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Abstract

Color of retail fresh beef is the most important quality influencing the consumers' purchase decisions at the point of sale. Discolored fresh beef cuts are either discarded or converted to low-value products, before the microbial quality is compromised, resulting in huge economic loss to meat industry. The interinfluential interactions between myoglobin, small biomolecules, proteome, and cellular components in postmortem skeletal muscles govern the color stability of fresh beef. This review examines the novel applications of high-throughput tools in mass spectrometry and proteomics to elucidate the fundamental basis of these interactions and to explain the underpinning mechanisms of fresh beef color. Advanced proteomic research indicates that a multitude of factors endogenous to skeletal muscles critically influence the biochemistry of myoglobin and color stability in fresh beef. Additionally, this review highlights the potential of proteomic components and myoglobin modifications as novel biomarkers for fresh beef color.

Significance:

Color of retail fresh beef is the most important quality influencing the consumers' purchase decisions at the point of sale. Discolored fresh beef cuts are either discarded or converted to low-value products, before the microbial quality is compromised, resulting in huge economic loss to meat industry. The interinfluential interactions between myoglobin, small biomolecules, proteome, and cellular components in postmortem skeletal muscles govern the color stability of fresh beef. High-throughput tools in mass spectrometry and proteomics are exploited to elucidate the fundamental basis of these interactions and to explain the

underpinning mechanisms of fresh beef color. Advanced proteomic research also highlights the potential of proteome components and myoglobin modifications as novel biomarkers for fresh beef color. The novel findings from proteomic studies would help engineering innovative strategies to improve fresh beef color, minimize food waste, and maximize the economic competitiveness of global meat industry.

Keywords: beef color; mitochondrial proteome; myoglobin; post-translational modifications; proteome biomarkers; sarcoplasmic proteome

1. Introduction

Color of retail fresh meat is the most important quality trait influencing the consumers' purchasing decisions at the point of sale [1, 2]. Fresh red meats inevitably undergo discoloration during refrigerated retail display and storage. Consumers often consider discolored meats as unwholesome, leading to rejection and condemnation of otherwise safe and nutritious food products [2]. Discolored red meats are often discarded or converted to low-value products causing huge economic loss to meat industry. Approximately 2.5% of retail fresh beef in the US is discarded due to discoloration leading to annual revenue loss of USD 3.73 billion to the beef industry [3]. In addition to the revenue loss and food waste, this leads to wastage of valuable resources (land, water, feed, labor, and energy) used to raise cattle and thus negatively affects environment.

A multitude of exogenous and endogenous factors in the production, processing, and retailing value chain influence the color and color stability of fresh red meats [4, 5, 6]. The reciprocal and interinfluential interactions of myoglobin with cellular components and small biomolecules in the muscle food matrix govern the color and appearance of fresh meats [7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18]. Additionally, ultrastructure and biophysical properties of muscle contribute to meat color [19, 20].

Numerous qualitative and quantitative methods (biochemical, chemical, physical, and optical) are applied to evaluate color and changes in color of meat and meat products [21]. In this perspective, the biochemical mechanisms governing the interactions of myoglobin with small biomolecules, sarcoplasmic proteome, and mitochondria in fresh beef were extensively studied in the last two decades employing cutting-edge analytical tools in proteomics and mass spectrometry. Integration of data-driven proteomic approach and traditional meat color

evaluation strategies has provided valuable means to explain fundamental bases of color phenomena in fresh meats [2, 18, 22, 23, 24]. Furthermore, the abundance (increased or decreased) of various proteome components can be correlated with meat quality traits through metabolic pathways, thus allowing the innovative use of these proteins as biomarkers for meat color [25, 26, 27]. This review will focus on the applications of mass spectrometry and proteomics to explain the emerging roles of myoglobin modifications, sarcoplasmic proteome, and mitochondria in fresh beef color stability.

2. Myoglobin modifications and beef discoloration

The role of lipid oxidation in off-flavor and rancidity development in meat has been known for more than a century, whereas its role in fresh meat discoloration is relatively new. Oxidation of muscle lipids generate reactive products such as aldehydes and ketones resulting in rancidity in fresh meats. The biological mechanisms preventing oxidative damage of macromolecules cease at the harvest of food animals, whereas the oxidative reactions continue in the complex matrix of postmortem skeletal muscle [17]. The oxidation of lipids and myoglobin in muscle foods are interinfluential [13, 14, 28], and the oxidation of one promotes the other leading to deterioration of both flavor as well as color quality [12].

The advances in proteomics and mass spectrometry have characterized the mechanistic interactions between reactive lipid oxidation products and myoglobin leading to fresh meat discoloration. Research using model systems documented that the covalent modifications of histidine residues in myoglobins of beef [29, 30, 31, 32, 33, 34], horse [32, 35], and pork [30, 31, 36] by reactive aldehydes are responsible for lipid oxidation-induced meat discoloration. These

studies concluded that the adduction of reactive aldehydes at the proximal (i.e., position 93) and distal (i.e., position 64) histidines, which are critical to myoglobin functionality [14], compromises the heme protein's redox stability and accelerates the formation of brown metmyoglobin.

From a practical perspective, dietary antioxidants, such as vitamin E, have been exploited in animal production to prevent lipid oxidation and concomitant discoloration in fresh red meats [37]. As a free radical scavenger, lipid-soluble vitamin E inhibits oxidation of polyunsaturated fatty acids in cell membranes [38], and the increased tissue levels of vitamin E have been reported to minimize oxidation of membrane lipids and myoglobin. Numerous studies have documented increased color and lipid stability of fresh beef from cattle fed a supranutritional level of vitamin E in finishing diet [39, 40, 41, 42, 43, 44]. In contrast, a color stabilizing effect was not evident in vitamin E-supplemented pork, although lipid oxidation was minimized [45, 46, 47]. Pork generally has a greater proportion of unsaturated fatty acids than beef. Pork lipids, being more unsaturated, could undergo lipid oxidation more readily than beef lipids. This, in turn, could result in a decline in color stability in pork, which could be prevented by dietary vitamin E. The findings that vitamin E supplementation improved beef color stability [39, 40, 41, 42, 43, 44], but had no impact on pork color [45, 46, 47] indicated that the susceptibility of beef and pork myoglobins to lipid oxidation is different.

The species-specific effect of vitamin E on beef and pork color has been extensively studied using proteomic tools [30, 31] in model systems containing 4-hydroxynonenal (a reactive aldehyde product of lipid oxidation) and myoglobin. Suman et al. [30] examined the fundamental

basis of previously reported differences in pork and beef color stability in the presence of lipid oxidation products and observed that at meat storage conditions (pH 5.6 and 4°C), beef myoglobin is more susceptible to aldehyde adduction than its pork counterpart. Tandem mass spectrometric analyses indicated that while beef myoglobin formed both mono- and di-adducts with 4-hydroxynonenal after 3 days incubation at meat storage conditions, pork myoglobin formed only mono-adducts with 4-hydroxynonenal. Furthermore, while four histidines were adducted in beef myoglobin by 4-hydroxynonenal, only two histidine residues in pork myoglobin were adducted. Beef myoglobin was susceptible to nucleophilic attack by reactive lipid oxidation products at a greater degree than pork myoglobin. These findings suggested that the primary structure of beef myoglobin increases its susceptibility to reactive lipid oxidation products. Additional investigations [31] characterized the kinetics of 4-hydroxynonenal adduction in beef and pork myoglobins through quantitative proteomic techniques. The myoglobin peptides adducted by 4-hydroxynonenal were isotope-labelled and quantified, and mass spectrometric data indicated that while histidines 88 and 81 in beef myoglobin (in close vicinity to the heme pocket) were readily adducted by 4-hydroxynonenal, histidine 36 in pork myoglobin was the most reactive to the aldehyde. These findings suggested that preferential adduction of lipid oxidation products to histidines 88 and 81 in beef myoglobin can change the three-dimensional structure and conformation of the heme protein, making the heme accessible to the prooxidants and thereby leading to discoloration in fresh meats. Noticeably, these proteomics investigations [30, 31] demonstrated that lipid oxidation is more critical to color stability in fresh beef than in fresh pork and also explained why dietary vitamin E did not improve fresh pork color stability. A side-by-side comparison of 4-hydroxynonenal adduction sites identified in myoglobins from various meat species (Figure 1) indicated that at meat storage conditions (pH 5.6 and 4°C) beef

myoglobin is more susceptible to lipid oxidation products than the myoglobins from pig, horse, chicken, ostrich, and emu [29, 30, 31, 34, 48, 49, 50].

From a different perspective, mass spectrometric studies [51] reported that the adduction of 4-hydroxynonenal to lactate dehydrogenase inactivates this enzyme and can compromise metmyoglobin reduction, which in turn increases fresh beef discoloration. Subsequent investigations [52] demonstrated that pre-incubation of beef myoglobin with 4-hydroxynonenal resulted in covalent modifications of the heme protein and decreased non-enzymatic metmyoglobin reduction at meat storage pH (pH 5.6) and physiological pH (pH 7.4). Additionally, these results demonstrated that the covalent binding of 4-hydroxynonenal with myoglobin decreased the ability of heme to accept electrons and undergo reduction. Together, these studies indicated that the exposure to lipid oxidation products compromises metmyoglobin reduction systems in postmortem muscles and thus accelerate beef discoloration.

Canto et al. [53] identified multiple protein spots with similar molecular weight and different isoelectric pH in two-dimensional electrophoresis gels as beef myoglobin, suggesting the possibility of post-translational modifications in myoglobin during post-mortem aging. Post-translational modifications (such as phosphorylation, methylation, carboxymethylation, acetylation, and alkylation) influence structure and function of proteins in biological systems [54]. While previous investigations on myoglobin modifications focused on covalent modifications induced by lipid oxidation products in in-vitro model systems, Wang et al. [55] examined in-situ post-translational modifications in myoglobin and their impact on color stability of beef longissimus lumborum muscles during post-mortem aging, employing two-dimensional electrophoresis and tandem mass spectrometry. Multiple post-translational

modifications (phosphorylation, methylation, carboxymethylation, acetylation, and 4-hydroxynonenal alkylation) were detected in beef myoglobin. The amino acids susceptible to phosphorylation were serine, threonine, and tyrosine, whereas other post-translational modifications were detected in lysine, arginine, and histidine residues. Additionally, distal histidine (position 64), critical to heme stability, was found to be alkylated. Overall, post-translational modifications in myoglobin increased with aging with a concomitant decrease in surface redness of steaks. The aging-induced post-translational modifications, especially those occurring close to hydrophobic heme pocket, could disrupt tertiary structure of myoglobin and compromise color stability of fresh beef. Additional studies [56] examined the effect of dietary supplementation of vitamin E on myoglobin post-translational modifications in beef longissimus lumborum. Mass spectrometric data identified differential occurrence of post-translational modifications (such as acetylation, methylation, dimethylation, and trimethylation) in myoglobin from vitamin E-supplemented and control cattle. Moreover, a lower number of amino acids were modified in myoglobin from vitamin E supplemented animals than in the myoglobin from control animals suggesting that vitamin E supplementation decreased post-translational modifications in beef myoglobin, thereby improving myoglobin redox stability and beef color stability. The aforementioned studies [55, 56] highlighted the usefulness of myoglobin post-translational modifications as novel biomarkers for fresh beef color stability.

3. Sarcoplasmic proteome influences fresh beef color stability

Sarcoplasmic proteome comprises soluble proteins and enzymes interacting with myoglobin in muscle food matrix and represents about 30% of the total proteins in skeletal muscles. Due to their ability to interact directly with myoglobin, sarcoplasmic proteins play an

important role in color stability of fresh meats [57]. Last two decades witnessed extensive studies employing cutting-edge tools in proteomics to characterize the roles of sarcoplasmic proteome components in variations in fresh beef color stability due to muscle source, aging, and diet. These studies established a sound foundation for engineering novel (pre- and post-harvest) strategies for improving fresh beef color stability.

3.1. Muscle-specific beef color stability

Individual muscles in food animals have specific anatomical locations as well as physiological functions, leading to differences in biochemistry, structure, and metabolism. As a result, beef muscles demonstrate significant variations in post-mortem biochemistry and color stability. Myoglobin oxidation and surface discoloration in beef cuts are muscle-specific. Based on color stability and biochemical attributes, muscles in a beef carcass are categorized as color-stable and color-labile [58, 59]. Extensive studies have documented intermuscular [60, 61, 62, 63, 64, 65, 66, 67, 68, 69] and intramuscular [70, 71] variations in beef color stability.

3.1.2. Intermuscular variations

Joseph et al. [72] examined the differential abundance of sarcoplasmic proteome in beef longissimus lumborum (a color-stable muscle) and psoas major (a color-labile muscle) to explain how proteome components influence muscle-specific beef color stability. Sixteen differentially abundant proteins were identified in longissimus lumborum and psoas major, including glycolytic enzymes, antioxidant proteins and chaperones. The proteins demonstrating positive correlation with redness (aldose reductase, creatine kinase, and β-enolase) and color stability (peroxiredoxin-2, peptide methionine sulfoxide reductase, and heat shock protein-27 kDa) were

overabundant in longissimus lumborum compared to psoas major. The increased levels of glycolytic enzymes can regenerate reducing equivalents (NADH), which in turn can improve myoglobin redox stability and color stability in beef longissimus lumborum. The results also suggested that the overabundance of sarcoplasmic proteins functioning as antioxidants, to inhibit lipid and myoglobin oxidation, can be attributed to the greater color stability in longissimus lumborum than in psoas major. Similar results were reported in longissimus lumborum and psoas major from Holstein cattle [73], in which an overabundance of proteins with antioxidant, protection, and repair functions in longissimus lumborum contributed to its increased color stability, while the overabundance of proteins involved in tricarboxylic acid cycle and mitochondrial electron transport chain in psoas major led to a low color stability.

Clerens et al. [74] compared the proteomic and peptidomic profiles of four beef muscles (semitendinosus, longissimus thoracis et lumborum, psoas major, and infraspinatus) exhibiting differences in color stability and documented that 24 protein spots were different among the muscles. The differentially abundant proteins included myoglobin, hemoglobin, glycolytic enzymes, contractile proteins, and serum albumin. These findings on muscle-specific proteome profile agreed with the previous reports and reiterated the critical role of muscle proteome on beef color.

Wu et al. [75] examined the changes in the muscle-specific changes in the sarcoplasmic proteome during the aging of beef longissimus lumborum and psoas major from Chinese Luxi cattle. Muscle-specific changes were observed in color stability, with longissimus lumborum being more color stable than psoas major, along with proteome during aging. Several enzymes

associated with glycolysis were more in longissimus lumborum than in psoas major during aging and have contributed to the greater color stability of longissimus lumborum.

The relationship between proteome biomarkers and color traits in the longissimus thoracis and rectus abdominis muscles from the Protected Designation of Origin Maine-Anjou beef cattle has been examined [76]. While several biomarkers (metabolism enzymes, heat shock proteins, antioxidant proteins, and structural proteins) and color traits were correlated, a muscle-dependent relationship was evident with more proteins related to the color parameters in rectus abdominis than in longissimus thoracis muscle. In agreement with previous findings, heat shock proteins were strongly associated with color traits in both muscles. The association of metabolic enzymes, heat shock proteins, antioxidant proteins, and myofibrillar proteins with color traits highlighted the importance of biological pathways in beef color stability.

The proteome profiles of longissimus thoracis from forty-three Charolais bulls were examined to explore biological mechanisms governing beef color [78]. Variations in color existed among the carcasses and numerous proteins correlated with color attributes (lightness, redness, yellowness, chroma, and hue). Of specific, the proteins positively correlated to redness included aldehyde dehydrogenase, heat shock protein, superoxide dismutase, and Four and a half LIM domains 1. Among the 16 proteins correlated to color, Four and a half LIM domains 1 and tripartite motif-containing 72 were for the first time associated with beef color.

Nair et al. [78] examined the changes in the sarcoplasmic proteome of beef muscles with differential color stability (longissimus lumborum, psoas major, and semitendinosus) during

postmortem aging for 21 days. Color attributes and sarcoplasmic proteome profile were influenced by muscle source and aging. Proteomic profiling identified 135 protein spots differentially abundant between the muscles and aging time suggesting muscle-specific changes during aging. Of these spots, 49 protein spots were differentially abundant with aging, whereas 86 spots were differentially abundant in three muscles. Interestingly, multiple protein spots were identified as the same protein in suggesting possible post-translational modifications. The network of protein-protein interactions (Figure 2) generated using the STRING database [79] using the differentially abundant proteins in beef psoas major, longissimus lumborum, and semitendinosus [72, 78] at different postmortem aging periods highlighted the critical roles of glycolytic and metabolic enzymes as well as antioxidant proteins in muscle-specific variations in beef color.

3.1.2. Intramuscular variations

Beef semimembranosus is a large round muscle exhibiting intramuscular variations in color (i.e., two-toned color). Based on the visual appearance/color and the location within the carcass, this sizeable beef muscle can be separated to inside and outside regions [70, 71]. The outside region of semimembranosus is darker and color-stable, whereas the inside region is lighter and color-labile [70]. Due to its internal location within the beef carcass, the inside portion of semimembranosus chills at a slower rate than its outside counterpart, leading to variations in temperature and pH within the same muscle [70]. The differential rates of temperature fall and pH decline during postmortem chilling could compromise metmyoglobin reducing mechanisms leading to the intramuscular variations in color stability of beef semimembranosus [71].

Proteomic tools have been utilized to examine the fundamental basis of intramuscular variations in color stability of beef semimembranosus at 48 h postmortem [80] and after aging for 21 days [81]. The investigations by Nair et al. [80] documented an increased abundance of glycolytic enzymes (fructose-bisphosphate aldolase A, phosphoglycerate mutase 2, and beta-enolase) in the sarcoplasmic proteome of inside semimembranosus region (48 h postmortem), which could contribute to a rapid pH drop during early postmortem when the carcass temperature is still high. A high temperature-low pH combination in early postmortem skeletal muscles denatures muscle proteins resulting in a pale, soft, and exudative-like condition in beef [82, 83]. Additional proteomic investigations [81] on wet-aged beef semimembranosus observed that multiple proteins involved in glycolysis and energy metabolism (triosephosphate isomerase, β-enolase, and creatine kinase M-type) were more abundant in outside region than in inside region after 21 days. The impact of aging on sarcoplasmic proteome was greater extent in outside region than in inside region of beef semimembranosus. The network of interactions (Figure 3), generated using the STRING database [79], of 9 differentially abundant proteins in outside and inside regions of beef semimembranosus [80, 81] emphasized the critical roles of glycolytic and metabolic enzymes in intramuscular variations in beef color stability.

3.2. Aging- and storage- induced changes in sarcoplasmic proteome

The influence of cattle genetics and postmortem aging time on the changes in sarcoplasmic proteome and meat quality was studied in the longissimus muscles from Romagnola × Podolian, Podolian, and Friesian bulls [84]. While beef from Podolian cattle was

the darkest and the reddest, Friesian beef was the lightest and had the least redness. The proteomic data indicated that sarcoplasmic proteome underwent changes during aging and the aging-induced changes in proteome were influenced by breed. These findings suggested the potential of sarcoplasmic proteome components as biomarkers for breed-specific beef color stability.

While previous proteomic studies were focused on muscles on or after 24 h postmortem, Gagaoua et al. [85] evaluated the relationships between protein biomarkers and color development in early post-mortem (i.e., < 24 h) longissimus thoracis muscle of Young Blond d'Aquitaine Bulls. Principal component analysis and regression analysis revealed that color attributes at 24 h postmortem were correlated with protein biomarkers, which were involved in apoptosis, antioxidant, and chaperone activities. The positive correlation of redness with malate dehydrogenase, peroxiredoxin, and HSPs is explained by the antioxidant effect of these proteins on color by promoting myoglobin redox stability in post-mortem beef muscles.

The changes in color attributes and sarcoplasmic proteome during postmortem aging of semitendinosus from Chinese Luxi yellow cattle were evaluated in an attempt to identify predictors of discoloration [86]. Surface discoloration and changes in sarcoplasmic proteome components were observed during storage. Among the differentially abundant proteins, triosephosphate isomerase, L-lactate dehydrogenase A chain isoform, fructose-bisphosphate aldolase A isoform, peroxiredoxin-6, and pyruvate kinase isozymes M1/M2 isoform were highly correlated to meat color and were suggested as biomarkers for discoloration during post-mortem.

Poleti et al. [87] characterized muscle proteome of longissimus thoracis (from Nellore cattle) demonstrating variations in pH. The high (pH ≥ 6.0) and normal (pH < 5.8) ultimate pH muscle groups also exhibited variations in color during aging for 14 days, and more than 60 proteins (involved in metabolic processes and muscle contraction) were differentially abundant between the two groups. The protein Histone H2A.J exhibited strong correlations with redness and yellowness on day 7 of aging suggesting that this protein may be exploited as a biomarker for color in aged beef. Kim et al. [88] quantified the peptides derived from proteome of beef psoas major and longissimus lumborum muscles during 21 days aging in an attempt to identify proteins associated with meat color as well as other quality traits. More than 40 proteins, predominantly sarcoplasmic proteins (myoglobin, glycolytic enzymes, and antioxidant proteins), exhibited a muscle-specific degradation pattern during aging. These muscle-dependent proteolytic changes could contribute to the variations in color between the two muscles observed during aging.

3.3. Pre-harvest factors influence sarcoplasmic proteome profile and beef color

Pre-harvest factors such as animal genetics, management, and diet influence color stability of fresh beef, possibly through modulating the biochemical pathways in muscles. Investigations have attempted to characterize the changes in sarcoplasmic proteome to explain the underlying mechanisms for the diet and animal effects on beef color. The influence of genetics on beef color stability is well documented, and the animal-to-animal variations have been reported in color stability of longissimus lumborum [89, 90]. The proteome basis of animal-to-animal variations were studied by profiling the sarcoplasmic proteome of longissimus lumborum steaks (i.e. color-labile and color-stabile steaks) from beef carcasses demonstrating

variations in retail color stability [53]. Color-stable longissimus steaks exhibited over-abundance of multiple glycolytic enzymes, which positively correlated to redness and color stability. The over-abundance of glycolytic enzymes contributes to improved color stability in the color-stable longissimus steaks through regeneration of NADH, which is necessary for enzymatic and non-enzymatic metmyoglobin reduction in post-mortem beef muscles.

The impact of vitamin E supplementation on beef proteome profile was examined [91]. The sarcoplasmic proteome profiles of beef longissimus lumborum muscle from heifers that received 1,000 IU vitamin E supplementation for 89 days prior to harvest and those from control heifers receiving no vitamin E supplementation were compared. Mass spectrometric analyses identified 5 differentially abundant proteins that were over-abundant in control animals. The differentially abundant proteins were antioxidant proteins (thioredoxin-dependent peroxide reductase, peroxiredoxin-6, and serum albumin) and glycolytic enzymes (beta-enolase and triosephosphate isomerase). These proteomic data suggested that the strong antioxidant activity of vitamin E in beef skeletal muscles led to less expression of antioxidant proteins and antioxidant-related proteins.

Antonelo et al. [92] analyzed the proteome basis for the biological variations in color of longissimus thoracis muscle Angus x Nellore crossbred steers that differed in growth rate (high vs. low) and finishing system (feedlot vs, pasture). Finishing system and growth rate influenced the proteome profile, which correlated with color stability. Proteome profiling identified 16 protein spots differentially abundant among steers from different finishing and growth rate. The differentially abundant proteins were associated with in two major clusters (i.e., energy

metabolism and muscle structure) that are involved in glycolysis, carbon metabolism, amino acid biosynthesis, and muscle contraction pathways. Additionally, several differentially abundant proteins correlated with color, and these results also suggested the potential of two proteins (phosphoglucomutase-1 and phosphoglycerate mutase-2) as novel biomarkers for beef color.

4. Mitochondrial proteome contributes to beef color stability

The important role of mitochondria in fresh beef color stability became increasingly evident in the past 2 decades [15, 16]. In postmortem muscles, mitochondria remain functional and are involved in biological mechanisms governing myoglobin redox chemistry [93, 94, 95]. Mitochondrial content and degradation influence muscle-specific beef color stability, with color-stable longissimus lumborum having lower mitochondrial content than the color-labile psoas major at the beginning of aging and display [96, 97]. However, the decrease in mitochondrial content during retail display was more rapid in beef psoas than in the longissimus suggesting muscle-specific variations in mitochondrial degradation [97]. Additional studies [98] documented that oxygen consumption and metmyoglobin reducing activity decreased more rapidly in mitochondria isolated from beef psoas than those from longissimus. From a different perspective, Zhai et al. [99] documented that dietary supplementation of vitamin E, an antioxidant known to improve beef color, to heifers decreased the abundance of mitochondrial proteins involved in oxidative metabolism and ATP generation in post-mortem longissimus muscle. The strong antioxidant protection by vitamin E may have resulted in low expression of metabolic proteins in mitochondrial proteome, leading to low mitochondrial activity and low oxidative stress. Together, these studies indicated the criticality of mitochondrial proteome to biochemistry of fresh beef color [17].

The differential abundance of mitochondrial proteome and its influence on color stability was investigated in color-stable longissimus lumborum and color-labile psoas major beef muscles during retail display [100]. While seven differentially abundant proteins were identified on day 3 in the two muscles, mitochondria could not be isolated from psoas major on day 6 because of extensive degradation. Additionally, 7 mitochondrial proteins were more abundant in longissimus on day 3 than on day 6 of retail display. The differentially abundant mitochondrial proteins included enzymes, binding proteins, and proteins involved in biosynthesis pathways. The protein-protein interaction network (Figure 4), generated using the STRING database [79], of differentially abundant proteins in the mitochondrial proteome of beef psoas major and longissimus lumborum steaks indicated the importance of mitochondrial proteome and metabolic pathways in muscle- and aging-dependent variations in beef color stability.

5. Proteome biomarkers for color in beef longissimus muscle

For a better management of meat quality, proteomic strategies have been further exploited beyond the objective of unveiling the underlying mechanisms and post-mortem changes in relation with color development as discussed above. The tools in proteomics have been applied successfully for identifying potential candidate biomarkers to explain and/or predict the variability in various meat quality traits [101], including beef color stability [22, 26, 72]. The discovery of biomarkers is considered a major ongoing challenge as well as a valuable outcome, for which proteomics is used to evaluate, predict, and monitor meat quality near line or at line.

Studies employing proteomics revealed the correlation between differential abundance of several proteins and beef color traits. Previous studies revealed that numerous metabolic and antioxidant proteins in the sarcoplasmic and/or myofibrillar proteome are critically involved in the biochemical mechanisms governing fresh beef color stability and suggested that these proteins could be exploited as biomarkers for beef color [25, 26, 53, 76, 77]. Based on a literature data-mining review, the proposed protein biomarkers from 13 proteomic studies were gathered using an integromics approach in a repertoire of 79 candidate biomarkers of which 59 were from beef longissimus muscle (Gagaoua et al., 2020). The proteins were found to belong to six interconnected molecular pathways (Figure 5A): (1) catalytic, metabolism and ATP metabolic process; (2) contractile and associated proteins, (3) chaperones and heat shock proteins; (4) oxidative stress and cell redox homeostasis; (5) proteolysis and associated proteins; and (6) binding, cofactor and transport proteins, signaling or apoptosis. An in-depth analysis of this beef color proteomics repertoire that gathered the proteins correlated with instrumental color parameters (L^*, a^*, and b^* values) of fresh beef (Figure 5B) further allowed to shortlist 27 protein candidate biomarkers (indicated by solid black circles in the Figure 5A) for future evaluation. These shortlisted proteins were reported 3–8 times in 13 independent proteomics studies. The proteins have different string of correlations, from which certain can be correlated negatively, positively, or in both direction depending on a number of factors behind each experiment. Nonetheless, it is beyond the scope of this review to discuss the direction of the proteins' correlations with beef color.

From the shortlisted 27 proteins, the top biomarkers was β-enolase (ENO3) identified in 8 studies, followed by Peroxiredoxin 6 (PRDX6) in 7 studies, HSP27 (HSPB1) in 6 studies,

Phosphoglucomutase 1 (PGM1), Superoxide Dismutase (SOD1) and Calpain-1 catalytic subunit or μ-calpain (CAPN1) in 5 studies. Additional biomarkers included eight proteins reported 4 times: HSP40 (DNAJA1), HSP70-8 (HSPA8), HSP70-Grp75 (HSPA9), Malate dehydrogenase 1 (MDH1), Triosephosphate isomerase 1 (TPI1), Lactate dehydrogenase (LDH), Myosin-7 (MYH7) and Myosin-2 (MYH2); and 13 others reported 3 times: HSP20 (HSPB6), αB-crystallin (CRYAB), HSP72 (HSPA2), Pyruvate kinase M 2 (PKM), Creatine kinase M type (CKM), Fructose-bisphosphate aldolase A (ALDOA), DJ-1 (PARK7), Peroxiredoxin 2 (PRDX2), Myosin light chain 1 (MLC1), Myosin-1 (MYH1), α-Actin, (ACTA1), Myosin binding protein-H (MYBPH) and Phosphatidylethanolamine-binding protein 1 (PEBP1). These proteins are indicated by solid black circles in the protein-protein interaction network to highlight the pathways they belong to (Figure 5A).

An in-depth analysis of the biochemical pathways governing beef color demonstrated that similar mechanisms are shared with tenderness, however glycolysis and associated energy metabolic pathways are dominant in color while muscle structural and contractile proteins were central to tenderness [26, 102]. With respect to the enriched Gene Ontology (GO) terms using the 59 protein biomarkers, the bioinformatics analyses using Metascape® platform revealed 20 enriched and significant terms (Figure 5C). The dominating GO terms can be considered as molecular signatures driving beef color development and they are dominated by "generation of precursor metabolites and energy" and "muscle system process", followed by "response to oxidative stress", "protein refolding" and "hexose biosynthetic process" (Figure 5C). The comparison of the lists of protein candidate biomarkers of *L**, *a**, and *b** relevant to enriched GO terms generated a heatmap revealing the common pathways and specific pathway to each

color parameter (Figure 5D). This is the first time such a heatmap comparison is done for beef color parameters. The heatmap revealed that molecular signatures such as “protein refolding”, “muscle system process”, “generation of precursor metabolites and energy”, “response to oxidative stress”, and “regulation of intrinsic apoptotic signaling pathway” are common to *L**, *a**, and *b** values (Figure 5D).

These results highlighted the power of proteomics and integrative studies in revealing the underlying mechanisms of beef color stability. Furthermore, the findings suggested feeding the proposed biomarker proteins into the pipeline of biomarker discovery to proceed with their evaluation under experimental and real-life conditions. These novel biomarkers would further provide future opportunities for pre- and post-harvest interventions that could improve the visual appearance and color stability critical to the consumer acceptance, while addressing problematic issues in beef color relevant to global beef producers, processors, and retailers.

6. Conclusions

Postmortem skeletal muscles are biologically active during aging as well as retail display, and the components of muscle proteome are critically involved in the cellular mechanisms governing meat color. Numerous endogenous and exogenous factors modulate proteome profile and myoglobin stability in beef muscles, ultimately contributing to variations in fresh color. The applications of novel tools in proteomics and mass spectrometry have enabled researchers to explain the role of proteome in beef color stability. Innovative results highlighted the criticality of myoglobin modifications, muscle proteome components, metabolic pathways and proteome biomarkers in beef color biochemistry. The findings from proteomic studies would help

engineering innovative processing strategies to improve fresh beef color, minimize food waste, and maximize the economic competitiveness of global meat industry. Furthermore, coupling the other emerging disciplines in omics (such as genomics, metabolomics, integromics, and bioinformatics) with proteomics will contribute to expanding the body of knowledge in meat color biochemistry. Future research need to focus on strategies to seamlessly integrate powerful analytical tools in various omics disciplines to a single platform to address the perpetual problems in beef color.

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Figure 1: Heat map on species-specificity of 4-hydroxynonenal alkylation at histidine residues in myoglobin at pH 5.6. The red color indicate histidines adducted by 4-hydroxynonenal, green color represents unadducted histidines, and the white color represent missing histidine residues due to species-specific differences in myoglobin primary structure. The heat map was constructed using data from mass spectrometric studies on beef (Alderton et al., 2003, Suman et al., 2006, Suman 2007, Nair et al., 2014, and Viana et al., 2020), pork (Suman et al., 2006), horse (Kiyimba et al., 2019), chicken (Naveena et al., 2010), emu (Nair et al., 2014), and ostrich (Nair et al., 2014) myoglobins.

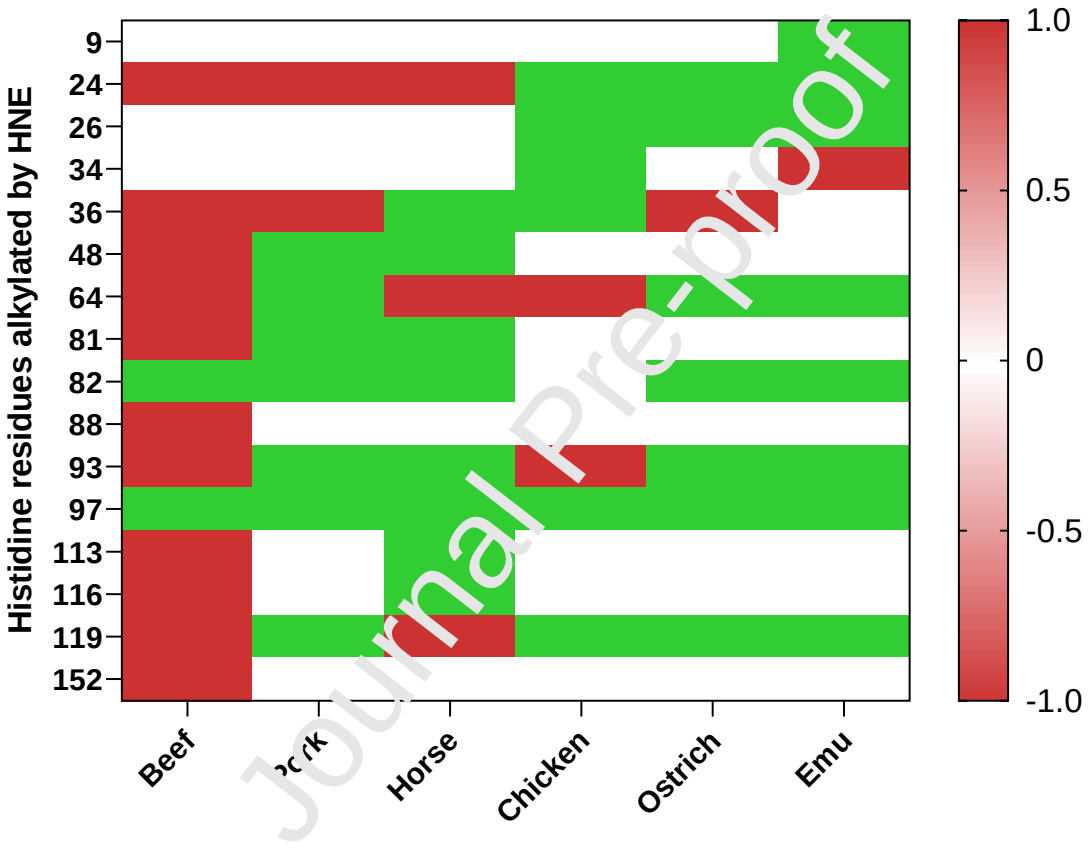


Figure 2: Protein-protein interaction network of differentially abundant proteins in the proteome of beef psoas major, longissimus lumborum, and semitendinosus muscles. The interacting proteins were identified using STRING 11.5 software (Szklarczyk et al., 2015). The nodes represent proteins from a *Bos taurus* database, whereas the lines (teal= evidence from curated databases; pink= experimentally determined evidence; green = gene neighborhood evidence; dark blue= gene co-occurrence evidence; light green = text mining evidence; black = co-expression evidence) indicate predicted functional annotations. ENO1= alpha-enolase; HSPD1= mitochondrial heat shock protein (60 kDa); GAPDH= glyceraldehyde-3-phosphate dehydrogenase; MSRA= mitochondrial peptide methionine sulfoxide reductase; GPT= alanine aminotransferase 1; SOD3= superoxide dismutase [Cu-Zn]; MDH2= malate dehydrogenase (mitochondrial); PARK7= protein deglycase DJ-1; TPI1= triosephosphate isomerase; MB= myoglobin; LDHA= L-lactate dehydrogenase A; MDH1= malate dehydrogenase (cytoplasmic); PRDX2= peroxiredoxin-2; HSPB1= heat shock protein (27 kDa); TF= transferrin; HSPA8= heat shock cognate protein (71 kDa); HSPA1A= heat shock protein beta-1 (70 kDa); ATP5B= ATP synthase (beta subunit, mitochondrial); ENO3= beta-enolase; SOD2= superoxide dismutase [Mn], mitochondrial; CRYAB= alpha-crystallin B; TXNDC8= thioredoxin; STIP1= stress induced phosphoprotein-1; ALDH1B1= aldehyde dehydrogenase (mitochondrial); AKR1B1= aldehyde reductase; ALB= serum albumin; PGM1= phosphoglucomutase-1; DLAT= pyruvate dehydrogenase; PHPT1= phosphohistidine phosphatase-14 kDa; QDPR= dihydropteridine reductase; UBB= polyubiquitin-B; AK1= adenylate kinase isoenzyme 1; ACO2= mitochondrial aconitase 2; CKM= creatine kinase M-type.
The interaction network was constructed using data from proteomic studies on beef psoas major, longissimus lumborum, and semitendinosus (Joseph et al., 2012; Nair et al., 2018a).

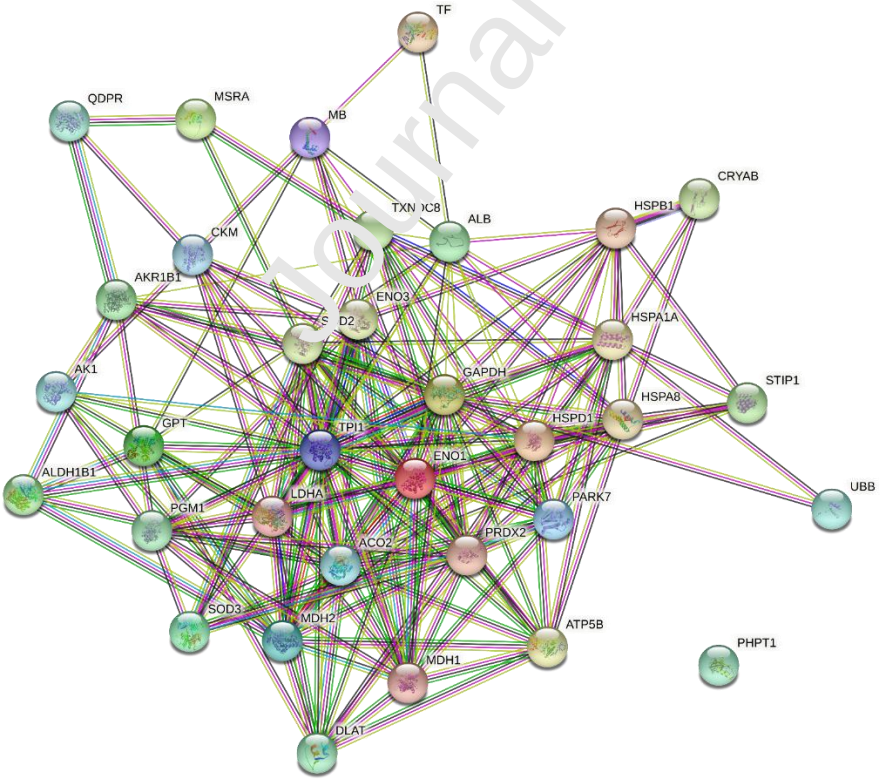


Figure 3: Protein-protein interaction network of differentially abundant proteins in the proteome of beef inside and outside semimembranosus muscles. The interacting proteins were identified using STRING 11.5 software (Szklarczyk et al., 2015). The nodes represent proteins from a *Bos taurus* database, whereas the lines (teal= evidence from curated databases; pink= experimentally determined evidence; green = gene neighborhood evidence; dark blue= gene co-occurrence evidence; light green = text mining evidence; black = co-expression evidence) indicate predicted functional annotations. TPI1= triosephosphate isomerase; PGAM2= phosphoglycerate mutase 2; ENO3= beta-enolase; MB= myoglobin; CKM= creatine kinase M-type; ALDOA= fructose-bisphosphate aldolase A; PEBP1= phosphatidylethanolamine-binding protein 1; UBB= polyubiquitin-B; PARK7= protein deglycase DJ-1.
The interaction network was constructed using data from proteomic studies on beef inside and outside semimembranosus (Nair et al., 2016; Nair et al., 2018b).

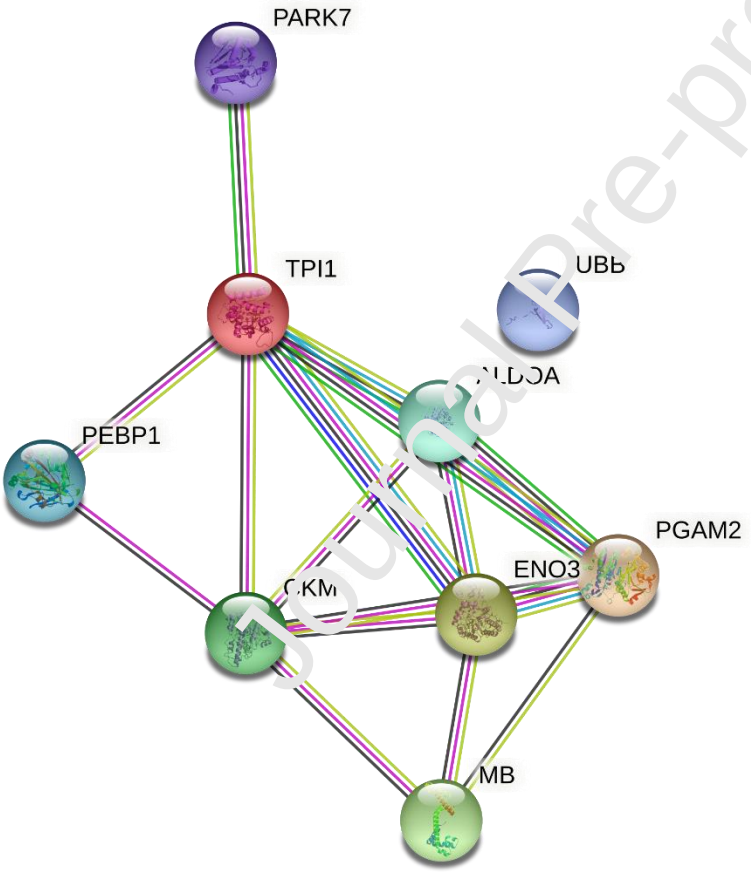


Figure 4: Protein-protein interaction network of differentially abundant proteins in the mitochondrial proteome of beef psoas major and longissimus lumborum muscles. The interacting proteins were identified using STRING 11.5 software (Szklarczyk et al., 2015). The nodes represent proteins from a *Bos taurus* database, whereas the lines (teal= evidence from curated databases; pink= experimentally determined evidence; green = gene neighborhood evidence; dark blue= gene co-occurrence evidence; light green = text mining evidence; black = co-expression evidence) indicate predicted functional annotations. ACADVL = very long-chain specific acyl-CoA dehydrogenase; ATP5B = ATP synthase subunit beta; C1H21orf33 = ES1 protein homolog; CA3 = carbonic anhydrase 3; COQ9 = ubiquinone biosynthesis protein COQ9; GOT2 = aspartate aminotransferase; SUCLA2 = succinyl-CoA ligase subunit beta; ACO2 = mitochondrial aconitase 2; ATP2A1 = sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, chain A; CKM = creatine kinase M-type; NDUFV1 = NADH dehydrogenase (ubiquinone) flavoprotein 1; OGDH = 2-oxoglutarate dehydrogenase; PDHA1 = pyruvate dehydrogenase E1 component subunit alpha, somatic form; TF = transferrin.
The interaction network was constructed using data from proteomic studies on beef psoas major and longissimus lumborum (Ramanathan et al., 2021).

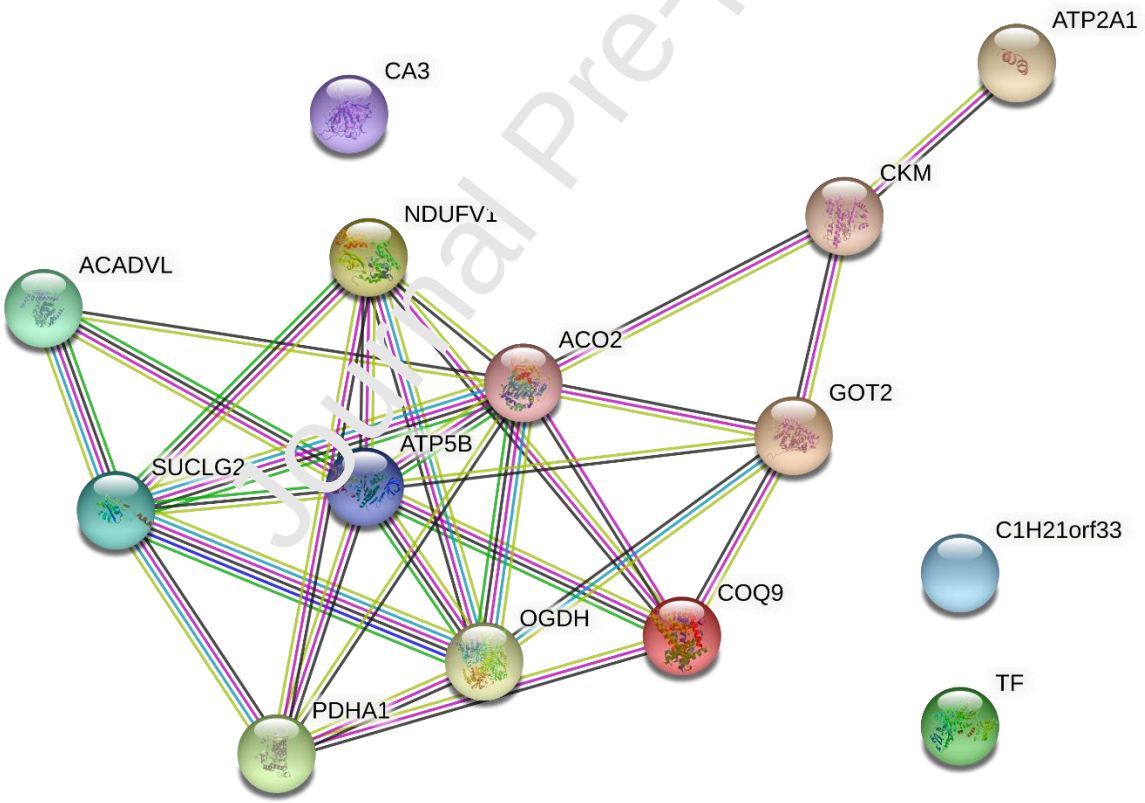
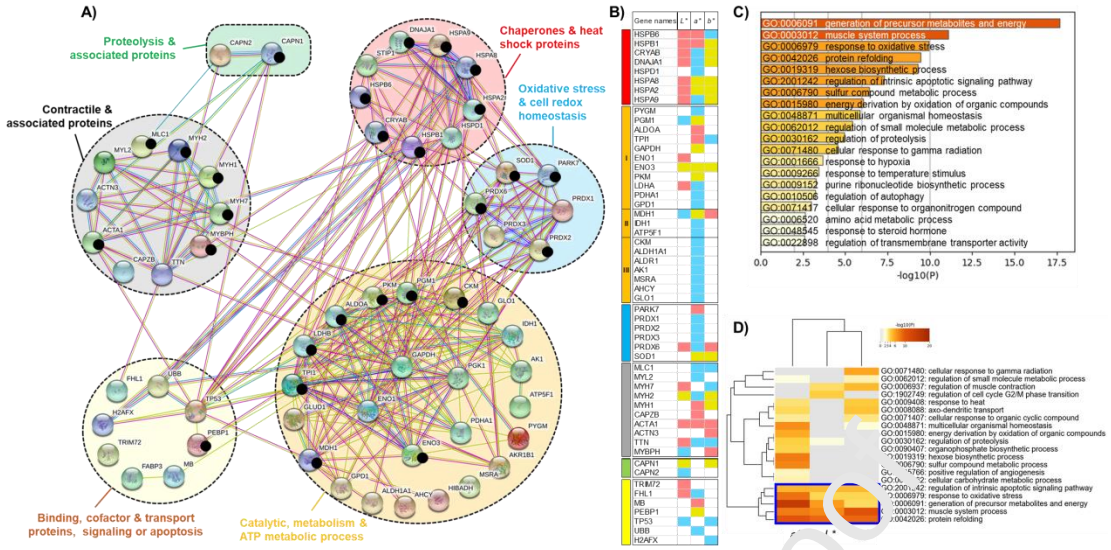


Figure 5: List of the 59 putative biomarkers of beef color and related pathways identified in longissimus muscle using proteomics. The graphs were constructed using data from the previous proteomics integromics meta-analysis study conducted by Gagaoua et al. [26]. (A) String protein-protein interaction network linking the 59 proteins organized into 6 majors biological pathways. The common proteins suggested in the integromics study of Gagaoua et al. [26] as robust putative biomarkers (more than 3 times) and based on significant correlations with beef color traits are marked with solid black circles. (B) List of the 54 biomarkers by biological family found to be correlated with *L**, *a**, and *b** values. The direction of the correlations in the boxes are further given: blue = positive; red = negative; orange = both directions (positive and negative). The complete names of the proteins based on their gene names can be retrieved from UniProt (https://www.uniprot.org/). (C) Gene Ontology (GO) analysis on the 59 putative biomarkers built using Metascape® (https://metascape.org/). The bar graphs 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. (D) Hierarchical heatmap clustering comparing the significantly enriched GO terms found for *L**, *a**, and *b** values based on the putative protein biomarkers. 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 common GO terms common to the *L**, *a**, and *b** values are shown by a blue rectangle.



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.