015; Ouali et al., 2013; Picard & Gagaoua, 2020a; Te Pas, Hoekman, & Smits, 2011) and, (iii) to understand the causes of quality defects in certain fresh and processed meat products and the associated underlying biological mechanisms (López-Pedrouso et al., 2020; Schilling et al., 2017). The proteomics techniques used have typically involved gel-based and gel-free approaches coupled with mass spectrometry (MS), array-based methods and sophisticated statistical approaches (Gagaoua, Couvreur, Le Bec, Aminot, & Picard, 2017; Gagaoua, Monteils, et al., 2018; Picard et al., 2017; Zhu, Gagaoua, Mullen, Kelly, et al., 2021; Zhu, Gagaoua, Mullen, Viala, et al., 2021).

In cattle, the recent advances in meat proteomics (Gagaoua, Terlouw, Richardson, Hocquette, & Picard, 2019; Hollung et al., 2014; Zhu, Gagaoua, Mullen, Viala, et al., 2021) have also allowed the discovery of certain proteins and associated molecular pathways behind the important eating quality traits of beef such as tenderness (Gagaoua, Terlouw, et al., 2021) and color (Gagaoua, Hughes, et al., 2020). The valuable knowledge gained from previous studies was due in a large way to the data-mining and integrative studies of public proteomics datasets which enabled repertoires of proteins to be proposed; certain protein candidates were curated as robust and of pivotal interest for further evaluation following the pipeline of meat quality biomarkers discovery (Gagaoua, Bonnet, et al., 2020; Picard & Gagaoua, 2020a). For example, the integromics meta-analysis conducted by Gagaoua, Hughes, et al. (2020) on beef

with normal color, without considering dark, firm, and dry (DFD) beef, otherwise termed dark-cutting beef, led to the identification of seventy-nine proteins belonging to six biological pathways: energy metabolism, responses to stress, oxidative stress, muscle structure, signaling, proteolysis and apoptosis. Based on this first integrative meta-analysis that targeted normal beef color proteomics, a list of twenty-seven putative biomarkers consistently identified among independent proteomic datasets for beef color parameters was proposed. The first objective of the present study aimed to follow the same approach by reviewing proteomics studies of dark-cutting beef condition to create the first repertoire of protein biomarkers of this beef quality defect. The dark-cutting condition usually arises after cattle experience physical and psychological stress prior to slaughter such that muscle glycogen is depleted, hence impacting the rate of pH decline resulting in abnormally dark muscles (Ponnampalam et al., 2017; Tarrant, 1989; Terlouw et al., 2021). As a result, dark-cutting carcasses are discounted during meat grading and downgraded in value, leading to economic losses for the beef sector. The second objective of using publicly available data aimed performing an integrative meta-analysis on the first list of proteins and investigating the extent to which the available proteomics datasets will allow the identification of potential biomarkers of dark-cutting beef. Initially we have reviewed the main causes of dark-cutting beef, the effect of high pH on the color of meat, and the new insights of muscle biology on dark-cutting beef. Subsequently, we present the first repertoire list of putative protein biomarkers in cattle and their analysis using several bioinformatics tools. The analysis of this repertoire allows us to gain more insights, at the proteome level, into the key biological pathways and mechanisms underlying dark-cutting beef. Finally, we discuss the directions that future proteomics (and omics) studies could take to broaden our understanding about this beef quality defect.

## 2. Brief overview of dark-cutting beef and main causes

Dark-cutting meat is generally defined as meat with high ultimate pH (pHu). In many countries, carcasses with a pHu threshold  $\geq 6.0$  in the loin are defined as dark-cutting, including in industry and for laboratory and proteomics experiments (**Table 1**). There are exceptions; for example, the Australian beef industry specifies pHu  $> 5.7$  cut-off for dark-cutting and failing to grade, due not only to the toughness of intermediate pH ( $5.8 < \text{pHu} < 6.2$ ) meat measured using instruments and sensory panels (Grayson et al., 2016) but also because experienced meat buyers discriminate against meat with a pHu  $\geq 5.8$  (Truscott, 1988). For Chinese consumers, the threshold can be higher ( $> 6.2$ ) (Y. Zhang et al., 2021). Although many references indicate that the main factor influencing the dark-cutting condition is muscle

glycogen content at slaughter. Although many references refer to the main factor influencing the pHu being muscle glycogen content at slaughter, the inherent determinant is the concentration of hydrogen ions, which are predominantly generated through glycolysis and the formation of lactate from glycogen, but also from ATP hydrolysis, which is reviewed in several studies (Apaoblaza et al., 2020; Scheffler & Gerrard, 2007). There are several other aspects of dark-cutting meat that warrant attention including (i) time of grading, (ii) rate of pH and temperature decline and (iii) muscle type, which influences amongst others glycogen content.

### 2.1. Time of grading

Color and pH at the time of grading of beef carcasses, and hence occurrence of dark-cutting meat, are pivotal for meat to pass or fail grading scheme such as Meat Standards Australia, where grading can occur as early as 8 – 12 h *post-mortem*. Failure to grade due to high pHu can have serious economic consequences as the carcass can drop in value. In a survey of 1,512 beef carcasses, when grading of beef carcasses occurred at ~14 h *post-mortem*, the incidence of unacceptable meat color scores was 8% and when the time of grading was delayed to ~31 h, the incidence dropped to 3% (Hughes, Kearney, & Warner, 2014). In another study (Steel et al., 2021), 1,200 beef carcasses were graded at 13 h *post-mortem*, then re-graded at ~22 h, when the average loin pH dropped from 5.6 to 5.5 and the carcasses non-compliant for meat color dropped from 8.5% to 5.8%. It is tempting to attribute the influence of time of grading on meat color to non-attainment of a final pH, but there appears to be some color development and lightening of the muscle surface that occurs around rigor, which is independent of pH, reviewed in several papers (Hughes, Clarke, Purslow, & Warner, 2020; Purslow, Warner, Clarke, & Hughes, 2020) and briefly summarized in the section 3 of this paper. Although the logical recommendation may be to grade carcasses at a later time, this is not always practical or feasible. Hence, time of grading for meat color, and pH, must be included in any consideration of determinants of dark-cutting.

### 2.2. Rate of pH and temperature decline

Very fast chilling, to below 0°C within 5 hours of exsanguination can increase the pHu of the loin by 0.1 – 0.2 units in beef (Aalhus, Robertson, Dugan, & Best, 2002; Sikes, Jacob, D'Arcy, & Warner, 2017). The cessation of glycolysis at higher pH with very cold temperatures is likely related to rapid glycolysis (Jacob et al., 2012) and early formation of inosine monophosphate (IMP) (Warner et al., 2015), which cannot re-enter the glycolysis

cycle. Also, beef carcasses that go through rigor at 15 – 25 °C have a much higher incidence of dark color scores at grading relative to carcasses going through rigor at 35 – 40 °C (Hughes, Kearney, et al., 2014; Warner, Dunshea, Gutzke, Lau, & Kearney, 2014). This phenomenon is not driven by muscle glycogen content, but due to premature cessation of glycolysis for other *post-mortem* reasons (section 4).

### 2.3. Muscle glycogen, phenotype and pre-slaughter management

Muscle glycogen content is influenced by inherent muscle characteristics, as muscles containing predominantly red fiber types have a lower muscle glycogen amount (Picard & Gagaoua, 2020b) and hence often have limited *post-mortem* glycolysis, a higher pHu and higher occurrence of dark-cutting. This is exemplified by a comparison between *Masseter* muscle which contains 100% type I red fibers and pHu = 6.3, hence limited glycolysis, compared to the *Cutaneous trunci* of the same animals which has 95% type II white/intermediate fibers and a pHu = 5.5, hence extensive glycolysis (Vaskoska et al., 2021). Hence a beef carcass may be classified as non-dark-cutting, but some predominantly red fiber type muscles such as *Infraspinatus* and *Supraspinatus* might have pHu >5.7 – 6.0 (Kenny & Tarrant, 1984). Conversely, when a beef carcass is classified as dark-cutting using a pHu measurement on the loin, this does not mean all the muscles in the carcass will be dark-cutting (Holman, Kerr, Morris, & Hopkins, 2020), due not only to fiber type differences and metabolic properties (Picard et al., 2014) but also to different sensitivity of muscles to stress (Terlouw et al., 2021), to the rate of cooling *post-mortem* of the carcasses (Hopkins, Ponnampalam, van de Ven, & Warner, 2014), to adrenaline and also whether contractile action has occurred (*e.g.* due to mounting behavior) (Tarrant, 1989). Hence, carcasses classified as dark-cutting because the *Longissimus* muscle has pHu > 6.0, can have low pHu and non-dark-cutting in leg muscles such as *Semimembranosus*, *Gluteus medius* and *Biceps femoris*, as well as the *Psoas major* (Kenny & Tarrant, 1984).

The animal and pre-slaughter factors determining dark-cutting, and high pHu, can be grouped into nutrition, season and stress (Ponnampalam et al., 2017; Terlouw et al., 2021). Grass-fed cattle on a low plane of nutrition due to low pasture quality have lower levels of muscle glycogen, which for instance in Australia is most evident in autumn and winter (Knee, Cummins, Walker, & Warner, 2004). In the USA and Canada, the incidence of dark-cutting in beef carcasses peaks at 0.72% (Boykin et al., 2017) and 2-2.5% (Bruce, Holdstock, Uttaro, Larsen, & Aalhus, 2021), respectively, in August-October, which is most likely an effect of



changes in temperature (Boykin et al., 2017). In France, an incidence of 3.36% or less was reported (Gagaoua, Picard, Soulat, & Monteils, 2018; Mounier, Dubroeuq, Andanson, & Veissier, 2006), but this is variable among breeds and was recently found to be lower (0.11%) in Charolais cattle (Gagaoua, Picard, & Monteils, 2018). Grain-fed cattle have a much lower incidence of dark-cutting than grass-fed cattle in Australia and other countries (Hughes, Kearney, et al., 2014). Heat stress, which at severe levels can induce substantial changes to feed intake and gut permeability, can also increase the incidence of dark-cutting in beef carcasses and is discussed in detail in Gonzalez-Rivas et al. (2020).

The stress factors that are involved in dark-cutting are many and include saleyards, handling, stress susceptibility/temperament, mixing of cattle, time in lairage (Ponnampalam et al., 2017; Steel et al., 2021) to name a few, but for each factor, some experiments have shown 'no' or 'variable' effects, which is particularly the case for time in lairage. An overview and discussion of these factors is provided in Ponnampalam et al. (2017) and these authors conclude that 'no single production factor causes dark-cutting, but that a range of factors or a combination of factors and interactions lead to its occurrence'. The fact that the heritability of pHu is so low (Ponnampalam et al., 2017) supports the multi-factor determination of dark-cutting and indicates its complexity, as evidenced at the proteome level by the integromics analysis presented here (see sections 5 and 6). It is clear from the data collected world-wide, over many years that on-farm nutrition during the 1 – 4 weeks pre-slaughter plays a pivotal role. Seasonality/time of year, which is shown in high incidences of dark-cutting at times in cold temperatures (likely due to redder quality in pastures), fluctuating temperatures and heat events also predominate in surveys/audits of the incidence of dark-cutting (Ponnampalam et al., 2017). Finally, data have consistently shown that carcasses of lower fat depth, lower carcass weight (Steel et al., 2021) and smaller eye muscle area (McGilchrist, Alston, Gardner, Thomson, & Pethick, 2012) are associated with a higher incidence of dark-cutting, most likely because each of these are considered indicators of low growth rates and/or inadequate on-farm nutrition. Importantly, muscle glycogen is rapidly depleted during stress events (Terlouw et al., 2021), but is slow to be replenished in the muscle (Tarrant, 1989) hence feeding regimes to restore muscle glycogen must be at least 1 – 3 weeks duration (Knee, Cummins, Walker, Kearney, & Warner, 2007).

### **3. Effects of high pH on the color of meat**



As mentioned above, insufficient glycogen reserves leading to cessation of metabolism *post-mortem* at pH's above 6.0 is one of the principal causes of the dark-cutting condition. In relation to meat color, there are two sets of mechanisms that determine the abnormal color of meat in the dark-cutting condition: (i) Effects of high *post-mortem* pH on the redox state of myoglobin and the stability of that state. (ii) Effects of high *post-mortem* pH on the spacing of the myofilament lattice and on the denaturation of sarcoplasmic proteins, which in turn affect light scatter in the dark-cutting meat.

### 3.1. Effects of high *post-mortem* pH on the redox state of myoglobin and its stability

A role in the redox state of myoglobin in the color of dark-cutting meat can be related to two notable effects. First, the dark color due to the deoxygenated state of myoglobin in dark-cutting meat will not change to the bright red oxymyoglobin state when a cut surface is exposed to air (*i.e.* dark-cutting meat will not bloom; Egbert and Cornforth (1986)). Further, Cornforth and Egbert (1985) demonstrated that the dark color of dark-cutting beef could be transformed into a redder color by inhibiting mitochondrial respiration with rotenone (an organic compound that interrupts complex I of the electron transport chain). Second, dark-cutting beef cooked to the same internal temperature as normal-pH beef has a redder color and appears undercooked (Gašperlin, Žlender, & Abram, 2000).

The tricarboxylic acid (TCA) cycle and the electron transport chain (ETC) processes occurring within mitochondria require NAD and NADH (nicotinamide adenine dinucleotide reduced form), respectively. Maintenance of a favorable NAD/NADH ratio is necessary for efficient mitochondrial metabolism (Stein & Imai, 2012). The NADH level in *post-mortem* muscle plays an important role in metmyoglobin reduction. Metmyoglobin reductase activity (MRA) is the result of several pathways, all of which requiring NADH as coenzyme acting as an electron donor, and NADH therefore has an important role in the reduction of metmyoglobin in *post-mortem* meat (Mitacek et al., 2019; X.-Q. Zhang, Jiang, Guo, Bai, & Zhao, 2020). Reddy and Carpenter (1991) showed that MRA activity was greater at pH 5.8 than at pH 6.4, and varied between muscles. Using a model system, Tang et al. (2005) directly demonstrated that mitochondrial respiration led to a shift from oxygenated to deoxygenated forms of myoglobin. Limited glycolysis in dark-cutting meat, resulting in a high pH, encourages a high mitochondrial respiration (further evidence is shown thanks to our integromics study at the proteome level in section 6), affecting both the prevalence of the purplish deoxymyoglobin and, oxymyoglobin formation during blooming, with a knock-on effect on the changes in the

states of myoglobin during cooking. The comprehensive and integrative overview of the underlying mechanisms at the *post-mortem* muscle proteome level presented below supports this view (see section 6).

### 3.2. *Effects of high post-mortem pH on the spacing of the myofilament lattice and on the denaturation of sarcoplasmic proteins*

When specifically discussing dark-cutting meat, several reviews of meat color concentrate mainly on the role of water retention in the muscle at high pH and its effects on light scatter (Mancini & Hunt, 2005; Ponnampalam et al., 2017; Ramanathan, Hunt, et al., 2020; Suman & Joseph, 2013). Achromatic sources of variations in the lightness or darkness of meat are principally due to variations in the microstructure (see section 6.3 for more evidence at the proteome level), causing differences in the amount of light scattering (Hughes, Clarke, Li, Purslow, & Warner, 2019). Recently, Purslow et al. (2020) indicated that the main structural changes are variations in the spacing between thick and thin filaments in the sarcomere (myofilament lattice spacing), with resultant effects on myofibril diameter and myofibril-myofibril spacing within the muscle fiber. Other sources of variations include sarcomere length (Torres-Burgos et al., 2019), which may cause changes in myofibril and muscle fiber diameter, and variations in the state and distribution of sarcoplasmic proteins.

For example, the role of sarcoplasmic protein denaturation and precipitation in PSE pork has been highlighted by Liu, Arner, Puolanne, and Ertbjerg (2016), who infer that sarcoplasmic protein denaturation within myofibrils (together with some denaturation of myosin heads) at low pH is associated with a decreased myofilament lattice spacing, whereas coagulated sarcoplasmic proteins in the extracellular space between muscle fibers are thought to trap water exuded from the myofibrils. In studies on beef muscles, Hughes, Clarke, Purslow, and Warner (2017) showed that manipulation of the spacing between thick and thin filaments in the myofilament lattice due to cycling between high and low pH values resulted in more light scattering from single muscle fibers at low pH than at high pH. When starting with a pH of 6.1 (equivalent to dark-cutting beef) and taking the fibers to a pH of 5.4 and then back up to pH 6.1, not all of the changes in light scattering were reversible. In further work, Hughes et al. (2019) investigated the possibility that this irreversible increase in light scatter on lowering the pH was due to the denaturation of some sarcoplasmic proteins that were normally partially denatured at normal and low *post-mortem* pH's. The authors further demonstrated that *post-*

*mortem* muscle with high pH<sub>u</sub> and dark-cutting appearance had a wider myofilament lattice spacing, more sarcoplasmic proteins adhering to the thin filaments, more degradation of some cytoskeletal proteins, including titin, and disorganization of the Z-disc. The latter two effects could be due to increased proteolysis at high pH<sub>u</sub>, but it may contribute to lower light scatter if proteolytic disruption of the regular sarcomeric structure results in a reduction of sharp refractive index changes between A- and I-bands.

#### 4. Muscle biology of dark-cutting beef

In addition to the mechanisms highlighted above, the occurrence of dark-cutting beef is also related to stress before slaughter, reducing muscle glycogen content and thus, limiting muscle pH decline (Kiyimba et al., 2021; Mahmood, Turchinsky, Paralis, Dixon, & Bruce, 2018; Poleti et al., 2018; Ponnampalam et al., 2017). As indicated above, greater *post-mortem* muscle pH is a conducive factor for enhanced mitochondrial oxygen consumption, limiting oxygen availability for myoglobin, and allows muscle fibers to hold more water (Ramanathan, Mancini, Suman, & Cantino, 2012; Tang et al., 2005). The latter decreases oxygen diffusion beneath the surface. Their combined effects explain the darker color and greater deoxymyoglobin levels in dark-cutting than normal meat (English et al., 2016; Hughes, Oiseth, Purslow, & Warner, 2014).

Several studies have shown differential profiles of genes expression, metabolites, and proteins in dark-cutting beef (see section 6 for further details about the first proteome repertoire) compared with normal pH beef. For example, a genomic experiment showed that high-pH beef has a greater abundance of genes involved in stress-related signaling pathways such as protein-lysine methyltransferase (*METTL21C*), amide oxidase [Flavin-containing] A (*MAOA*), and growth arrest and DNA damage-inducible beta (*GADD45B*) (Jerez-Timaure et al., 2019). Over-abundance of these genes is an indicator of high pre-slaughter stress, often a contributing cause of dark-cutting beef.

Metabolomics and proteomic characterization of dark-cutting beef profiles suggested down-regulation of several metabolites such as; glucose-1-phosphate, glucose-6-phosphate, glycerol-3-galactoside, gluconic acid, and fructose-6-phosphate (Cônsole et al., 2021; Ramanathan, Kiyimba, Gonzalez, Mafi, & DeSilva, 2020), and enzymes involved in glycogen metabolism, including glycogen phosphorylase, bis-phosphoglycerate mutase, phosphorylase kinase b, UTP--glucose-1-phosphate phosphate uridylyltransferase, and adenosine monophosphate

deaminase (Kiyimba et al., 2021; Poleti et al., 2018). Reduced glycolysis could be due to downregulation of glycolytic enzymes coupled with pre-slaughter stress and low glycogen.

## 5. Data-mining applied to different published dark-cutting beef proteomics datasets

### 5.1. Data collection and preparation of the dark-cutting beef proteome repertoire

Meta-analysis and data-mining are very useful tools to gather and compare meat eating quality omics studies performed by various platforms (Boudon, Henry-Berger, & Cassar-Malek, 2020; Gagaoua, Hughes, et al., 2020; Gagaoua, Terlouw, et al., 2021) or even from a single laboratory using the same proteomic platform (Picard & Gagaoua, 2020a) in the frame of integrated animal systems biology. To gain comprehensive knowledge on the currently identified candidate protein biomarkers, the underlying pathways and the mechanisms of dark-cutting beef at the proteome level, research papers on dark-cutting beef proteomics (proteome datasets of normal-pH *versus* high-pH) were therefore identified. Selection was based on a computerized search strategy using Web of Science (Clarivate Analytics), Scopus and Google Scholar knowledge databases. The keywords used were “proteom\*”, “protein”, “biomarker”, “pH”, “ultimate pH”; “high pH”, “DFD”, “dark-cutting” and “color”, in combination with “meat”, or “beef” or “muscle”. Only research papers published in English and in peer-reviewed journals were considered, to ensure the methodological quality of the studies. Papers published up to 15 January 2021 were identified, screened independently and organized into a spreadsheet reference manager. The papers for inclusion in this meta-analysis were examined following an established process (Gagaoua, Hughes, et al., 2020) to determine whether they are eligible to be included in the database, based on the following criteria: (i) publications that considered solely bovine *Longissimus thoracis* (LT) muscle; (ii) studies that used “proteomics” based-methods; and (iii) papers that compared differentially abundant proteins between high-pHu muscle samples to those of normal-pHu beef. This inclusion/exclusion criteria step allowed the removal of publications that applied other “omics” methods such as phosphoproteomics, metabolomics or genomics. A total of 8 research papers (Franco et al., 2015; Fuente-Garcia et al., 2019; Fuente-Garcia, Sentandreu, Aldai, Oliván, & Sentandreu, 2020; Hughes et al., 2019; Kiyimba et al., 2021; Mahmood et al., 2018; Poleti et al., 2018; S. Wu et al., 2020) met the inclusion criteria as eligible proteomic studies on dark-cutting beef (**Table 1**). In addition to the details given in the main text of the papers, supplementary files, if available, were further screened and any relevant data were used in this integromics review.

Data on the selected dark-cutting beef proteomic studies are listed in **Table 1**. Briefly summarized are the experimental designs concerning the animal types, the conditions and pHu thresholds used to categorize the normal from dark-cutting meat samples, the number of samples used together with the proteomics platforms and type of protein extracts used. Of these studies listed in Table 1, 7 studies used a threshold of pHu > 6.0 with mean pHu values of dark-cutting samples varying from 6.15 to 6.86, and one study used pHu > 5.9 but with a higher mean pHu value of the dark-cutting group ( $6.61 \pm 0.09$ ). The sampling of *Longissimus thoracis* muscle was performed at 24 h *post-mortem* (for 5 studies) or at  $\geq 48$  h (for 3 studies) (**Table 1**). The muscle proteome was characterized on total protein extracts (4 studies), sarcoplasmic proteins (2 studies), myofibrillar proteins (1 study) or mitochondrial muscle protein extracts (1 study). Among the 8 studies, three of them used label-free quantitative proteomics, two used liquid isoelectric focusing (OFFGEL) method at pH ranges of 3–10 or 4–7 coupled to mass spectrometry, two used two-dimensional electrophoresis (2-DE at pH ranges of 3–10 or 4–7) and one focused on protein bands using 1D SDS-PAGE (**Table 1**). Variations in using proteomics approaches and reporting analytical details among studies were in accordance with recent observations (Gagaoui Teriouw, et al., 2021). However, in this case, this variation did not affect the final outcomes of this review from a qualitative point of view, which consists of data integration from multi-platform proteomics datasets with a focus on the pathways and identity of the unique gene names (proteins) that differ between normal and dark-cutting beef. Therefore, such variations are not a major hindrance, as the list and direction of the differential proteins between dark-cutting and normal-pH is the main information needed.

A total of 130 proteins (total number of animals = 122 from which 48 were dark-cutting, minimum of dark-cutting carcasses per group = 4, and maximum = 8) were gathered from the 8 studies and analyzed in this integromics meta-analysis as being differential in dark-cutting *versus* normal-pH beef (**Table 2**). Key information regarding the protein biomarkers candidates were annotated in the database using their unique gene names including the consideration of proteoforms, the bovine UniprotID accession numbers, the full Uniprot names of the proteins and the direction of change (Up ( $\uparrow$ ) in dark-cutting and Down ( $\downarrow$ ) in dark-cutting) reported in each study. Accordingly, 69 proteins were found to be up-regulated, 53 were down-regulated and 8 proteins were common in at least two studies and went in both directions (**Table 2**). In the case of a protein being up- and down-regulated in two or more independent studies, it was considered in both lists for the bioinformatics analyses.

## 5.2. Bioinformatics analyses applied to the dark-cutting beef proteome repertoire

The dark-cutting beef proteome repertoire was mined using different bioinformatics and software tools following recently described methods (Gagaoua, Hughes, et al., 2020; Gagaoua, Terlouw, et al., 2021; Gagaoua, Troy, et al., 2021).

(i) All the bovine UniProtIDs were converted into human identifiers based on the orthologous and homologous links, to avoid any limitation of the Gene Ontology (GO) annotations on bovine proteins, so taking advantage of the current and most complete annotation available for human gene names. Thus, both bovine and human UniprotIDs were indexed in the database and used.

(ii) Pathway and clustering enrichments, network analysis, comparative heat maps (hierarchical clustering of pathways) and circos plots were performed using Metascape<sup>®</sup> (<https://metascape.org/>, date last accessed February 2021). We used the Benjamini–Hochberg *P*-value correction algorithm and hypergeometric test to display the first statistically significant enriched ontology terms. This provided a comprehensive gene list annotation curated via Gene Ontology (GO) Biological Processes, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Reactome gene sets (Y. Zhou et al., 2019). The hierarchical clustering was performed with the enriched terms involving the association of gene names (proteins) with similar expression patterns by calculating and classifying data based on similarities. This clustering allowed the prediction of unknown gene functions and whether they are involved in similar metabolic processes or cellular pathways. Thus, pairs of terms with a kappa score > 0.3 were considered as a cluster, and a cluster was represented by the most significant term. Subsequently, network layouts of the enriched terms on each dataset were visualized in Cytoscape software (V3.8.2).

(iii) The STRING database (Search Tool for Retrieval of Interacting Genes, ver. 11.0 at <https://string-db.org/>) was used to construct Protein–Protein Interactions (PPI) among specific protein datasets using construction default settings with minimum interaction score confidence of 0.700 (high confidence).

(iv) The molecular complex detection (MCODE) algorithm available at Metascape<sup>®</sup> was used to detect densely connected regions in the protein interaction network (neighborhoods). The cut-off criteria were the default values: degree cut-off = 2, node score cut-off = 0.2, Max



depth = 100, and  $K$ -score = 2. Pathway and process enrichment analyses were subsequently performed independently to each MCODE component.

(v) The computational prediction of the putatively secreted proteins by bovine muscle (“secretome” investigation) through classical (involving a signal peptide) or non-classical pathways was performed using ProteINSIDE web tool (<https://www.proteinside.org/>).

## 6. Main findings of the dark-cutting beef proteome integromics meta-analysis and discussion

Proteomics has provided substantial data in terms of new proteins related to or explaining the development of dark-cutting beef and allowed in this integromics meta-analysis the description of the main biological pathways that are involved in the phenomenon. This work synthesizes the current knowledge and existing literature on dark-cutting beef at the *post-mortem* muscle proteome level and generated the first repertoire of protein candidates’ biomarkers of this quality defect (**Table 2** and **Fig. 1**). Such repertoires (databases) serve as efficient references and may be enriched with newly identified proteins in future work. Importantly, integromics meta-analyses give insights into the biological pathways involved in meat quality traits (Boudon et al., 2020; Gagaoua, Hughes, et al., 2020; Gagaoua, Terlouw, et al., 2021; Purslow et al., 2021; Welzenbach et al., 2016).

### 6.1. Strong disparity among dark-cutting beef proteomics datasets

The first notable finding revealed by this integromics meta-analysis is the strong disparity among the 8 studies in terms of common proteins (**Fig. 1A,B** and **Table 2**). From the list of 130 proteins, the circo plots representing the between-group differences showed that very few proteins were simultaneously categorized in more than 1 study (**Fig. 1A**). Thus, a cut-off  $\geq 2$  revealed that only 10 proteins (highlighted by a network) have been identified in more than one investigation (**Fig. 1C**), these being ACTA1, DES, ACTN2, MYLPP, MYH1, CRYAB, HSPB1, MDH1, UGP2 and YWHAG. The overlap represents around 7.69 % of the proteins gathered in the database which is significantly lower than the high scores previously reported for beef tenderness (51.61% from 124 proteins of LT muscle) (Gagaoua, Terlouw, et al., 2021) or normal beef color (46.83% from 79 proteins of 5 muscles or 50.84 % from 59 proteins of LT muscle) (Gagaoua, Hughes, et al., 2020). This discrepancy may also explain the identification of high number of proteins ( $n = 130$ ) from 8 experiments compared to other meta-analyses. The disparity in dark-cutting beef proteomics studies may be partly explained by three reasons, despite the existence of unknowns around the multi-factor determinants.



Firstly, the proteomics approaches used were not always conducted equivalently leading to strong differences in the number of differential proteins (from 5 to 57). Further, due to the nature of the protein identification algorithms, false-positives may be present in datasets because not all datasets were equally filtered. Indeed, the recent rapid pace of technical development in the field of proteomics has resulted in relatively poor equivalency of data across the current body of literature. In particular, it can be confusing to compare 2-DE gel data that resolves proteins to their constituent species or proteoforms with label-free LC-MS profiling methods (data-independent acquisition and software quantitative informatics tools) that reports the overall abundance of all species of each protein. We expect that a wide spread application of (shotgun) label-free LC-MS (Kiyimba et al., 2021; Zhu, Gagaoua, Mullen, Viala, et al., 2021), as a robust platform, will produce in the future parallel data with greater equivalency of proteome coverage across studies from different laboratories.

Secondly, the dynamic changes of *post-mortem* muscle proteome (Gagaoua, Troy, et al., 2021; Jia et al., 2007) and post-translational modifications (PTM) such as phosphorylation (Mato et al., 2019) or acetylation (Jiang, Liu, Shen, Zhou, & Shen, 2019) may also be a source of variation.

Thirdly, pre-slaughter stress status and stress reactivity further related to the PTM of the *post-mortem* muscle proteome (Zhou, Shen, Liu, Wang, & Shen, 2019) could be a great source of variation that is unfortunately not often considered in such studies (Terlouw et al., 2021). Reporting stress levels and slaughter context in dark-cutting beef studies is another important recommendation for future work to facilitate comparison of experiments.

From the 10 common proteins, 5 belong to the myofibrillar and structure pathway, followed by 3 heat shock proteins (HSPs) involved in protein folding and apoptosis and two energy metabolism proteins. The interaction of the two small HSP proteins (CRYAB:  $\alpha$ B-crystallin and HSPB1: Hsp27) with structural proteins (**Fig. 1C**) is of importance and supports previous knowledge on the protective role of HSPs proteins on structural proteins (Gagaoua, Terlouw, Boudjellal, et al., 2015; Gagaoua, Terlouw, Micol, et al., 2015; Lomiwes, Hurst, et al., 2014). The pathway enrichment analysis on these 10 common proteins confirmed the importance of muscle contraction (GO: 0006936) as the top ontology term in dark-cutting beef development, followed by others related to the regulation of apoptosis, carbohydrate biosynthetic and protein folding processes (**Fig. 1D**). On the other hand, 8 of the common proteins had different directions, being up-regulated in one study and down-regulated in another and vice versa (**Table 2**). These incongruities were already reported in previous studies (Gagaoua, Hughes, et

al., 2020; Gagaoua, Terlouw, et al., 2021; Ouali et al., 2013; Picard & Gagaoua, 2020a), possibly due to either different isoforms of the proteins (proteoforms) or different experimental procedures and conditions, related to pre-slaughter stress levels, sampling time, breed, gender, age, rearing practices, muscle fibers (myofibre phenotype and basal metabolic rate), amongst others.

Two structural proteins, ACTA1 (actin, from thin filament and Z-disc) and DES (desmin, an intermediate filament from the periphery of the myofibrillar Z-disk) were consistent in the direction of their change, being up-regulated and down-regulated in dark-cutting beef, respectively. The identification of actin (mostly as proteolytic fragments) may be related to its key regulator role of apoptosis (Ouali et al., 2013). Apoptosis was revealed in this study to be up-regulated in dark-cutting beef (see section 6.4). The identification of desmin (decrease of its abundance by aging) can be explained by its proteolytic degradation mostly by  $\mu$ -calpain (Huff-Lonergan, Zhang, & Lonergan, 2010), and hence would impact on light scattering. It is important to note that desmin was documented to be degraded more rapidly in myofibrils from tender samples and higher water content (Huff-Lonergan et al., 2010), a typical characteristic of dark-cutting meat. For further details on the pathways involving apoptosis and structural proteins on dark-cutting beef refer to section 6.3.

YWHAG (14-3-3 protein gamma) was the only protein identified in 3 studies; it was down-regulated in 2 of them (Kiyimba et al., 2021; Mahmood et al., 2018) and up-regulated in the third (Hughes et al., 2019). The 14-3-3 family members are involved in a wide variety of processes such as protein trafficking, apoptosis and intracellular signaling (Aghazadeh & Papadopoulos, 2016). In skeletal muscle of cattle, YWHAG was reported to differ between low and high residual feed intake groups and was confirmed to be associated to energy metabolism (Carvalho et al., 2019). A previous phosphoproteomics study reported that 14-3-3 proteins are able to regulate glucose homeostasis in response to insulin or to energetic stress (Ogihara et al., 1997), which may explain its identification as a biomarker of dark-cutting beef. Its epsilon isoform (YWHAE) was earlier identified as a robust biomarker of beef tenderness (Gagaoua, Terlouw, et al., 2021). Rodrigues et al. (2017) suggested that the phosphorylation of 14-3-3 family members may also play a role in the muscle structure integrity and apoptosis (Haydon et al., 2002).

Besides the few number of proteins and molecular pathways that are shared among studies (**Fig. 1E**), some common features emerge and the unbiased nature by which these were collected provides some validation of their biological importance. For example, the

identification of “muscle system process” ontology term (GO: 0003012) as a major pathway in dark-cutting beef (significantly enriched in 6 studies, **Fig. 1E**) is of high importance and supports the recent hypothesis stating that meat color can be determined by the physical structure and achromatic light scattering properties of the muscle (Hughes et al., 2020; Purslow et al., 2020). Furthermore, the consistently appearing proteins during enrichment may provide key mechanistic insights or be surrogates for mechanisms that are more difficult to detect using conventional hypothetico-deductive methods (Purslow et al., 2021).

## 6.2. Mitochondria and associated pathways are the first drivers of dark-cutting beef

The pathway process enrichment on the 130 proteins permitted the construction of the first interconnected and robust biological network of dark-cutting beef (**Fig. 2** and **Fig. 3**) and identified the main significantly enriched pathways related to its development (**Table 3**). In relation to the current study, all of the GO terms mentioned in **Fig. 2** that relate to oxidation-reduction processes, mitochondrial and cellular respiration, tri-carboxylic acid cycle (TCA) cycle and electron transport chain, aerobic respiration, energy metabolism and mitochondrial organization have obvious links with the mechanisms known to impact pH decline, color stability and *post-mortem* metabolism. These findings indicate that aerobic metabolism plays a pivotal role in the development of dark-cutting beef. Thus, the up-regulation expression of a great number of aerobic energy metabolism proteins and associated pathways could be ascribed to the elevated ATP production in *post-mortem* muscle (Jia et al., 2006), whereas the down-regulated expression of glycolytic-responsive proteins decreased anaerobic metabolism, lactic acid production, hence impacting the rate of pH decline, ultimate pH and blooming. Dark-cutting meat is known to present low carbohydrate storage levels, but appears also to be associated with greater mitochondrial oxygen consumption, and an increased use of energy and mitochondrial respiration rate. For example, we can observe from the studies retained in this integromics meta-analysis a greater abundance of certain proteins involved in the electron transport, including NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2 (NDUFA2), succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial A and B (SDHA and SDHB), cytochrome c oxidase subunit 6A2, mitochondrial (COX6A2), and ATP synthase subunit e, mitochondrial (ATP5ME) (Kiyimba et al., 2021), and proteins involved in the TCA cycle such as; pyruvate dehydrogenase kinase 4, and malate dehydrogenase (S. Wu et al., 2020).

Earlier studies reported that greater pHu (> 6.0) in dark-cutting beef can sustain mitochondrial function *post-mortem* (Ashmore, Doerr, Foster, & Carroll, 1971). The *post-mortem* muscle cells turn to other energy sources to sustain its energy needs by using lipids and amino acids (Ouali et al., 2013) through, for example, oxidative phosphorylation, hence impacting mitochondrial oxygen consumption and respiration (S. Wu et al., 2020). A recent metabolic pathway analysis revealed that the majority of metabolites discriminating dark-cutting from normal-pH beef were associated with cell energy production pathways, likely the pentose phosphate pathway, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, galactose metabolism and glycolysis/gluconeogenesis (Cônsole et al., 2021). Using a metabolomics approach, Cônsole et al. (2021) further evidenced an important production of ATP in dark-cutting beef compared to normal pHu beef. This is in line with a previous meta-analysis (Gagaoua, Hughes, et al., 2020) that identified “ATP metabolic process” as a prominent pathway driving beef color. Mitochondrial respiration is supported by increased availability of TCA metabolites and increased mitochondrial protein content. According to recent studies, greater mitochondrial content in dark-cutting beef than normal-pH beef was reported (McKeith et al., 2016; Ramanathan, Kiyimba, et al., 2020). Metabolomics studies further showed an increased abundance of TCA metabolites, such as fumaric, malic, and citric acid, in dark-cutting beef (Cônsole et al., 2021; Ramanathan, Kiyimba, et al., 2020). Many of these metabolites are important in mitochondrial respiration and oxygen consumption, mainly through the synthesis of NADH and flavin adenine dinucleotide (FADH). The greater abundance of TCA metabolites coupled with greater mitochondrial content therefore suggests that dark-cutting beef has greater mitochondrial oxygen consumption. Increased mitochondrial respiration *post-mortem* can decrease oxygen availability for muscle blooming producing dark-colored muscles (English et al., 2016; Ramanathan & Mancini, 2018; Tang et al., 2005). Taken all together, the findings of this integrative highlighted that a better understanding of dark-cutting beef biochemistry requires further investigations into mitochondrial roles and pathways.

### 6.3. A key role of muscle structure in the development of dark-cutting beef

Interestingly, “muscle system process” and “striated muscle tissue development” were identified in the top 5 enriched ontology terms (**Fig. 2**), hence validating muscle structure as a major pathway underpinning the development of dark-cutting meat. This can be partly related to the extent of *post-mortem* degradation, in a pH-dependent manner, of structural proteins (Gagaoua, Troy, et al., 2021; G. Wu, Farouk, Clerens, & Rosenvold, 2014). For example,

degradation of higher molecular weight proteins in elevated pHu meat was reported (Lomiwes, Farouk, Wu, & Young, 2014). The enriched terms “striated muscle contraction” and “filament sliding process” revealed in this study as main components of the network (**Fig. 3**), were also found by Gagaoua, Troy, et al. (2021) as major pathways related to the release, in a pH-dependent manner, of major *post-mortem* proteolytic fragments. Earlier studies evidenced the role that *post-mortem* muscle microstructure plays on the surface meat color (Hughes et al., 2020; Swatland, 2012) including the identification of several structural proteins (Gagaoua, Hughes, et al., 2020; Ramanathan, Hunt, et al., 2020; Ramanathan, Suman, & Faustman, 2020). However, the mechanisms by which structural proteins and pH decline impact color are not fully elucidated and further investigations are needed. For instance, we suppose that the extent of muscle protein denaturation might play a role in oxygen penetration and myoglobin status (Sammel, Hunt, Kropf, Hachmeister, & Johnson, 2012). In fact, the diffusion of oxygen into the meat surface including its consumption by muscle is one of the pivotal factors impacting color (Hughes et al., 2020), which are mostly influenced as detailed above, by mitochondrial functionality (Ramanathan, Mancini, Naveena, & Konda, 2010). A high pHu value reduces muscle lightness and increases oxygen consumption (Kiyimba et al., 2021), which can be partly related to differences in muscle fibers types mainly to an increased percentage of type I fibers (Gagaoua, Hughes, et al., 2020; Picard & Gagaoua, 2020b). It is worthy to note that mitochondria are not evenly distributed in muscle fibers: slow-contracting muscle fibers (type I, oxidative) possess a greater mitochondria concentration than do fast-contracting fibers (Picard & Gagaoua, 2020b), which can further add a level of complexity to the role of myosin fibers and muscle structure to the mechanisms underpinning dark-cutting development.

On another hand, the oxygen penetration depth and formation of oxymyoglobin (red myoglobin form) is dependent upon the partial pressure of oxygen ( $pO_2$ ) and its ability to diffuse into the muscle structure. The speed of oxygen penetration into the muscle (known as the oxygen consumption rate, OCR) has an inverse relationship to oxygen penetration depth. Accordingly, Hughes et al. (2020) suggested that changes in the structure and spacing of the muscle could alter the oxygen penetration depth and hence the OCR. The comprehensive review of Hughes and co-workers exemplified this specific point. Briefly, in high pH dark muscle, the lack of spaces between cells and muscle bundles prevented oxygen diffusion into the tissue and there is a greater demand for oxygen by myoglobin in the interior, hence at the surface there is less oxymyoglobin and more purple deoxymyoglobin (Hughes et al., 2020;

Krzywicki, 1979). In contrast to this, lower pH muscles with more spaces between cells and muscle bundles in the structure have a greater ability to undergo oxygenation and oxidation, resulting in greater redness and browning closer to the surface of the muscle. Dark meat samples are also known to have structural differences at various positions of the sarcomere compared to either pale or medium light meat (Hughes et al., 2019; Hughes, Clarke, Purslow, & Warner, 2018). Ultrastructural changes would then impact the light scattering arising from the structural elements (Hughes, Oiseth, et al., 2014; Swatland, 2008). The increase in pH<sub>u</sub> may also be accompanied by higher water-holding capacity, hence inducing swelling of muscle fibers and shrinkage of the space between the fibrils. This decreases light scattering and increase light absorption by myoglobin and as a consequence, the muscle surface color appears darker (Hughes et al., 2017; Hughes et al., 2020; Purslow et al., 2020). Accordingly, Hughes and co-workers suggested the involvement of three main changes in microstructural components of *post-mortem* muscle: (i) transverse shrinkage of the structural lattice of the myofilaments, myofibrils, and muscle fibers, which would create light scattering between adjacent transverse elements; (ii) longitudinal shrinkage of the sarcomere, whereby changes in the protein density in the A/I-bands would impact light scattering; and (iii) different protein composition of the surrounding medium that alter the refractive index or optical protein density of the fluid (Hughes et al., 2020).

#### 6.4. Similarities and divergences in the up- and down-regulated proteome of dark-cutting beef

The comparison of the Top20 significantly enriched GO terms between the up- and down-regulated proteome datasets (**Fig. 4** and **Table S2**) revealed seven common ontology terms, these being tight junction (KO: 04530), response to inorganic substance (GO: 0010035), cellular amino acid metabolic process (GO: 0006520), muscle system process (GO: 0003012), striated muscle tissue development (GO: 0014706), oxidation-reduction process (GO: 0055114) and purine ribonucleotide metabolic process (GO: 0009150). Among these pathways, the oxidation-reduction process seemed to be more abundant in the up-regulated dataset (**Fig. 4**) in agreement with the above statements and available literature (Kiyimba et al., 2021; Ramanathan, Hunt, et al., 2020). Three terms related to glycogen biosynthetic process (GO: 0005978), IMP biosynthetic process (GO: 0006188) and response to activity and external stimuli (GO: 0014823) were significantly and exclusively specific to the down-regulated proteome dataset. This means that the proteins involved in glycogen homeostasis are likely to be down-regulated in dark-cutting meat, therefore suggesting (i) a down-regulation of the enzymes/proteins responsible of glycogen breakdown and (ii) a decrease in glycogen



mobilization/use. Muscle glycogen content is not the sole determinant of dark-cutting beef (Apaoblaza et al., 2015), as meat with higher pH<sub>u</sub> can have residual muscle glycogen and this has variously been related to mitochondrial function (Hudson, 2012) but essentially glycolysis ceases for reasons other than a lack of glycogen as a substrate. Particularly, the down-regulation of IMP biosynthetic process in dark-cutting beef may be a consequence of down-regulation of glycolytic enzymes (**Fig. 5**), associated with decreased glycolysis and lactic acid production (Eric M. England, Matarneh, Scheffler, Wachet, & Gerrard, 2014; E. M. England, Matarneh, Scheffler, Wachet, & Gerrard, 2015). *Post-mortem* muscle pH decline rate influences phosphorylation of proteins involved in glycolysis, which in turn impacts *post-mortem* metabolism (Huang et al., 2011). Thus, a decreased abundance of IMP biosynthetic and glycogenolytic enzymes reduces *post-mortem* hydrogen ion production leading to elevated pH<sub>u</sub>.

Ten GO terms were found to be specific to the up-regulated proteome dataset that are mainly related to mitochondria, cellular respiration and associated pathways, among which the TCA cycle and respiratory electron transport are significant terms. The results further evidenced that pathways such as oxidative phosphorylation and the TCA cycle (in line with the above), were up-regulated in dark-cutting beef, whereas those associated with glycolysis were down-regulated. The TCA cycle is an important pathway of energy and substance metabolism, accompanied by the production of energy and reduction equivalents (NADH) that promote mitochondrial electron transfer chains and influence beef color stability during storage or display period. This suggests that an enhanced generation of NADH, a precursor mentioned earlier to play a role in the reduction of metmyoglobin and oxygen consumption (Tang et al., 2005), would lead to greater MRA and dark color (myoglobin is predominantly deoxymyoglobin). In dark-cutting condition, more NADH was found to induce higher oxygen consumption (Kiyimba et al., 2021) and MRA (Tang et al., 2005), which are inherent biochemical properties that influence meat color.

It is interesting to observe that the regulation of the apoptotic signaling pathway was significantly up-regulated in dark-cutting meat (**Fig. 4**). Taken together, all these effects are in agreement with the suggested roles of mitochondria in meat color (Hudson, 2012; McKeith et al., 2016; Ramanathan & Mancini, 2018; Ramanathan, Suman, et al., 2020; Sierra & Oliván, 2013) and of apoptosis in meat tenderization through proteolytic enzymes (Ouali et al., 2013). The latter may in part explain, although other mechanisms including higher water-holding capacity, limited *post-mortem* cross-linking, etc. may also be contributing, the greater



tenderness of dark-cutting beef (Cônsole et al., 2021; Franco et al., 2015; Grayson et al., 2016; Jeremiah, Tong, & Gibson, 1991). The over-abundance of “mitochondrion organization” term (GO: 0007005) supports the earlier mentioned relationship on one hand between dark-cutting and the cleavage of cytochrome b-c1 by caspases (Mato et al., 2019) and on another hand with biomarkers of autophagy (Beclin-1 and LC3-II/LC3-I) and apoptosis (caspase-3) (Díaz-Luis et al., 2021). The significant enrichment of the “regulation of ion transmembrane transporter activity” term (GO: 0032412) in the up-regulated proteome dataset adds further support for the existence of these mechanisms. This can to some extent be related to calcium release known for its pivotal role in the muscle to meat conversion and consequences on the quality of meat (Purslow et al., 2021). Therefore, *post-mortem* energy metabolism and pH decline rate could be also affected by mitochondria through calcium regulation, related to their ability to store calcium in their matrix (Matarneh, Yen, Bodmer, El-Kadi, & Garrard, 2021; Ouali et al., 2013). Indeed, a disturbed regulation of calcium is known to enhance the metabolic pathways and ultimately can lead to inferior meat quality (Küchenmeister, Kuhn, & Ender, 2000). However, muscle activities are initiated by changes in intracellular calcium amount, and this concentration is dependent on proper sarcoplasmic reticulum function (Purslow et al., 2021). Specifically, if the calcium flux is elevated the integrity of mitochondria will be destroyed, leading to the release of cytochrome c and other pro-apoptotic factors, triggering apoptosis (Dang et al., 2020; Ouali et al., 2013; Sierra & Olivan, 2013; Zhu et al., 2012) and the decreased free calcium may result in dark-cutting beef (Torres-Burgos et al., 2019). Further research is required to better understand the role of calcium and its pre-slaughter supplementation on meat quality and incidence of dark-cutting condition.

#### 6.5. Comparison of dark-cutting and normal beef color proteomes to gain further mechanistic insights on the underpinning pathways

To understand better the above findings, we performed another functional annotation comparison between the dark-cutting beef proteome repertoire to that of normal beef color (**Fig. 5** and **Fig. 6**) described in a previous meta-analysis (Gagaoua, Hughes, et al., 2020). Despite the low number of proteins in the normal beef color dataset (n = 59) compared to dark-cutting beef, the analyses revealed key findings (**Table 4**). First, the protein overlap of the two proteome repertoires showed 20 common proteins (**Fig. 5A**), mainly belonging to three biological pathways: energy metabolism proteins (n = 9) with 6 glycolytic enzymes, myofibrillar and structural proteins (n = 7) and three small HSPs (**Fig. 5B**). These findings together with the previous conclusions from the integromics meta-analysis on normal beef

color (Gagaoua, Hughes, et al., 2020) underline the importance of muscle contraction, NADH metabolic and carbohydrate catabolic processes, regulation of cell growth and protein folding on the color of meat (**Fig. 5C**). It further confirmed the importance of stress proteins in the determination of meat color (Gagaoua, Hughes, et al., 2020) including the dark-cutting condition. Second, the comparison of the enriched pathways between the two datasets (**Fig. 5D** and **Fig. 6A-C**), indicated (i) deficiency of lactate biosynthetic and metabolic processes in dark-cutting beef, and (ii) enrichment of fatty acid oxidation ( $\beta$ -oxidation) and glycogen catabolic process, explaining the limited capacity of dark-cutting beef to form *post-mortem* lactate, and associated, hydrogen ions. The greater  $\beta$ -oxidation in dark-cutting beef supports the above discussion in terms of ATP production, involvement of mitochondria and use of oxygen, in line with the study of Yu et al. (2018).

Interestingly, the heatmap revealed a deficiency of “response to oxidative stress” pathway in dark-cutting compared to normal meat color. The dynamic changes that occur in early *post-mortem* muscle, especially those related to mitochondria and ATP production through oxidative phosphorylation and  $\beta$ -oxidation amongst others are accompanied by the accumulation of several waste metabolites such as the reactive oxygen species (ROS) (Gagaoua, Terlouw, et al., 2021; Malheiros et al., 2019; Purslow et al., 2021). The excessive amounts of ROS lead to the oxidation of lipids and proteins including myoglobin (Domínguez et al., 2019), that can trigger apoptosis and other cell death pathways. If the endogenous scavenger proteins (*e.g.* peroxidases, (Gagaoua, Hughes, et al., 2020; Malheiros et al., 2019)) are inhibited or less efficient, the consequence can be deleterious and result in inferior meat quality. In this case the high levels of ROS, the lack of response to oxidative stress as well as a weak protein folding response (less HSP proteins) may be other pivotal pathways driving dark-cutting beef. Again, the heatmap of **Fig. 5D** confirms that “muscle system process” and ‘myofibril assembly’ are significantly enriched in dark-cutting beef, supporting the key role of this pathway in the development of this quality defect.

#### 6.6. Deciphering the secretome to better understand dark-cutting beef development

Skeletal muscles have been suggested to be a source of secreted proteins, conceptualized as myokines that can influence metabolism and other biological processes (Henningsen, Rigbolt, Blagoev, Pedersen, & Kratchmarova, 2010). Based on this, we investigated in this integromics meta-analysis the secretome of dark-cutting proteome dataset to gain more insights on the cell–cell communication and extent of involvement of secreted proteins in the development of this

meat quality defect. The secretome is also considered as a strategy to validate certain biomarkers among those most often identified by discovery proteomics in different biological fields (Henningsen et al., 2010; Stastna & Van Eyk, 2012) including meat quality (Boudon et al., 2020; Gagaoua, Hughes, et al., 2020; Picard & Gagaoua, 2020a). Surprisingly, our analyses (**Fig. 7**) revealed a great percentage of putatively secreted proteins (51.5%,  $n = 67$  proteins) through classical or non-classical secretory pathways (**Fig. 7A**). The 67 proteins belong to different cellular components (**Fig. 7B**), of which the Top5 terms were “extracellular exosome”, “cytosol”, “cytoplasm”, “nucleus” and “extracellular region”. The 7 proteins secreted through a signal peptide were mostly down-regulated and grouped in four enriched GO terms (**Fig. 7C**), and the first one being collagen-activated tyrosine kinase receptor signaling pathway (GO: 0038063). The analyses of the up- and down-regulated proteome datasets, confirmed that the main pathways described above having a pivotal role in dark-cutting beef are mainly driven by secreted proteins (**Fig. 7D,E**). Further, from the ten common proteins listed in **Table 2** and **Fig. 1**, nine were found to be secreted, including actin and desmin, showing consistent directions across studies (**Fig. 7**). It is beyond the scope of this meta-analysis to detail all the mechanisms around the dark-cutting secretome and its role in driving this quality defect. However, these first findings highlight the importance of in-depth consideration of the secretome to better understand dark-cutting development, the relationship with light scattering and its role in apoptosis and autophagy.

## 7. Conclusions and future directions

In this integromics study we summarize the results of available proteomics data on dark-cutting beef using a dataset of 130 proteins. This created dark-cutting proteome repertoire may further serve as a one-stop reference for future studies. Despite a relatively strong disparity among the studies in the identified proteins, several proteins and pathways were common across studies. The literature described different mechanisms associated with the development of dark-cutting meat, and the pathways associated with the enriched terms in this integromics analysis were all in keeping with this existing knowledge. The large heterogeneity both in the proteins and the mechanisms described for the development of dark-cutting meat could suggest that different independent pathways may lead to the dark-cutting condition, but more detailed analysis shows that many of them are closely related. Furthermore, the comparison of this integromics study of dark-cutting beef with a previous meta-analysis of (normal) variations in beef meat color (Gagaoua, Hughes, et al., 2020) reveals two things. Firstly, despite a good consensus amongst various studies of the protein biomarkers for normal variations in beef

color, there is a striking disparity amongst dark-cutting beef studies. This indicates that there is no simple “main pathway” to dark-cutting, and that the conditions can be a result of different interactions between several mechanisms and pathways that are induced by a variety of causal factors. Secondly, the disparity between the integromics results for normal beef meat color and dark-cutting indicates that dark-cutting is not simply one extreme end of a “normal” spectrum of variations in beef color, but that different mechanisms seem to be involved.

The present study proposes, in a robust manner, an integrated view of certain of possible mechanisms associated with dark-cutting beef, based on a meta-analysis of a large dataset of identified proteins. Specifically, increased mitochondrial respiration, reduced glycolysis and increased use of alternative energy metabolic processes were found to be central events. These shifts lead to high oxidative pressure and partly failing anti-oxidative defense as well as that of response to cellular stress through heat shock proteins (protein folding), enhancing activation of defense pathways including apoptosis and autophagy, and to modifications in structural proteins. The study shows for the first time that the main mechanisms involved in dark-cutting beef driven at a certain extent by the secretome, which is a very interesting question for further research, specifically in relation to light scattering phenomenon and apoptosis and autophagy cell death pathways.

In terms of future studies, the focus given by this integromics analysis on the strong role of mitochondrial metabolism on the incidence of dark-cutting emphasizes the need for future research on mitochondrial functions and pathways in peri-mortal and *post-mortem* events. From this integromics, it seems that most proteomics studies to date have looked at dark-cutting in association with a high pH. However, it could be quite valuable to look at dark-cutting defined by color standards in comparison to pHu, as the causative mechanisms at play are likely to be different. In this way, one can expect distinguish the effects due to mitochondrial OCR from those due to pH/light scattering described in this study. Mitochondrial OCR and pH anaerobic metabolism are clearly linked to both the characteristic alteration in myoglobin biochemistry and the variations in myofilament lattice spacing seen in dark-cutting. Any new hypothesis and strategy to manipulate or control the incidence of dark-cutting should take variations in mitochondrial function as a central focus. Furthermore, the multi-omics approaches by combining both genomics, proteomics and metabolomics will further validate certain mechanisms proposed in this study and allow to gain more insights on dark-cutting beef.

## Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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## Appendix A. Supplementary data

**Table S1 and Table S2.**

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**CRedit author statement**

Mohammed Gagaoua: **Conceptualization, Methodology, Software, Formal analysis, Resources, Data curation, Visualization, Investigation, Writing- Original draft preparation and Project administration.** Robyn D. Warner, Peter Purslow, Ranjith Ramanathan, Anne Maria Mullen, Maria López-Pedrouso, Daniel Franco, José M. Lorenzo, Igor Tomasevic, Brigitte Picard, Declan Troy and E.M. Claudia Terlouw: **Writing- Reviewing and Editing.** All authors have read and agreed to the published version of the manuscript.

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**Declaration of Competing Interest**

The authors declare that there is no conflict of interest.

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## Figure captions

**Fig. 1.** Functional annotations, Gene Ontology (GO) pathway enrichment, clustering and overlap among the eight eligible dark-cutting beef proteomics studies. **A)** Circos plot showing how proteins from the input protein lists ( $n = 130$ ) overlap across the eight studies (**Table 2**) and degree of disparity among dark-cutting beef proteomics studies. Each outside arc represents one study with a different color. On the inside, the dark orange color represents the proteins that appear in multiple lists and the light orange color represents proteins that are unique to that protein list; and purple lines link the same protein (gene name) that are shared by the input. The length of the outside arcs is related to the number of proteins in each list. **B)** Extended Circos plot indicating the amount of functional overlap among the input protein lists. The new blue lines link the different proteins (gene names), where they fall into the same ontology term (the term has to be statistically significantly enriched and with size no larger than 100). **C)** String network built using the 10 common proteins across the eight studies (ACTA1, ACTN2, CRYAB, HSPB1, DES, MYLPF, MYH1, MDH1, UGP2 and YWHAG) and identified in more than one study (see details in **Table 2**). **D)** Significant enriched TOP 5 GO terms found for the 10 proteins shared among the eight studies (one row per term cluster). The bar graphs highlight the top enriched terms and colored according to  $P$ -values: terms with a  $P$ -value  $< 0.01$ , a minimum count of 3, and an enrichment factor  $> 1.5$ . **E)** Hierarchical Heatmap clustering indicating the first TOP 20 enriched GO terms (further details in **Table 3**) and confirming the disparity among studies. The enrichment of GO and KEGG pathways were analyzed by Metascape® (<https://metascape.org/>) and compared among the eight studies. In the heatmap, colors from grey to brown indicate  $P$ -values from high to low; and grey cells indicate the lack of significant enrichment.  $P$ -value was derived by a hypergeometric test. The two analyses based on the proteins or the biological pathways illustrate the little consistency among studies in terms of the gathered proteins and pathways involved in the production of dark-cutting beef compared to what is known for normal beef color (Gagaoua *et al.* 2020b) or tenderness (Gagaoua *et al.* 2021a).

**Fig. 2.** Enriched ontology network based on the 130 dark-cutting beef protein biomarkers repertoire and TOP 20 Gene Ontology terms. Each enriched cluster term is presented with the corresponding color, where nodes that share the same cluster ID are typically close to each other. The sizes of the nodes reflect the enrichment significance of the terms. The bar graphs on the right highlight the TOP 20 enriched terms and colored according to  $P$ -values: terms with a  $P$ -value  $< 0.01$ , a minimum count of 3, and an enrichment factor  $> 1.5$  (see details in **Table 3**

and **Cytoscape file at GONetwork Fig. 2.cys**: doi:10.17632/j34smf76f3.1). The TOP 5 enriched terms: “Oxidation-reduction process”, “Muscle system process”, “Striated muscle tissue development”, “Purine ribonucleotide metabolic process” and “Small molecule catabolic process” are further highlighted in the network layout.

**Fig. 3.** Molecular complex detection (MCODE) enrichment analysis by Metascape® (<https://metascape.org/>). The MCODE algorithm was applied to clustered enrichment ontology terms to identify neighborhoods where proteins are densely connected. **A)** Key most modular MCODEs identified from the network of **Fig. 2** using the 130 proteins (see details in **Cytoscape file at MCODE\_PPI Fig. 3.cys**: doi:10.17632/j34smf76f3.1). Each node represents a protein, and the edge between nodes represents the interaction between two connected proteins. **B)** Description of the significant seven modules from the PPI network forming the MCODEs clusters.

**Fig. 4.** Functional annotations comparing the up- and down-regulated proteins related to dark-cutting beef development. **A)** Enriched ontology network using Metascape® (<https://metascape.org/>) based on the comparison of the Up (n = 77) and Down (n = 61) regulated dark-cutting beef protein biomarkers. The eight common proteins that were in both directions were included in both lists. Each enriched cluster term is presented with the corresponding color, where nodes that share the same cluster ID are typically close to each other. The sizes of the nodes reflect the enrichment significance of the terms. **B)** Hierarchical Heatmap clustering comparing similarities and differences between highly significant process and pathways among the TOP 20 Gene Ontology terms and colored (ranked) according to *P*-values: terms with a *P*-value < 0.01, a minimum count of 3, and an enrichment factor >1.5. In the heatmap, colors from grey to brown indicate *p*-values from high to low; and grey cells indicate the lack of significant enrichment. *P*-value was derived by a hypergeometric test. The terms in blue color are significantly enriched and specific to down-regulated proteins, those in red are for up-regulated proteins and those in black are significant and common to both protein lists. The darker is the cell, the more significant is the enriched term for that group. For example, the term “GO:0055114: Oxidation-reduction process” is more significant and abundant for the list of up-regulated proteins compared to the list of down-regulated proteins.

**Fig. 5.** Comparison of the protein datasets and enriched pathways of dark-cutting (DC) beef (n = 130 proteins) with normal beef color (n = 59 proteins from the database of Gagaoua *et al.* 2020b, **Table S1** and **Table S2**). **A)** Circos plot showing the degree of overlap between the



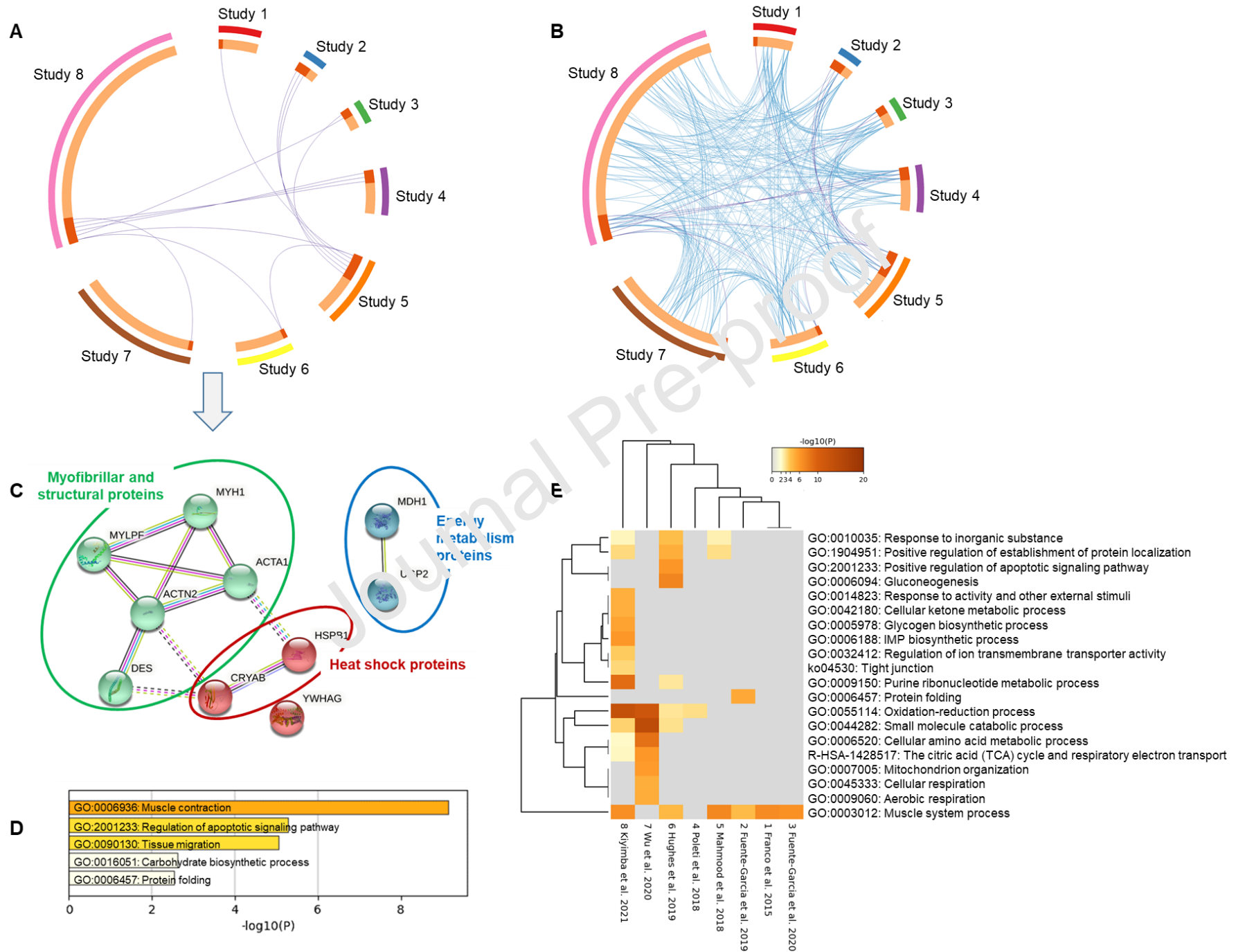
protein lists of dark-cutting and normal beef color. The gene names of the proteins are shown in red (up-regulated), blue (down-regulated) or orange (in both directions). The proteins in bold font and underlined are those identified in more than one study (common proteins shown in **Fig. 1A,C**). **B**) String network built using the 20 common proteins between dark-cutting and normal beef highlighting two main sub-networks: “muscle structure” and “energy metabolism” proteins. **C**) Enriched GO terms of the 20 common proteins showing the TOP 6 significant and enriched term clusters (one row per cluster). The enriched terms are colored according to *P*-values: terms with a *P*-value < 0.01, a minimum count of 3, and an enrichment factor >1.5. **D**) Hierarchical Heatmap clustering comparing similarities and differences between highly significant process and pathways between dark-cutting and normal beef for the TOP 20 enriched GO terms (see details in **Table 4** and **Fig. 6**). Color from grey to brown indicate *P*-values from high to low; and grey cells indicate the lack of significant enrichment. *P*-value was derived by a hypergeometric test.

**Fig. 6.** Enriched ontology networks based on the two protein lists of dark-cutting beef (*n* = 130) and normal beef color (*n* = 59) in support of **Fig. 5D** using the TOP 20 Gene Ontology terms. **A**) Enriched ontology clusters colored by cluster ID. Each enriched cluster term is presented with the corresponding color, where nodes that share the same cluster ID are typically close to each other. **B**) Enriched ontology clusters colored by *p*-value. The sizes of the nodes reflect the enrichment significance of the terms. **C**) Enriched ontology clusters pieced by gene counts between dark-cutting and normal beef color.

**Fig. 7.** Functional annotations of dark-cutting beef secretome and associated enriched pathways. **A**) Distribution of the predicted secreted proteins from the repertoire of 130 proteins using ProteINSIDE tool (<https://www.proteinside.org/>) through classical pathways (yellow, *n* = 7) or non-classical pathways (green, *n* = 60). **B**) Enriched GO Cellular Component terms performed on the full list of the potentially secreted proteins (*n* = 67) either by classical or non-classical pathways. The number of proteins from each component are further given. **C**) Significant and enriched TOP 4 GO terms of the 7 proteins secreted through a signal peptide and colored (ranked) according to *P*-values: terms with a *P*-value < 0.01, a minimum count of 3, and an enrichment factor >1.5 using Metascape® (<https://metascape.org/>). **D**) Significant and enriched GO terms of the Up regulated proteins potentially secreted by *Longissimus thoracis* muscle through pathways that do not involve a signal peptide. The bar graphs highlight the whole TOP 17 enriched GO terms for the 35 potentially secreted proteins and colored (ranked) according to *P*-values. **E**) Significant and enriched GO terms of the Down

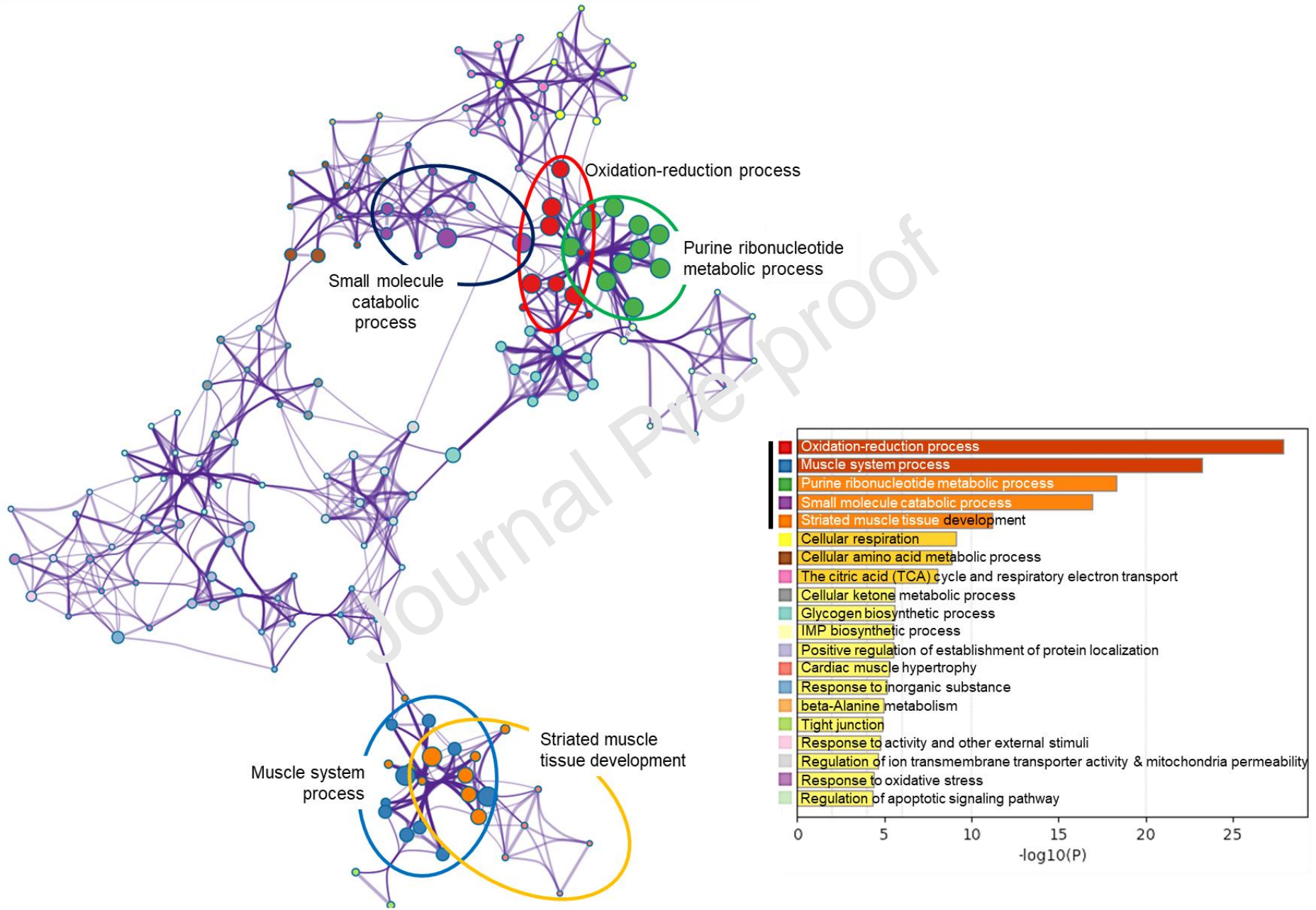
regulated proteins potentially secreted by *Longissimus thoracis* muscle through pathways that do not involve a signal peptide. The bar graphs highlight the whole TOP 7 enriched GO terms for the 30 potentially secreted proteins and colored (ranked) according to *P*-values. The gene names are shown in red (up-regulated), blue (down-regulated) or orange (common proteins in both directions). The proteins in bold font and underlined are those identified in more than one study (common proteins ACTA1, ACTN2, CRYAB, HSPB1, DES, MYLPF, MYH1, MDH1, UGP2 and YWHAG shown in **Fig. 1A,C**).

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**Fig. 1.**

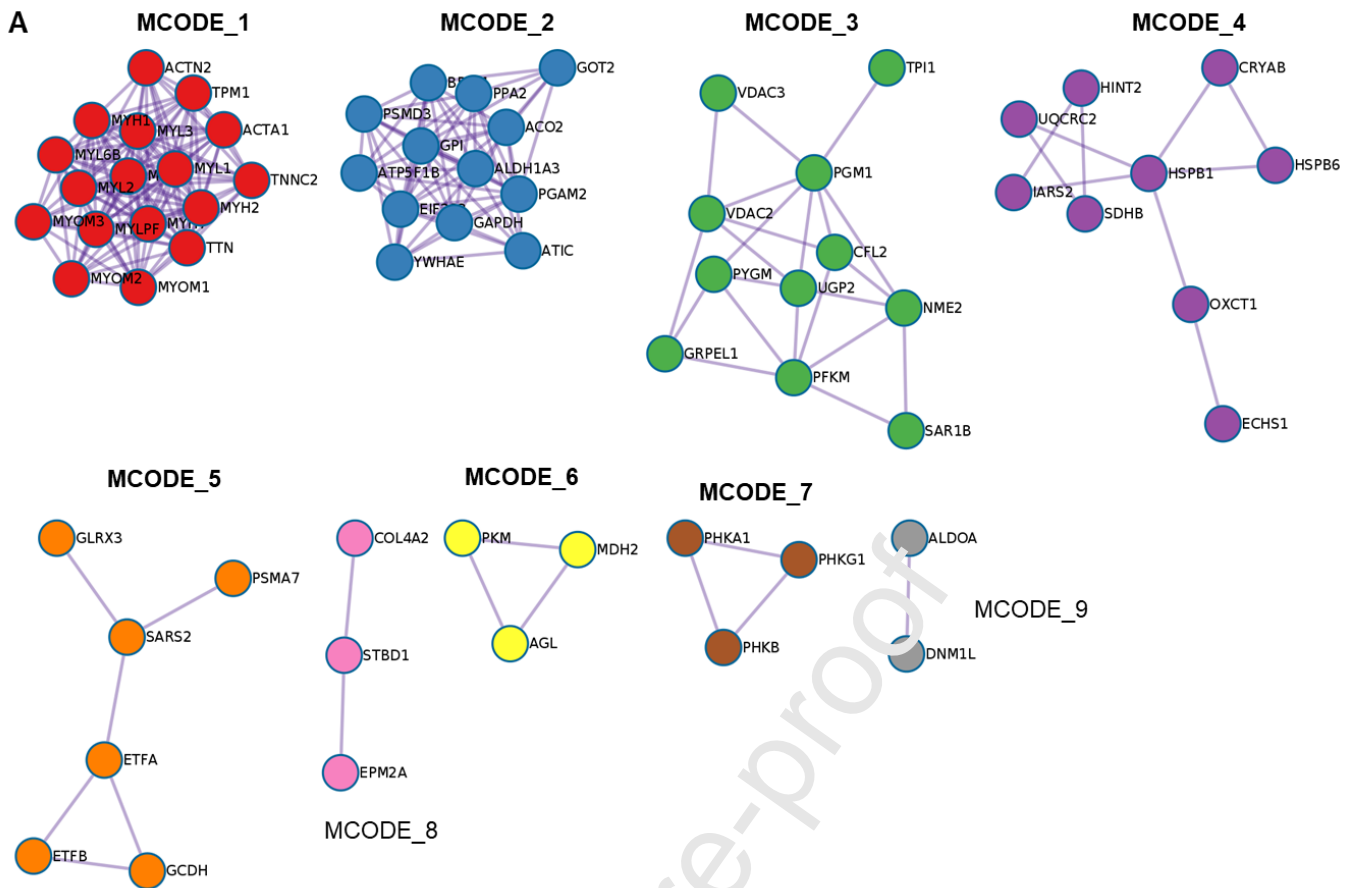
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**Fig. 2.**

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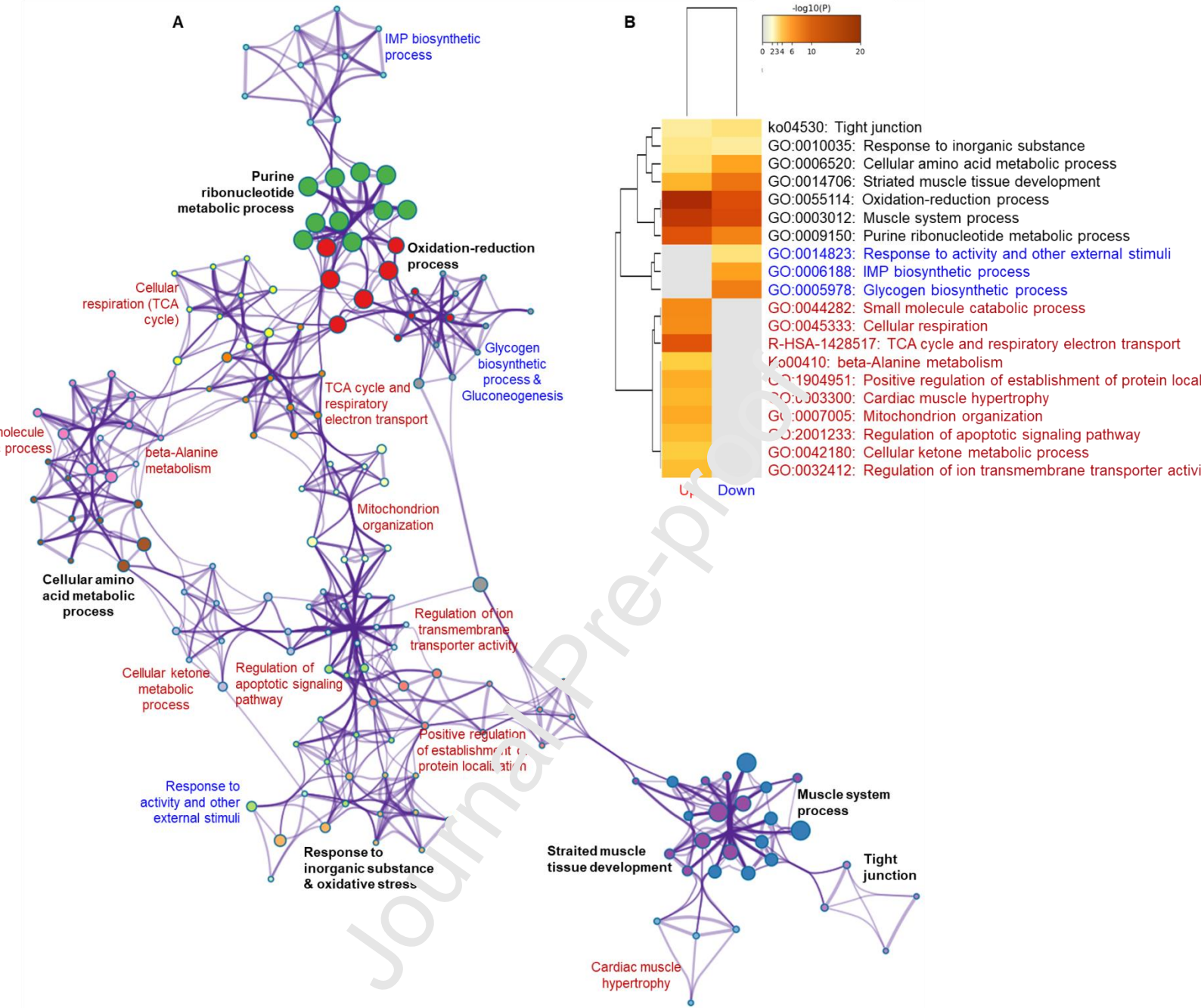




**B**

MCODE	GO	Description	Log10(P)
<b>MCODE_1</b>	GO:0006936	muscle contraction	-32.6
	GO:0033275	actin-myosin filament sliding	-31.3
	GO:0030049	muscle filament sliding	-31.3
<b>MCODE_2</b>	ko00010	Glycolysis / Gluconeogenesis	-10.1
	GO:0009168	purine ribonucleoside monophosphate biosynthetic process	-9.9
	GO:0009127	purine nucleoside monophosphate biosynthetic process	-9.9
<b>MCODE_3</b>	R-HSA-71387	Metabolism of carbohydrates	-7.3
	GO:0006165	nucleoside diphosphate phosphorylation	-6.8
	GO:0046939	nucleotide phosphorylation	-6.8
<b>MCODE_4</b>	GO:0006457	protein folding	-4.3
	GO:0055114	oxidation-reduction process	-3.3
	GO:0006091	generation of precursor metabolites and energy	-3.3
<b>MCODE_5</b>	GO:0033539	fatty acid beta-oxidation using acyl-CoA dehydrogenase	-9.1
	GO:0006635	fatty acid beta-oxidation	-6.4
	GO:0019395	fatty acid oxidation	-6.0
<b>MCODE_6</b>	R-HSA-71387	Metabolism of carbohydrates	-5.9
	GO:0055114	oxidation-reduction process	-5.2
	GO:0006091	generation of precursor metabolites and energy	-5.2
<b>MCODE_7</b>	CORUM:6640	Phosphorylase kinase complex	-12.0
	R-HSA-70221	Glycogen breakdown (glycogenolysis)	-9.7
	GO:0005980	glycogen catabolic process	-9.3

Fig. 3.



**Fig. 4.**

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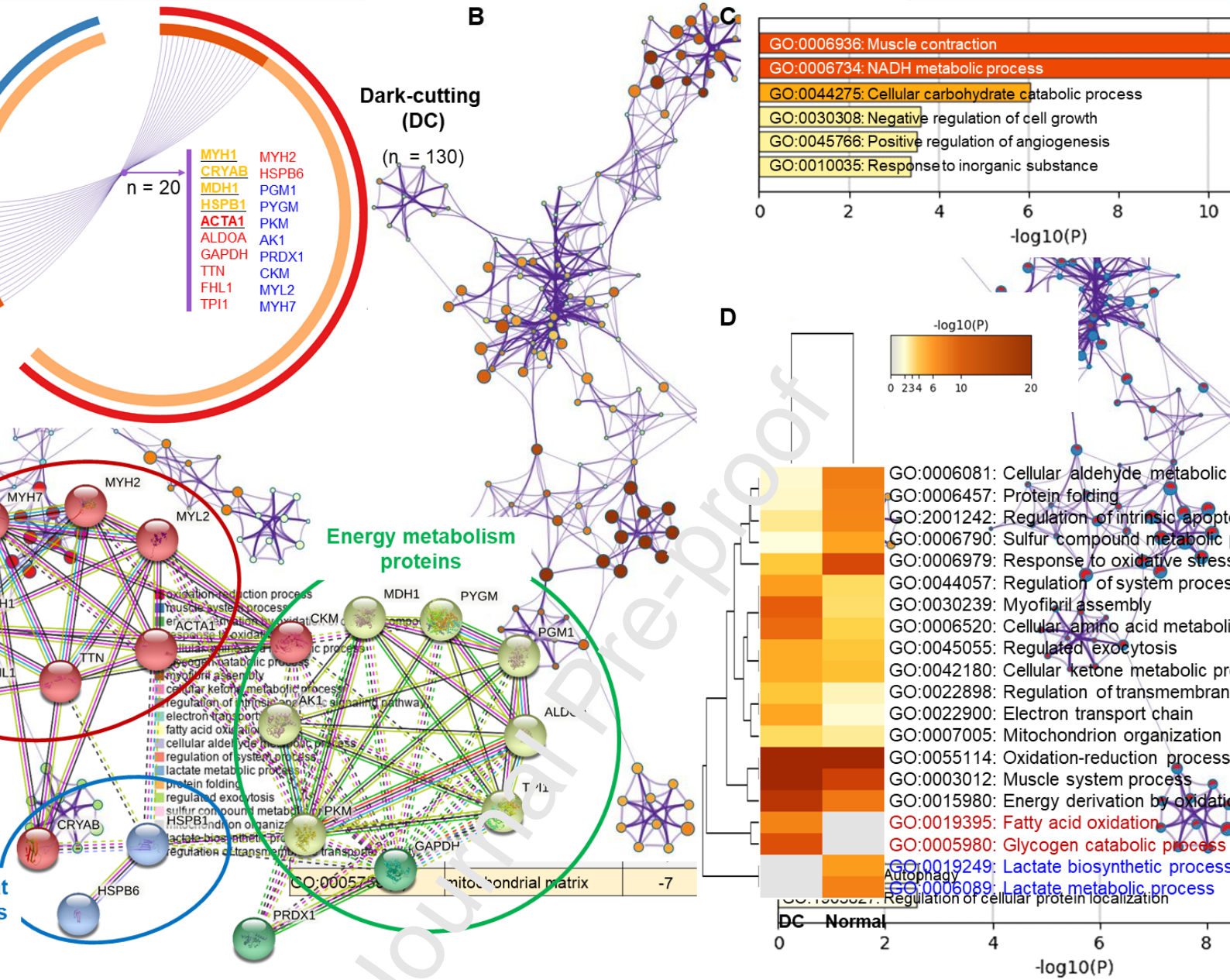


Fig. 5.

Fig. 6.

Fig. 7.



**Table 1.** Description of the eight publicly proteomic studies used to build the dark-cutting (DFD) beef proteome. All the studies used in this integromics meta-analysis were on *Longissimus thoracis* muscle only.

ID	Author (reference)	Animal type   Breed   Gender   Age   Sample types	Number of samples	Proteomics platform	pHu threshold and mean values of groups	Sampling time	Protein extracts	Number of DEPs
1	Franco <i>et al.</i> 2015	Male calves of Rubia Gallega breed, 10 months	8 (4 per group)	2-DE coupled to MS analysis (LC-MS/MS and MALDI-TOF)	DFD: pHu $\geq 6.0$ • Normal: $5.61 \pm 0.01$ • DFD: $6.37 \pm 0.13$	24h	Total protein extract	<b>9</b> Up ( $\uparrow$ ) = 5 Down ( $\downarrow$ ) = 4
2	Fuente-Garcia <i>et al.</i> 2019	Asturiana de los Valles (AV) and crossbreds cattle (AV x Friesian), 12 - 18 months (yearling bulls)	20 (14 normal and 6 DFD)	Liquid isoelectric focusing (OFFGEL) pH range 3–10, and mass spectrometry	DFD: pHu $\geq 6.0$ • Normal: NA • DFD: NA	24h	Sarcoplasmic proteins	<b>5</b> Up ( $\uparrow$ ) = 4 Down ( $\downarrow$ ) = 1
3	Fuente-Garcia <i>et al.</i> 2020	Asturiana de los Valles x Friesian yearling bulls, 14 - 15 months	12 (6 per group)	Liquid isoelectric focusing (OFFGEL) pH range 4–7, and mass spectrometry	DFD: pHu $\geq 6.0$ • Normal: $5.53 \pm 0.14$ • DFD: $6.56 \pm 0.25$	24h	Myofibrillar proteins	<b>5</b> Up ( $\uparrow$ ) = 2 Down ( $\downarrow$ ) = 3
4	Poleti <i>et al.</i> 2018	Male Nellore cattle, 24 $\pm$ 1.2 months of age	12 (6 per group)	Label-free quantitative proteomic using nanoESI-HDMSE technology	DFD: pHu $\geq 6.0$ • Normal: $5.57 \pm 0.01$ • DFD: $6.19 \pm 0.05$	24h	Total protein extract	<b>10</b> Up ( $\uparrow$ ) = 2 Down ( $\downarrow$ ) = 8
5	Mahmood <i>et al.</i> 2018	Canada AA (normal), typical dark cutting Canada B4 (pH > 5.9) and atypical dark cutting Canada B4 (pH < 5.9) samples from heifer and steer	23 AA (n=8), AB4 (n=8) TB4 (n=7)	2-DE coupled to LC-MS/MS analysis	DFD: pHu > 5.9 • Grade AA: $5.68 \pm 0.08$ • Grade AB4: $5.74 \pm 0.08$ • Grade TB4: $6.61 \pm 0.09$	24 h	Sarcoplasmic and myofibrillar proteins	<b>15</b> Up ( $\uparrow$ ) = 4 Down ( $\downarrow$ ) = 11

6	Hughes <i>et al.</i> 2019	Carcasses were allocated to 3 meat color groups (light, medium or dark) as defined by AUSMEAT color scores	19 Light, n = 7 Medium, n = 7 Dark, n = 5	Mono-dimensional SDS-PAGE and LC-MS/MS analysis	DFD: pHu $\geq$ 6.0 • Light: 5.47 • Medium: 5.52 • Dark: 6.15	72 – 96h	Sarcoplasmic proteins	12 Up (↑) = 12 Down (↓) = 0
7	Wu <i>et al.</i> 2020	Beef carcasses were selected from a commercial abattoir	16 (8 per group)	Label-free quantitative proteomics using LC-MS/MS	DFD: pHu $\geq$ 6.1 • Normal: 5.49 • DFD: 6.86	48h	Muscle mitochondrial proteins	28 Up (↑) = 21 Down (↓) = 7
8	Kiyimba <i>et al.</i> 2021	Loins collected at the same time from commercial abattoir consisting of USDA Low Choice and dark-cutting beef	12 (6 per group)	Label-free quantitative proteomics using LC-MS/MS	DFD: pHu $\geq$ 6.0 • Normal: 5.6 • DFD: 6.4	72h	Total protein extract	57 Up (↑) = 28 Down (↓) = 29

**Abbreviations:** NA: Not available; **2-DE:** Two-dimensional electrophoresis; **LC-MS/MS:** Liquid Chromatography with tandem mass spectrometry; **MALDI-TOF:** matrix-assisted laser desorption ionization time-of-flight mass spectrometry; **nanoESI:** nano Electrospray Ionization; **nanoESI-MS/MS:** nanoESI-MS/MS; **nanoESI-HMSE:** Bi-dimensional Nano Ultra-Performance Liquid Chromatography (nanoUPLC) tandem Nano Electrospray High Definition Mass Spectrometry.







**Table 3.** TOP 20 clusters with their representative enriched terms (one per cluster) using the list of 130 dark-cutting beef protein biomarkers across the eight proteomics studies (see **Table 2** for the full list of the proteins and **Fig. 2** for the corresponding network layout).

Studies <sup>a</sup> 1 2 3 4 5 6 7 8	GO	Category	Description	Count <sub>b</sub>	Log <sub>10</sub> (P) <sub>c</sub>	Log <sub>10</sub> (q) <sub>d</sub>
■■■■■■■■	GO:0055114	Biological Processes	Oxidation-reduction process	33	-27.92	-23.57
■■■■■■■■	GO:0003012	Biological Processes	Muscle system process	28	-23.26	-19.39
■■■■■■■■	GO:0009150	Biological Processes	Purine ribonucleotide metabolic process	26	-18.34	-14.83
■■■■■■■■	GO:0044282	Biological Processes	Small molecule catabolic process	23	-16.91	-13.92
■■■■■■■■	GO:0045333	Biological Processes	Cellular respiration	7	-9.09	-6.82
■■■■■■■■	GO:0006520	Biological Processes	Cellular amino acid metabolic process	14	-8.86	-6.60
■■■■■■■■	R-HSA-1428517	Reactome Gene Sets	The citric acid (TCA) cycle and respiratory electron transport	10	-8.07	-5.85
■■■■■■■■	GO:0006094	Biological Processes	Gluconeogenesis	3	-7.40	-4.88
■■■■■■■■	GO:2001235	Biological Processes	Positive regulation of apoptotic signaling pathway	4	-6.18	-3.86
■■■■■■■■	GO:0009060	Biological Processes	Aerobic respiration	5	-6.10	-3.96
■■■■■■■■	GO:0006188	Biological Processes	IMP biosynthetic process	3	-6.05	-3.75
■■■■■■■■	GO:0007005	Biological Processes	Mitochondrion organization	3	-5.94	-3.66
■■■■■■■■	GO:0042180	Biological Processes	Cellular ketone metabolic process	9	-5.60	-3.49
■■■■■■■■	GO:0005978	Biological Processes	Glycogen biosynthetic process	15	-5.56	-3.46
■■■■■■■■	GO:1904951	Biological Processes	Positive regulation of establishment of protein localization	10	-5.53	-3.44

Studies <sup>a</sup> 1 2 3 4 5 6 7 8	GO	Category	Description	Count <sup>b</sup>	Log <sub>10</sub> (P) <sup>c</sup>	Log <sub>10</sub> (q) <sup>d</sup>
■■■■■■■■	GO:0006457	GO Biological Processes	Protein folding	3	-5.25	-3.06
■■■■■■■■	GO:0010035	GO Biological Processes	Response to inorganic substance	12	-5.13	-3.05
■■■■■■■■	GO:0014823	GO Biological Processes	Response to activity	4	-4.96	-2.81
■■■■■■■■	ko04530	KEGG Pathway	Tight junction	7	-4.88	-2.81
■■■■■■■■	GO:0032412	GO Biological Processes	Regulation of ion transmembrane transporter activity	8	-4.62	-2.58

<sup>a</sup> The colour code used to distinguish the protein lists among the 8 dark-cutting beef proteomics studies: ■ study 1 (Franco *et al.* 2015); ■ study 2 (Fuente-García *et al.* 2019); ■ study 3 (Fuente-García *et al.* 2020); ■ study 4 (Poletti *et al.* 2018); ■ study 5 (Mahmood *et al.* 2018); ■ study 6 (Hughes *et al.* 2019); ■ study 7 (Wu *et al.* 2020) and ■ study 8 (Kiyimba *et al.* 2021), where the term is found statistically significant, *i.e.*, multiple colours indicate a pathway/process that is shared across multiple lists.

<sup>b</sup> The number of protein names from the list of protein biomarkers with membership in the given ontology term.

<sup>c</sup> The p-value in log base 10.

<sup>d</sup> The multi-test adjusted p-value in log base 10. An adjusted (Benjamini–Hochberg corrected) p-value <0.05 was considered as the threshold for statistical significance.

**Table 4.** TOP 20 clusters with their representative enriched terms (one per Gene Ontology (GO) cluster) from the comparison of the list of 130 dark-cutting beef protein biomarkers to the list of 59 protein biomarkers of normal beef color from Gagaoua *et al.* 2020b. This table is related to Fig. 5.

Category <sup>a</sup>	GO	Description	Count <sup>b</sup>	Log <sub>10</sub> (P) <sup>c</sup>	Log <sub>10</sub> (q) <sup>d</sup>
■■	GO:0055114	oxidation-reduction process	47	28.14	-41.62
■■	GO:0003012	muscle system process	34	20.36	-27.04
■■	GO:0015980	energy derivation by oxidation of organic compounds	25	14.97	-21.59
■■	GO:0006979	response to oxidative stress	21	12.57	-12.43
■■	GO:0006520	cellular amino acid metabolic process	19	11.38	-12.13
■■	GO:0005980	glycogen catabolic process	7	5.47	-11.10
■■	GO:0030239	myofibril assembly	8	6.25	-9.57
■■	GO:0042180	cellular ketone metabolic process	14	8.38	-9.40
■■	GO:2001242	regulation of intrinsic apoptotic signaling pathway	11	6.59	-8.48
■■	GO:0022900	electron transport chain	11	6.59	-7.99
■■	GO:0019395	fatty acid oxidation	9	5.39	-7.87

Category <sup>a</sup>	GO	Description	Count <sup>b</sup>	Log <sub>10</sub> (P) <sup>c</sup>	Log <sub>10</sub> (q) <sup>d</sup>
■ ■	GO:0006081	cellular aldehyde metabolic process	6	10.17	-7.86
■ ■	GO:0044057	regulation of system process	18	10.78	-7.71
■ ■	GO:0006089	lactate metabolic process	5	2.99	-7.70
■ ■	GO:0006457	protein folding	8	13.56	-7.49
■ ■	GO:0045055	regulated exocytosis	20	11.98	-7.35
■ ■	GO:0006790	sulfur compound metabolic process	14	8.38	-7.15
■ ■	GO:0007005	mitochondrion organization	16	9.58	-6.68
■ ■	GO:0019249	lactate biosynthetic process	3	5.08	-6.31
■ ■	GO:0022898	regulation of transmembrane transporter activity	11	6.59	-6.19

<sup>a</sup> The color code used to distinguish the protein lists among ■ Dark-cutting (DFD) beef (red) and ■ normal beef color (bleu), where the term is found statistically significant, *i.e.*, multiple colors indicate a pathway/process that is shared across multiple lists.

<sup>b</sup> The number of protein names from the list of protein biomarkers with membership in the given ontology term.

<sup>c</sup> The p-value in log base 10.

<sup>d</sup> The multi-test adjusted p-value in log base 10. An adjusted (Benjamini–Hochberg corrected) p-value <0.05 was considered as the threshold for statistical significance.

**Highlights**

- 130 protein biomarkers for DFD beef from 8 proteomics studies were gathered in a unique repertoire
- Few DFD proteins were indicated several times by different proteomics studies
- DFD proteomics studies are not always following similar conditions and protocols
- Oxidation-reduction, TCA cycle and muscle structure are pivotal pathways behind DFD beef
- A high number of the biomarkers are secreted proteins constituting the first DFD beef secretome
- Understanding the pathways underpinning DFD beef is important to reduce wastage

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