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Proteomic approach to investigate the impact of dietary supplementation on lamb meat quality

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GENERAL INTRODUCTION

In 2050, according to the report "The World Population Prospects 2019: Highlights," of United Nations, the world population will reach 9.7 billion, leading to a shift in consumption habits and a rising demand for quality food. The term "quality" for meat has always been used to define a product with specific characteristics of color, appearance, and texture. However, in recent years, consumers have exhibited a growing interest in a broader idea of quality, which includes the characteristics of healthy, safe, ethical, and sustainable food. In this context, the main goal of the livestock sector should be to promote the sustainable development of farming systems, with a focus on the efficient use of natural resources. Ruminant farming systems, mostly focused on pastures, may benefit from the use of agro-industrial by-products. From a circular economy perspective, maximizing the use of agro-industrial by-products as feeding supplement represents an important challenge aiming to preserve natural resources, guarantee environmental sustainability and improve the chemical, physical, and sensory characteristics of animal products.

Omics sciences could represent a powerful tool to understand the overall changes in muscle metabolism and therefore predict the effects induced by ante- and post-mortem factors. Indeed, feeding is one of the ante-mortem factors that most influence the quality of the animal product as it can significantly modify: the production and reproductive efficiency, the composition, the healthiness, the organoleptic, and technological characteristics of the meat.

The present thesis aimed to investigate the effects of the inclusion of agro-industrial by-products, specifically hazelnut skin, in the lambs' diet on meat quality and on protein changes. Particularly, it is composed of three trials. In the first trial, the effect of the inclusion of hazelnut skin by-product on meat color characteristics and sarcoplasmic proteome has been assessed while the meat tenderness and the myofibrillar proteome profile were examined in the second trial.

A proteomic approach combined with bioinformatics tools has been applied to a deeper understanding of the molecular mechanisms involved in the post-mortem processes related to feeding strategies through the discovery of several biological pathways involved in the development of meat quality traits.

CHAPTER 1
WHAT IS MEAT QUALITY?

1.1. Definition of Meat Quality

The EU regulation No. 853/2004 specifies the definition of fresh meat in Europe as “all the edible parts coming from the carcass of an animal, as well as its offal and blood”.

The concept of "quality" in the agri-food system is very complex. The *quality of meat* can be defined by the set of properties that affect the consumer's choice of purchase. To these, it is appropriate to consider aspects such as ethics and sustainability, which influence the consumer's *appreciation* of meat. Therefore, based on societal changes in consumer preferences and appreciation, the definition of meat quality can change over time. When we talk about meat quality, we normally refer to its organoleptic characteristics (perceived quality), particularly its flavor and texture.

This chapter provides an overview of the factors that influence meat quality in terms of both nutritional and organoleptic aspects.

1.2. Meat nutritional properties

Proteins

As a "protein-rich food", meat is unquestionably an excellent supply of high biological value proteins. Meat proteins provide a high proportion of essential amino acids, indispensable for the correct functioning of the human body that cannot be directly synthesized by the body but must necessarily be taken through food (Ahmad, Imran, & Hussain, 2018).

Meat proteins can be easily assimilated and digested (Pereira, & Vicente, 2013); the degree of digestibility depends on the age of the animal, and the method of meat preparation (Bax, et al., 2013).

Meat proteins have a plastic function since they contribute to muscle synthesis, but they are also essential in many metabolic processes.

Meat proteins are classified into three groups: *myofibrillar, sarcoplasmic*, and *stromal* (Guo, & Greaser, 2022).

Myofibrillar proteins constitute about 60% of total muscle tissue proteins and are represented by proteins with a structural, regulatory, and cytoskeletal function of relaxation and contraction of the myofibril. Myofibrillar proteins mainly affect the organoleptic quality of meat such as water retention or tenderness characteristics.

Myosin, actin, titin, tropomyosin, troponins (subtypes C, I, and T), desmin, nebulin are the principal myofibrillar proteins, here reported in decreasing order of their relative abundance.

About 45% of myofibrillar proteins are represented by myosin (approximately 520 kDa) which constitutes the thick filaments of myofibrils. Myosins in skeletal muscle are hexamers composed of 6 polypeptide chains: two myosin heavy chains (MHCs) and four myosin light chains (MLCs).

Myosin together with actin constitute the major contractile proteins; by creating the actomyosin complex modulates the physical state of the muscle. Actin (approximately 43 kDa) represents about 22% of myofibrillar proteins; with a double helix filaments structure, it constitutes the thin filaments of myofibrils.

Located in the actin helix thin filaments, tropomyosin and troponin represent the principal regulatory proteins of the

actomyosin complex and muscle contraction. Tropomyosin and troponin account for around 10% of myofibrillar proteins. Tropomyosin is a dimeric protein consisting of 2 distinct subunits known as α and ß tropomyosin; while troponin is composed of three subunits, troponin C (calcium-binding), troponin I (inhibitory), and troponin T (tropomyosin-binding) each respectively equipped with fast and slow isoforms (Wei, & Jin, 2011). Tropomyosin and troponin, also interacting with each other, are involved in calcium-ion-induced contraction regulation.

Furthermore, cytoskeletal proteins maintain the myofilaments in their relative locations inside the myofibril but also preserve the architecture of the myofibrils within the myofiber.

The remaining 40% of muscle proteins are made up of 30-35% by a fraction mainly represented by mitochondrial nucleoproteins, oxidative enzymes, lysosomal proteases, and myoglobin, normally present in the sarcoplasm.

Sarcoplasmic proteins are a heterogeneous group of hundreds different proteins soluble in water and low concentrated salt solutions.

Sarcoplasmic proteins include most of the enzymes responsible for the regulation of carbohydrate and protein metabolism in animal cells (Pearson, 2012). The main sarcoplasmic proteins include glyceraldehyde phosphate dehydrogenase, aldolase, enolase, creatine kinase, lactate dehydrogenase, pyruvate kinase, and myoglobin.

Among all, myoglobin is of considerable importance both from a nutritional and organoleptic point of view. Myoglobin is a globular protein composed of a single peptide protein, globin, and the prosthetic heme group whose oxidation state of iron contributes to the color change in meat (Suman, Ramanathan, & Nair, 2022). Instead, its nutritional value is mainly related to the heme protein group (see Paragraph 1.2.4.).

Both the myofibrillar and sarcoplasmic fractions impact the organoleptic properties of the meat. Huff-Lonergan, Zhang, & Lonergan (2010) reported that changes in the myofibrillar fraction are directly involved in the conversion processes of muscle into meat while the denaturation of sarcoplasmic proteins indirectly affects the quality of the meat by influencing color and water-holding capacity.

Stromal proteins account for a mere 10%–15% of total protein content mainly represented by collagen, elastin, and reticulin. They have limited nutritional interest since they have a low biological value due to a lack

of necessary amino acids and are poorly digested.

Collagen is the main protein constituting the connective tissue and generally, high collagen levels are associated with tough meat (Purslow, 2005). Weston, Rogers, & Althen, (2002) provide an overview of the correlation between collagen and meat tenderness and the link with the animal's age. Indeed, collagen molecules are bonded together through intermolecular cross-links which help provide structure and strength. These bonds, initially reducible, with advancing age, are replaced by mature, thermally stable, and less soluble bonds capable of influencing the toughness of the meat.

Fats

Fat represents a good indicator of the animal's health and nutrition.

The fat percentage of meat and meat products depends on the species, age of the animal, and part of the carcass considered (Pereira, & Vicente, 2022, Wood, et al., 2008).

Meat contains several lipid classes including neutral or non-polar lipids (triglycerides), polar lipids (phospholipids, cholesterol, and sphingomyelin), non-esterified fatty acids (NEFA), and essential fatty acids (EFAs).

Some of the physio-chemical characteristics of meat fat such as the melting point, the susceptibility to oxidation, and the nutritional value are highly dependent on the type of fatty acids (monounsaturated fatty acids, MUFA; saturated fatty acids, SFA; and polyunsaturated fatty acids, PUFA). Monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) constitute the majority of meat fats. Palmitic (C16: 0), stearic (C18: 0), and oleic (C18: 1) acids are the most prevalent meat SFA and MUFA fatty acids.

Meat represents also a great dietary source of PUFA particularly of linoleic acid (C18:2) and alpha-linolenic acid. In addition, meat from ruminants stands out for the presence of docosapentaenoic acid (DPA, C22: 5 n-3), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and conjugated linoleic acid (CLA), trans fatty acids that have been associated with various health advantages such as cardiovascular disease, diabetes, and obesity prevention (Dilzer, & Park, 2012).

Furthermore, cholesterol is a significant nutritional component of meats. The cholesterol level of meats ranges between 30 and 120 mg/100 g (Valsta, Tapanainen, & Männistö, 2005).

Minerals

Meat has excellent mineral content. The most important, as it is characteristic, is iron but potassium, sodium, phosphorus, zinc, and selenium supply is also relevant.

Meat is the best source of heme-iron because more than half of the iron in meat is of the heme type (Buzała, Słomka, & Janicki, 2016). Heme iron, indeed, is the one most usable by the body as it is absorbed as such and most effectively compared non-hemic iron (Uzel, & Conrad, 1998). Meat and meat products can contribute up to 18% of daily iron requirements, an important intake in a healthy and balanced diet.

The amount of minerals in meat varies significantly between species (Lawrie, & Ledward, 2006).

Generally, adult beef has the highest heme-iron content. Conversely, both heme and non-heme iron were found in significant lower amounts in poultry meat. However, pork, despite being red meat, may contain the same amount or even less iron than chicken or turkey (Lombardi-Boccia, Martinez-Dominguez, & Aguzzi, 2002).

Vitamins

Thiamine (vitamin B1), riboflavin (vitamin B2), pantothenic acid, folic acid, niacin (vitamin B3), and vitamin B6 and B12 are the most representative meat vitamins. Fat-soluble proteins (A, D, and K) are abundant in some fatty cuts and offal.

Red meat contains around two-thirds of the daily requirement for vitamin B12 in the same meal (Williams, 2007) while chicken breast is a particularly rich source of niacin (Pereira, & Vicente, 2022). Therefore, also for vitamins, the content is species-specific.

1.2.1. Healthy value of meat molecules

Due to its unique composition, meat is considered a very important food for maintaining a healthy and balanced diet; essential for achieving optimal growth and human development. Indeed, meat is an excellent source of essential nutrient and bioactive molecules that exerts a beneficial role in human health.

The nutrients provided by meat are very important, especially in some stages of life. The high protein content stimulates the secretion of growth hormones (Joslowski, et al., 2013), making this food essential and primary in the nutrition of children and adolescents. Additionally, its contribution to protein synthesis for building and maintaining skeletal muscle mass is significant for

preserving both physical function and metabolic health. In accordance with this, meat is an important element of the elderly's diet to avoid age-related muscular weakness (sarcopenia) (Naseeb, & Volpe, 2017), as well as in athletes' muscle recovery (Van Vliet, et al., 2018; di Corcia et al., 2022).

Minerals, especially iron, as essential components of many enzymes, are precious for children, the elderly, pregnant women, and those suffering from anemia.

Vitamin B12, the vitamin most present in meat, is involved in various vital functions of our body, especially related to the correct functioning of the nervous system and to that of red blood cells, to the synthesis of nucleic acids, and the use of fats (Stabler, 2013).

Although red meat has been demonized for years due to their saturated fat and cholesterol amount, the content in long-chain n-3 fatty acids EPA and DHA known to have anti-inflammatory, antiarrhythmic, and antithrombotic activities, is important in the prevention of cardiovascular diseases. Furthermore, bioactive molecules such as taurine, carnitine, carnosine, ubiquinone, glutathione, creatine, lipoic acid, and CLA, despite being present in small quantities, have numerous physiological properties (Vongsawasdi, & Noomhorm, 2014). These molecules can interact with one or more components of live tissue, producing a wide range of possible health consequences such as immune-modulating, antihypertensive, antimicrobial, and antioxidative activities (Kulczyński, Sidor, & Gramza-Michałowska, 2019).

1.3. Meat organoleptic properties

The physical qualities of meat perceived by the sense organs contribute to defining its organoleptic quality.

Physical characteristics such as color, shape, or marbling depend mainly on sight and affect the consumer in terms of perception of the freshness and state of conservation of the meat, thus resulting rather objective.

Chemical properties, on the other hand, are mostly taste-related and include flavor, juiciness, and tenderness.

1.3.1. Color

Color has a significant impact on meat purchase decisions because customers consider discoloration as a signal of product deterioration and wholesomeness (Mancini, 2013; Suman, Ramanathan, & Nair, 2022).

As stated above, myoglobin is a sarcoplasmic protein containing heme as a prosthetic group, with a centrally positioned ferrous iron atom that determines the color of meat.

The iron within the heme ring can form six bonds. Four of these bonds bind the iron to the heme group, whereas the fifth and sixth respectively connect the prosthetic heme group to the apoprotein and reversibly with other ligands. Meat color is determined by the ligand (oxygen, carbon monoxide…) present at this sixth coordination site and the redox state of iron, which results in the formation of four different chemical forms of myoglobin: deoxymyoglobin, oxymyoglobin, carboxymyoglobin, and metmyoglobin.

The type of binding and the oxygen tension determines the color variation of the meat, as illustrated in **Figure 1** (Mancini, 2013).

Oxymyoglobin, which causes the reddish surface of the meat, is associated with high oxygen tension. Low oxygen tensions increase

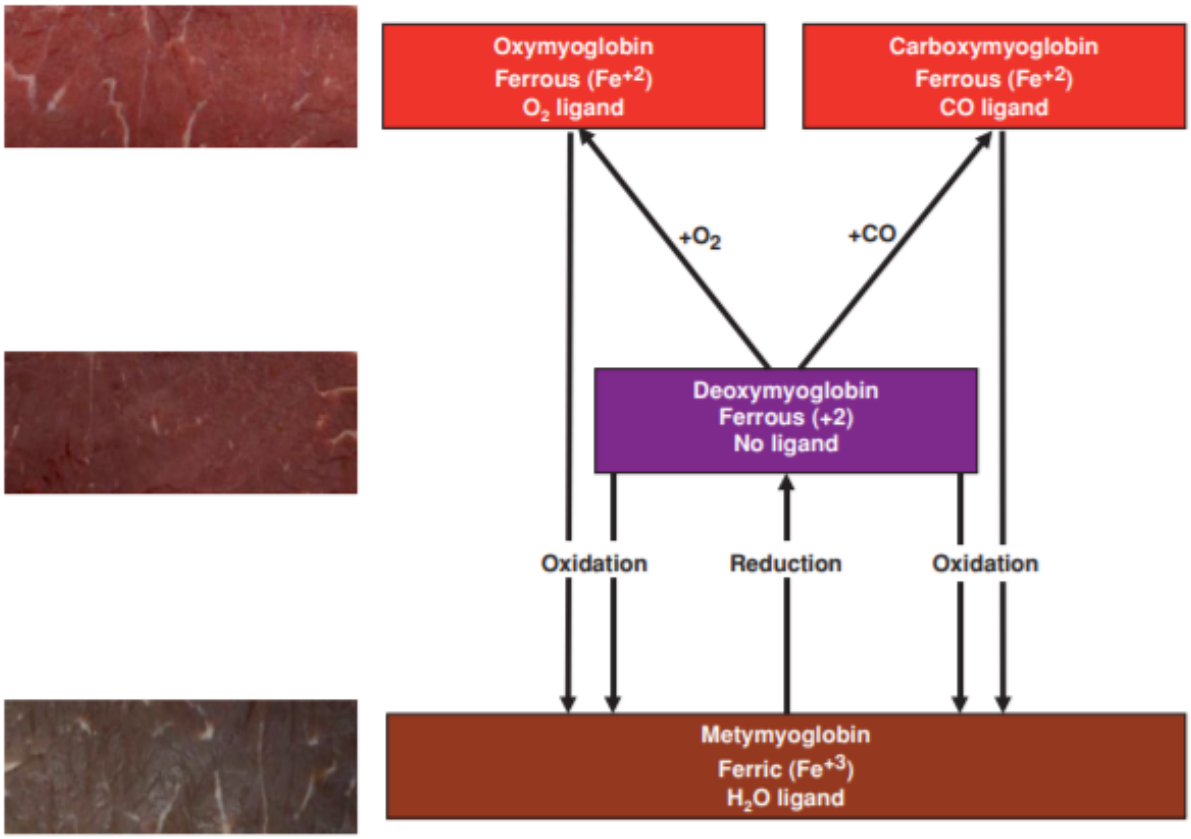


Figure 1. Changes in meat surface color as affected by myoglobin redox state, ligands, and iron valence (Mancini, 2013).

the synthesis of metmyoglobin, resulting in a brownish discoloration. High temperature, low pH, salt, low oxygen atmospheres, and aerobic microorganisms, all contribute to myoglobin oxidation (Tomasevic et al., 2021).

Meat color intensity is also thought to be linked to animal activity and differences in muscle fiber types. According to Listrat et al., (2016), muscles from an animal used for locomotion like cattle, sheep, and horses appear darker in color due to the large proportions of type I fibers rich in myoglobin and prone to metmyoglobin production and color stability loss.

The color of meat is evaluated using both chromatic and achromatic attributes and also includes the measurement of its stability by studying the variation over time of the predominant forms of myoglobin.

Chromatic and achromatic characteristics are related to lightness (L*), redness (a*), and yellowness (b*) measurements in the CIE-L*a*b* color space and may thus be described using Chroma (S*, saturation index) or hue angle (h*) (Warner, 2014). To this are added the reflectance measurements on a meat surface described by the absorption (K) and dispersion (S) coefficients (Macdougall, 1970).

1.3.2. Flavor

In meat, flavour is a fundamental quality attribute. Taste and aroma or smell are the two components of flavor. Since raw meat is generally almost flavorless, its distinctive flavors develop only by the cooking process. The flavors of meats are defined by a non-specie-specific component, common to all meat cuts, and from a species-specific component that determines the differences between beef, lamb, pork, chicken, etc. Free sugars, sugar phosphates, sugars bound to nucleotides, free amino acids, peptides, and nucleotides gives the meat its non-specie-specific taste while fats, particularly phospholipids, and to a lesser extent triglyceride (Meinert et al., 2007), contribute to defining the specie-specific component.

Two main cooking processes contribute to the generation of the characteristic "meaty" flavor. The first is the reaction between reducing sugars and amino acids or other amino compounds such as peptides according to the Maillard reaction. The Maillard reaction produces the desirable flavor in cooked meat through the generation of numerous compounds following the occurrence of cascade reactions and Amadori and Heyns rearrangement (Kanokruangrong, Birch, &

Bekhit, 2019). Strecker degradation represents the final step of the Maillard reaction and is the most important in the production of heterocyclic compounds such as pyrazines, pyrroles, furans, oxazoles, thiazoles, and thiophenes by reacting intermediate carbonyl compounds with each other, with amino compounds, and with amino acid degradation products such as hydrogen sulfide, and ammonia.

The second important contribution to the aroma is given by the oxidative degradation of lipids. Therefore, the fat composition of the meat, particularly the intramuscular fat, can significantly affect its flavor and palatability. Saturated and unsaturated fatty acids are distributed differently in intramuscular fat and adipose tissues. Both types of molecules can be degraded and oxidized, resulting in a large number of volatile flavor chemicals such as aliphatic hydrocarbons, aldehydes, ketones, alcohols, carboxylic acids, and esters.

Finally, the flavor of the meat is influenced by the thermal degradation of other chemicals, such as thiamine (Herrera, & Calkins, 2022).

1.3.3. Juiciness

Meat juiciness is a perceived characteristic during chewing. The juiciness depends on the amount of muscle juice released in the mouth at the beginning of chewing.

In meat, water represents about 70% of the total composition and its retention capacity varies considerably. Based on its mobility, five different types of water in meat are distinguished: constitutional, bound, immobilized, free, and extracellular. Meat juiciness is mostly defined by the free, and extracellular type representing the forms of water that escape the muscle cell structure as purging after the rigors process and for convectional heating.

The ability of meat to retain its intrinsic water after force application and/or processing (i.e., cutting, pressing, grinding, packaging, curing, thermal processing, etc.) has been defined as water-holding capacity (WHC) (Honikel, & Hamm, 1994) and is associate with drip loss during storage and with cooking loss during the cooking of meat.

It is widely accepted that post-mortem proteolysis may be responsible for the improved WHC of aged meats; however, the most important factor affecting the juiciness of meat is represented by the end-point temperature during cooking. A higher end-point temperature is associated with a high

cooking loss and therefore lower juiciness (Aaslyng et al., 2003; Aaslyng, 2009).

Lipids, on the other hand, indirectly influence the perception of juiciness (Puolanne, 2022). Fats can increase the water-holding capacity of meat, lubricate muscle fibres during cooking, stimulate salivary flow during mastication, and therefore the apparent sense of juiciness.

1.3.4. Texture

Tenderness is the most important factor affecting the overall eating quality of meat; consumers are willing to pay more for tenderness (Miller, et al., 2001).

Meat tenderness can be due to several factors such as protein (influenced by the contractile state of the sarcomere and the amount of myofibrillar protein degradation during aging), connective tissue amount, and background effects (Kerth, 2013).

Tenderness associated with protein is a related trait in the post-mortem aging of meat. During post-mortem aging, proteolytic enzymes degrade the proteins and disrupt the structure of the meat (see Chapter 2).

Measuring the sarcomere length, the myofibril fragmentation (MF index), and the protein degradation by using proteomics techniques can help to describe tenderness variation.

Also, the amount of collagen is an important indicator for determining meat tenderness (Purlow, Gagaoua, & Warner, 2021). During cooking, the kind and quantity of collagen cross-links influence the degree of collagen solubilization (Lewis, Purslow, & Rice, 1991). Collagen fibrils shrink when heated during cooking, resulting in fluid loss and less tender meat.

Connective tissue is less affected by proteolysis than myofibrils. Although it was thought that intramuscular connective tissue did not undergo major changes it has recently been demonstrated the role of the matrix metallopeptidase (MMP) system in collagen degradation (Christensen, & Purslow, 2016).

To date, all these factors are evaluated after slaughter with sensory analyses, mechanical (Warner-Bratzler shear force WBSF), and chemical (sarcomere length, total collagen content, MFI determination) measurements.

However, in the last decade, the application of proteomic technique allowed identifying possible biomarkers of meat tenderness, (Gagaoua et al., 2021a; Picard, & Gagaoua, 2020) which can be used for rapid evaluation.

CHAPTER 2
FACTORS AFFECTING THE QUALITY OF MEAT

2.1. Introduction

Several intrinsic and extrinsic factors can positively or negatively influence the quality of meat.

It is noteworthy that poor management conditions and animal stress are the main factors responsible for low even poor-quality meat production. However, also slaughtering, and post-mortem conditions can produce detrimental effects on meat quality.

This chapter provides an overview of all factors affecting meat quality, with an emphasis on extrinsic ones that can be easily modified to enhance animal products and production.

2.2. Intrinsic factors

The main intrinsic factors include the animal genotype, as well as the species (e.g., cattle, sheep, goats, pigs, poultry) and the breed to

which it belongs (e.g., Friesian, brown cattle; Lecce sheep, gentile di Puglia; Maltese goats, Mohair) but also the sex, and the age of the animal.

Animal genetics plays an important role in the quality of meat considering that the biochemical traits that define it are an expression of the characteristic genetic heritage of each species. Meat quality traits are generally recognized as moderate to highly heritable (Kerry, Kerry, & Ledward, 2002) however, there are intra-species genetic variations for important meat quality attributes associated with the breed type. From the quantitative point of view, the productions provided by the different breeds of the same species are distinct and this is defined as the productive specialization of each animal breed.

Within the breed, sex and age play a significant role in determining several meat quality traits. It is well known that males have a higher growth rate and better food conversion indices than females. Castration, however, can affect meat quality; indeed, meat from whole males might have a distinct flavor, and tenderness than meat from castrated females or males. Furthermore, in animals, particularly in females, the proportion of fat and connective tissue also varies according to age and therefore according to the changes that occur at the endocrine level. A recent review of sheep meat (Prache, Schreurs, & Guillier, 2021) reported thicker and more compact carcasses as the animal grows and gets older, emphasizing the importance of the age of the animals at slaughter to produce high-quality meat.

2.3. Extrinsic factors

The extrinsic factors include pre- and post-slaughter production practices such as handling of animals, farming systems, animal nutrition, transformation, and storage technologies. The welfare of animals and consequently the quality of the derivates products largely depends on the management practices applied.

The farming system and housing of animals before slaughter can affect meat quality. Farm animals such as cattle, pigs, and poultry, but also sheep and goats, can be raised both "intensively" and "extensively".

The main differences in farming systems are correlated with the level of stress inflicted on the animal, which can have significant effects on reproductive, and immune functions (Alejandro et al., 2014) on the growth rate of animals, and on the increase in infectious and

parasitic diseases that worsen meat quality (Kerry, Kerry, & Ledward, 2002).

Variations in environmental conditions between farming systems, in particular, the temperature and the space availability, have a significant impact on the physical activity and nutritional needs of the animals.

Feeding is one of the factors that most influence the well-being of the animal as well as the first means to obtain quality production as it can significantly affect the productive and reproductive efficiency, nutrient use, immune system, and emissions and thus ensure animal welfare (Andersen, et al., 2005). Furthermore, feeding plays a role also in controlling physicochemical and metabolic processes of muscle growth in farm animals, which affect the nutritional, organoleptic, and shelf-life quality of meat (Geay, et al., 2001).

The influence of grazing on improving the quality of livestock production is well known. Numerous studies point out that pasture-finished meat exhibits a slightly higher pH, implying that it has a higher water retention capacity (Razminowicz, Kreuzer, & Scheeder, 2006; Sierra, et al., 2010). Considerable variations were also observed in the chromatic indices and the oxidative stability of the meat of animals fed on pasture thanks to the greater number of antioxidants, especially vitamin E (Gatellier, Mercier, & Renerre, 2004). Another major consequence is that grazing increases the meat's fatty acid profile (Fruet, et al., 2018; Horcada, et al., 2020), resulting in a greater unsaturated/saturated ratio, a reduction in the n-6/n-3 ratio, and an increase in conjugated linoleic acid (CLA) content. Therefore, the diet of animals has a significant impact on the overall quality of meat (Horcada et al., 2016).

In the last few years, the incorporation of agro-industrial by-products has been suggested as a good strategy to improve meat product quality thereby also increasing the environmental sustainability of meat production. Interestingly, some by-products contain bioactive compounds such as vitamins, phytochemicals, minerals, unsaturated fatty acids, and phytochemicals able to increase the nutritional and organoleptic value of meat (Salami et al., 2019). Among all, phytochemicals, due to their antioxidant properties, have been investigated for their ability to improve the oxidative stability of meat, with significant implications for color, and flavor (Valenzuela-Grijalva, et al., 2017) and the shelf-life of meat products (Vasta, & Luciano, 2011; Salami, et al., 2016). In addition, Salami et al., (2019) reported an enrichment in bioactive compounds in meat from animals fed by-products rich in

phytochemicals, highlighting how feeding strategies can contribute to the development of functional and healthy meat products.

Finally, transport and stunning represent the last stages of the slaughter process where animals' welfare can be severely compromised (Xing et al., 2018). Preslaughter practices (Lensink, et al., 2001) are also essential to obtain high-quality meat products. Stress conditions in terms of preslaughter handling, transport, and stunning operations are responsible for changes in plasma biochemical indicators and energy metabolism (higher heart rate, higher plasma cortisol concentration, and reduced level of glucose) (Caroprese, et al., 2020) which negatively impact animal welfare, yield, and meat quality (Adzitey, 2011; Ferguson, & Warner, 2008).

After transport and handling to the slaughterhouse, stunning is the final stage of the slaughter process where animals' welfare and consequently meat quality can be seriously compromised. Different stunning procedure (Xing et al., 2018) can be adopted according to the species to help relieve the pre-slaughtering stress and slows the post-mortem pH decline, thus contributing on improving the quality of the carcass and meat.

2.3.1 Post-mortem processes

Post-mortem events in the muscle that occur before and after the onset of rigor mortis are the main factors in determining the physical–chemical and sensory quality traits of meat (color, and water-holding of fresh meat, tenderness, and flavor); (Smulders, Hofbauer, & Geesink, 2014).

During the post-mortem period, significant metabolic, biochemical, and physical changes occur in muscles (Matarneh, et al., 2017).

Immediately after exsanguination and oxygen depletion in the muscle, the glycogen and high-energy phosphate compounds present in the muscle at the time of slaughter are metabolized anaerobically to produce ATP and preserve cellular homeostasis. Subsequently, due to the inefficiency of anaerobic metabolism, the synthesis of ATP does not counterbalance its rate of hydrolysis, resulting in ATP full depletion and the onset of rigor mortis. In the absence of ATP, myosin binds to actin permanently leading to the completion of rigor mortis and loss of muscle excitability and extensibility. Rigor mortis occurs between 1 hour and 12 hours after death, depending on the species, type of muscle fiber, and ante- and post-mortem conditions. Only during the post-mortem

phase, muscle tension is reduced following the proteolytic degradation of cytoskeletal proteins that act on muscle structural integrity (resolution of rigor mortis). Post-mortem proteolysis is linked to several endogenous enzyme groups (calpains, cathepsins, and proteasomes) among these, calpains by interacting with caspases, represent the main enzyme system involved in the meat tenderization rate (Kemp, & Parr, 2012). Post-mortem proteolysis is essential for the qualitative improvement of meat, especially in terms of tenderness.

Notable differences in post-mortem tenderization are observed in relation to the species (Dransfield, Jones, & MacFie, 1981; Etherington, Taylor, & Dransfield 1987); pork is characterized by a much faster tenderizing than beef (Koohmaraie et al., 1991).

However, several factors can influence the proteolysis of proteins in the post-mortem period. Temperature and pH are the two factors that have the greatest impact on the rate of enzyme processes (Braden, 2013). Particularly, variations in ionic strength (especially calcium), pH, and temperature can alter the structure of proteolytic enzymes by activating them, which allows for the hydrolyzing of the protein substrate (Melody, et al., 2003).

The rate of post-mortem acidification (pH) affects meat color, texture, water-holding capacity, and shelf life. The ultimate pH value of meat in most species ranges between 5.5, and 5.7, and meat within this range has the best quality features. As shown in **Figure 2**, meat with a pH of 6.0 or higher has a darker color, a shorter shelf life, and is associated with DFD condition, whereas meat with a pH of 5.4 has a light color and decreased water-holding capacity.

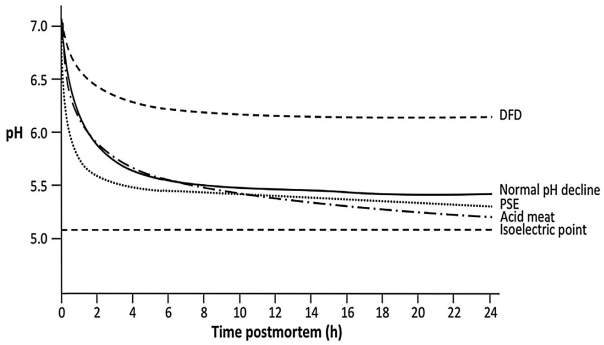
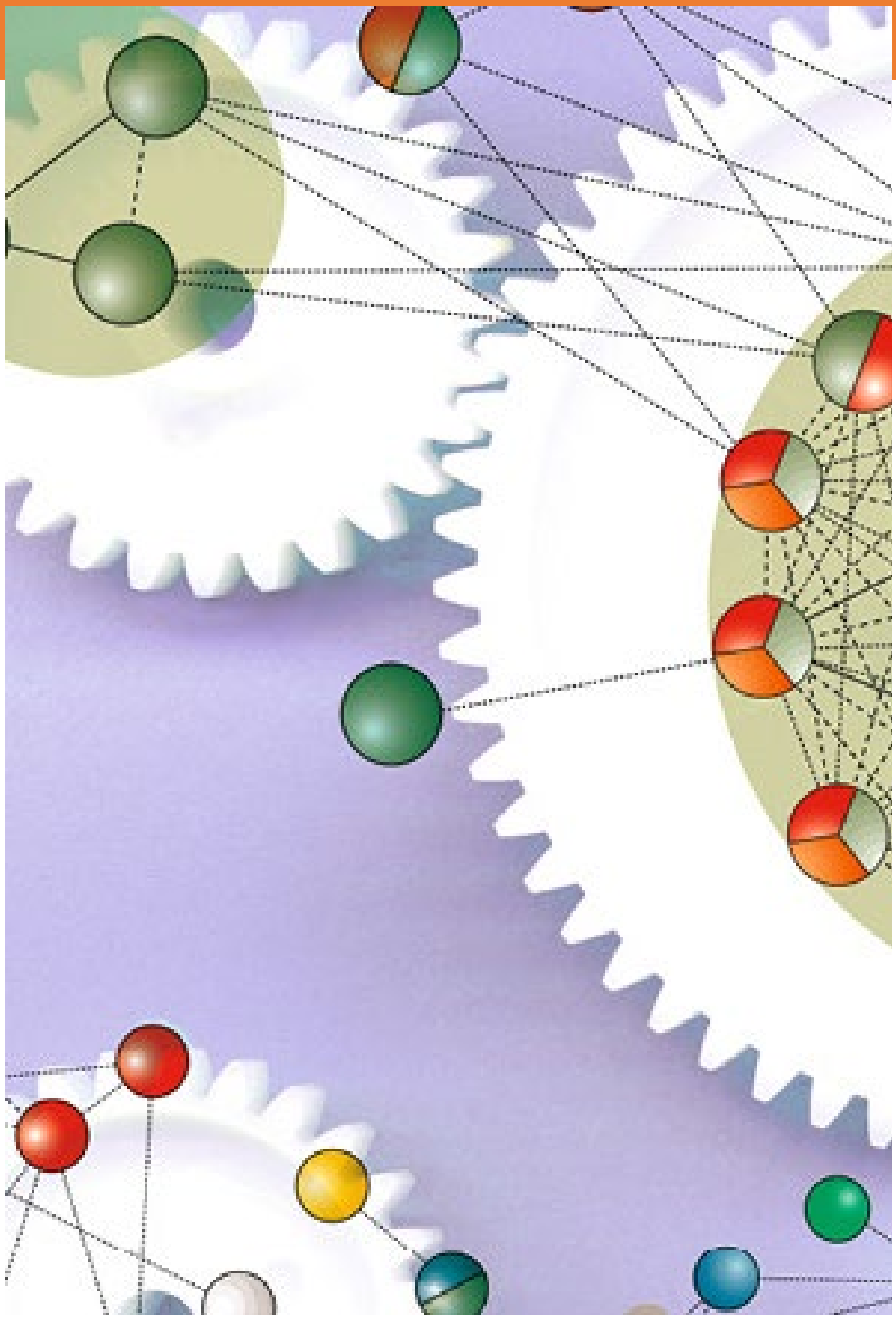


Figure 2. The impact of post-mortem pH drops rate and extent on meat quality attributes (Matarneh, et al., 2017).



CHAPTER 3
APPLICATION OF PROTEOMIC TOOLS IN MEAT QUALITY EVALUATION

3.1. Definition of Proteomics

Proteomics is the study of the proteome, particularly their expression patterns, structure and functions, to obtain information on the expression of cellular proteins, thus revealing the function of related genes (Bendixen, 2005).

Over 30 years ago, proteomic tools have been applied in meat science to overcome the limitations of conventional approaches and to deeply examine meat quality (Purslow, Gagaoua, & Warner, 2021), providing a large quantity of data for the systematic characterization of muscle proteome and meat post-mortem. Furthermore, the proteomic approach has allowed the identification of several protein biomarkers capable of predicting the quality of meat (Huang, et al., 2020; Gagaoua, & Picard, 2022; Gagaoua, et al., 2020).

Figure 3 by Gagaoua et al. (2022) summarize, the main objectives of meat proteomics:

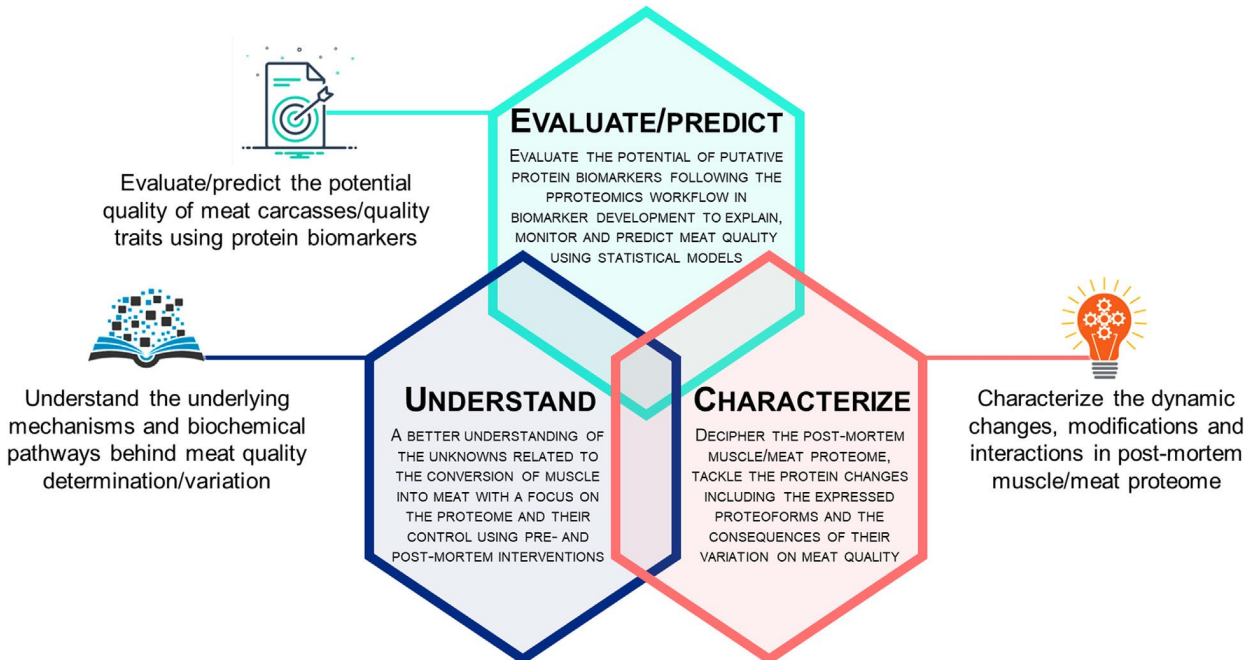


Figure 3. The major objectives of meat proteomics (Gagaoua, et al., 2022).

characterize the dynamic changes, modification, and interactions in post-mortem muscle proteome; understand the underling mechanisms and biochemical pathways behind meat quality variation; and evaluate the potential quality of meat traits using protein biomarkers. In general, the application of proteomics tools for meat quality evaluation and then genome characterization requires five steps (**Fig. 4**): protein extraction, separation, identification and quantification, validation, and bioinformatics analysis.

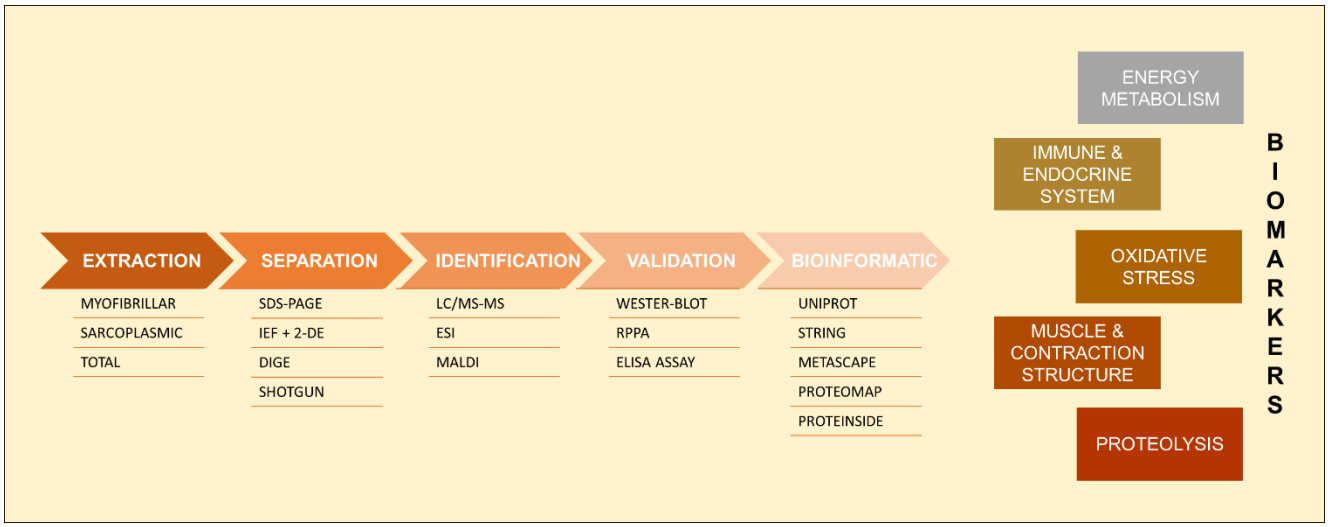


Figure 4. The roadmap of the main steps of proteomic analysis to discover biomarkers of meat quality traits.

3.2. Proteomic tools

3.2.1. Proteins' extraction

The most important steps in proteomic analysis for achieving reliable results is the sample preparation and extraction. Since muscle proteins are grouped into three categories (sarcoplasmic, stromal, and myofibrillar proteins) based on location in the skeletal muscle and solubility, the choice of the extraction method is essential for obtaining samples with high protein concentration.

To be separated and solubilized, myofibrillar proteins need denaturing conditions, such as a high ionic strength solution (Chen, et al., 2016), whereas sarcoplasmic proteins are soluble in water or solutions of low ionic strength. However, stromal proteins like collagen and elastin are reported to remain insoluble in high-salt solutions (Listrat, et al., 2016).

The most used method for extracting myofibrillar protein involves denaturing solutions containing urea, thiourea, reducing agents (DTT, beta-mercaptoethanol), detergents (SDS, sodium dodecyl sulfate), and salts (della Malva, et al., 2018). The efficiency of SDS as a protein extraction medium is related to its ability to create electrostatic interactions with proteins between cationic groups of the proteins and detergent anions, which are further stabilized by hydrophobic interactions of the alkyl chains of the detergent with non-polar moieties of the proteins (Takagi, Tsujii, & Shirahama, 1975).

3.2.2. Proteins' separation

The methods of protein separation can be broadly distinguished into two categories: gel-based, and gel-free approaches.

The *gel-based approaches* refer to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), simply known as one-dimensional electrophoresis (1-DE) and two-dimensional electrophoresis (2-DE).

The 1-DE is a technique that allows proteins to be separated only based on their molecular weight by using sodium dodecyl sulphate, a protein denaturing agent (Aslam, et al., 2017). However, 1-DE is a protein separation technique with very low gel resolution, since multiple proteins of similar molecular weight can occur in a single protein band.

Therefore, 2-DE represents a more successful approach since it can separate proteins based on their isoelectric focus (IEF) and molecular weights (Liebler, 2002).

In a 2DE analysis, the applied electric field allows for protein migration and the formation of a spot pattern, in which each spot represents a single protein species with

specific molecular weight and isoelectric point characteristics. The abundance of the proteins will thus be directly proportional to the intensity of a single spot.

Alongside the 2DE, the fluorescent 2DE differential gel electrophoresis (DIGE), has the benefit of separating two samples on a single gel. The tagging of two separate specific fluorophores, known as cyanines, distinguishes the spots of each sample (Viswanathan, Unlu, & Minden, 2006; Unlu, Morgan, & Minden, 1997).

2DE is an excellent preparatory tool for further characterization of proteins by MS-based methods (Scheler, et al., 1998), however, it has several limitations such as the inability to detect and isolate proteins of intermediate or low abundance or the discrimination of basic and hydrophobic molecules due to the chemical properties of IPG-based 2DE systems (Bendixen, 2005).

Several gel-free high-throughput proteome analysis methods have been developed in recent years to address some of these problems.

Gel-free approaches have seen widespread use in the field of meat research in recent years due to the high resolution, sensitivity, and efficiency in separating peptide segments.

The gel-free approach refers to a "bottom-up" proteomics technique known as shotgun proteomics when performed on a mixture of proteins (Zhang, et al., 2013) that allows for the study of hydrophobic proteins and peptides. Briefly, complex protein mixtures are digested into peptides by proteolytic enzymes, separated by chromatographic columns depending on distinct chemical characteristics, and then evaluated by MS/MS with subsequent protein identification (Nair, & Zhair, 2020).

The two approaches are characterized by the application of different protein quantification systems. Based on the procedures applied in the two trials proposed in this thesis, the next paragraph will focus on the identification and quantification techniques that follow the separation according to the gel-based approach.

3.2.3. Proteins' identification

After a gel-based separation, different techniques are used to visualize proteins.

Through acquisition systems, the 2-DE gel is acquired and digitized to allow image analysis and perform a screening of the proteins to be identified. Proteins of interest are identified and characterized based on their molecular

mass/charge (m/z) ratio through mass spectrometry (MS).

MS has numerous advantages such as high resolution, sensitivity, and accuracy (Burlingame, et al., 1976).

The most common technologies used for protein analysis and identification are liquid chromatography-MS/MS (LC-MS/MS), and electrospray ionization (ESI) (Fenn, et al., 1989; Whitehouse, et al., 1985) or matrix-assisted laser desorption ionization (MALDI) MS based-method alone or combined (Bodnar, et al., 2003). MALDI, combined with the time-of-flight (TOF) mass analyzer, and ESI are ionization techniques suited for protein identification using peptide mass fingerprinting (PMF).

PMF is a proteolytically digested protein analysis and represents the most widely used identification system in proteomics. Among enzymes (trypsin mostly) crude proteins in protein mixtures are digested and separated, resulting in tryptic peptides with the optimal size for analysis with MALDI (Bendixen, 2005).

The quality of PMF data depends primarily on the accuracy of the MS data, and the completeness of the genome sequencing data of a specific organism (Bendixen, 2005).

3.2.4. Proteins' validation

To identify potential biomarkers for meat quality, proteomic validation with qualitative-quantitative analysis such as western blot, reverse phase protein arrays (RPPA), and enzyme-linked immunosorbent assay (ELISA) is required.

These antibody-based techniques enable the separation, visualization, validation, and quantification of proteins extracted from cells or tissues.

Western blots are commonly used to detect the existence and integrity of a particular protein. Western Blot is a technique characterized by a high specificity but with negative aspects related to the times and costs, considerably high for its execution.

Western blot analysis is also limited to denatured proteins in the extract, unlike ELISA and RPPA which can be used to detect native proteins and protein interactions intact in their 3D structure.

The execution of an *ELISA* test involves the use of at least one specific antibody for each antigen and therefore, as for the blot, it will be possible to detect a protein per test. The basic principle (Aydin, 2015) is that one of these immunological components is immobilized on a solid phase, the cavities of a microplate. The

analyte taken from the sample interacts with the antibody-antigen system. This interaction can be visualized by enzymes, linked to antibodies or secondary antigens. The addition of a substrate capable of producing a color change measurable with a spectrophotometer allows for the quantification of the antigen on a calibration curve.

ELISA tests are technically less complex and expensive than western blot but nevertheless have lower specificity.

RPPA is a quantitative microformat Dot-Blot technique, emerged as a robust high-throughput method for quantifying and subsequently validating protein indicators related to meat quality (Gagaoua, et al., 2018a; Gagaoua, Bonnet, & Picard, 2020).

RPPA has a significant benefit in that it provides quantitative measurement of target protein abundance in huge sample sets while using just a small quantity of biological material.

3.2.5. Bioinformatic analysis

Bioinformatics represents an effective tool for understanding how biological mechanisms are associated and regulated by identified proteins (Kiyimba, et al., 2022a).

Mass spectrometry allow to identify proteins and related information on their isoelectric point, probability score, quantitative value, number of matched peptides, and sequence coverage. The following parameters play a key role in understanding the degree of accuracy of the identified data since the greater the number of peptides matched to a protein, the greater the sequence coverage (Baldwin, 2004).

To understand and interpret this data, the list of identified proteins must be further classified and filtered. Using globally recognized protein databases (SwissProt, TrEMBL, and UniProt) protein name is associated with a unique identifier (ID), thus preventing protein names to be different from database to database and even from version to version (Schmid, Forne, & Imhof, 2014).

Associate the identified protein with the term provided by the Gene Ontology database (http://www.geneontology.org) is the first step (known as *GO-term annotation*) towards functionally interpreting the resulting protein list (Ashburner, et al., 2000).

Online software such as METASCAPE (**Fig. 5**) (https://metascape.org/) directly combine genes with Gene Ontology and KEGG database and associates them with hierarchically grouped functional terms that

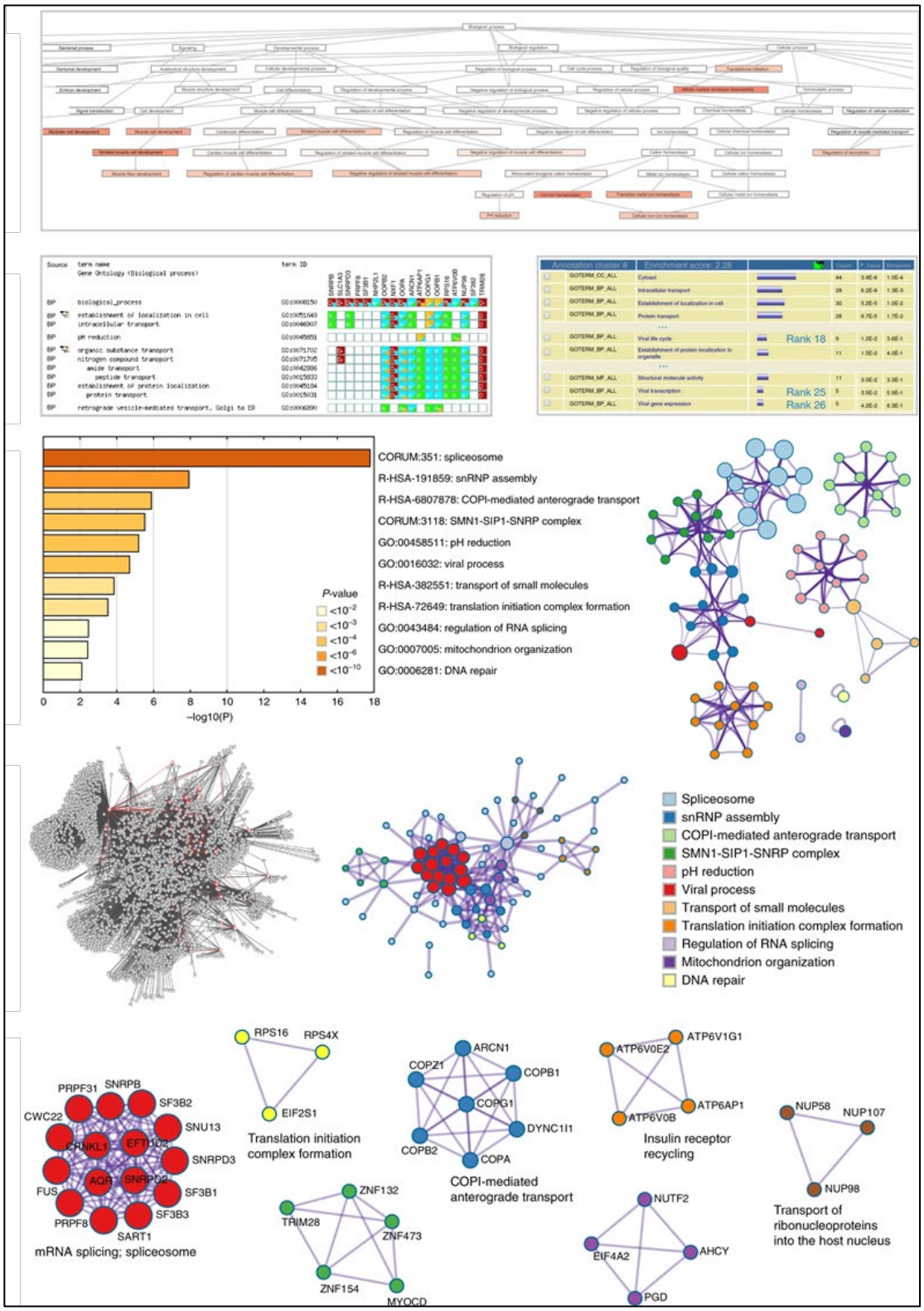


Figure 5. A typical visualization of functional enrichment and interactome analysis results in Metascape (Zhou, et al., 2019).

describe the "biological process," "molecular function," or "cellular component".

After GO-term annotation, a GO-term enrichment analysis is performed to compare the abundance of specific GO-terms in the dataset with the natural abundance (Malik, et al., 2010). To extract functions that are significantly enriched in one sample, a p-value is calculated which shows an overrepresentation of a specific GO term, thereby it is necessary to cluster related GO-terms (Schmid, Forne, & Imhof, 2014).

Analysis of protein complexes and the condition for their association and dissociation is fundamental to deeper understanding a biological system. Indeed, proteins form transitory but stable complexes with other proteins through which they regulate their activity in many biological processes in cells (Hu, et al., 2021).

Information on protein interactions in complexes are collected in interaction databases such as STRING (https://string-db.org/). This software is used to predict possible protein-protein interactions and infer the unknown functional information of proteins. Interaction results from sophisticated algorithms, that match software databases

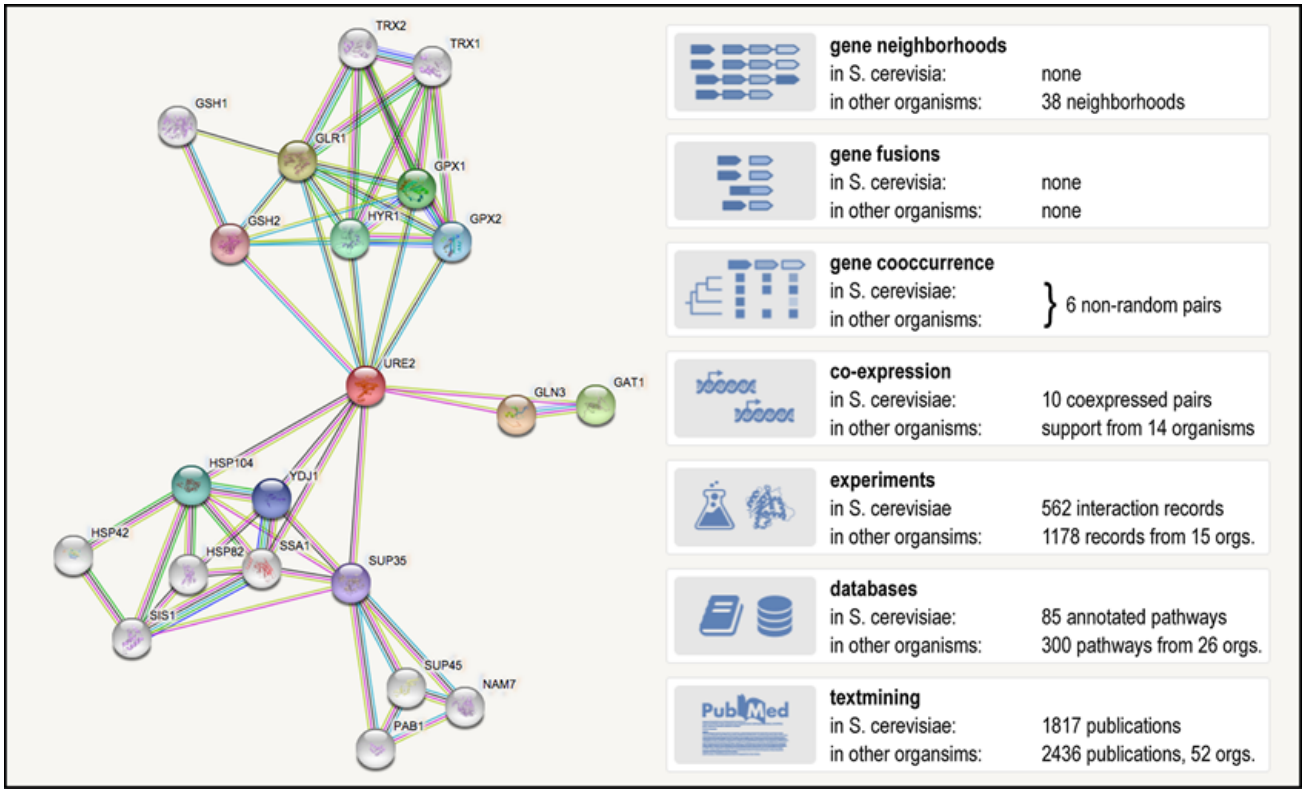


Figure 6. A typical association network in STRING (Szklarczyk, et al., 2019).

with several other data resources, thus providing a variety of visual representations (Fig. 6).

INTRODUCTION TO THE TRIALS

INTRODUCTION

The growing consumer interest in environmentally sustainable products has prompted farmers to identify potential farming systems suitable for ensuring quality products while preserving environmental sustainability. In this context, locally produced agro-industrial by-products employed as feed ingredients could represent a great resource in a sustainable perspective of meat production without compromising meat quality attributes (Salami, et al., 2019).

Hazelnut (*Corylus avellana L.*) skins account for about 2.5 % of the hazelnut processing by-products (Ivanovic et al., 2020); while being labeled as waste, stands out for the abundance of bioactive molecules (Del Rio, et al., 2011; Pelvan, et al., 2018; Ivanovic, et al, 2020), with proven antioxidant, antimicrobial, anti-inflammatory and immunomodulatory properties (Montella, et al., 2013). Recently, the effect of including tannin-rich hazelnut skins has been investigated on animal welfare and meat nutritional changes. Priolo et al. (2021) and Daghio et al. (2021) found that dietary hazelnut skin supplementation in growing lambs moderately affected rumen fermentation and enhanced their intramuscular meat fat with health-promoting fatty acids such as vaccenic acid and PUFA.

The effects of enriched tannins supplementation on meat nutritional quality and color stability are well known (Luciano, et al., 2009; Morales, & Ungerfeld, 2015; Valenti, et al., 2019; Zhao, et al., 2018), however, little is known about how they interact with post-mortem metabolic processes.

In recent years, with the rapid development of analytical techniques, proteomics combined with bioinformatics tools allowed a deeper understanding of the molecular mechanisms involved in the post-mortem processes induced by ante-mortem factors through the discovery of several biological pathways responsible of the development of meat quality traits (Picard, & Gagaoua, 2020; Gagaoua, et al., 2020). Proteomics-based techniques have been employed to investigate the dynamic changes and modifications occurring in post-mortem muscle during storage (D'Alessandro, & Zolla, 2013). However, to the best of our knowledge, few data are yet available on the use of proteomics to investigate the effect of dietary treatment on the post-mortem processes and changes underpinning the variation of meat quality through the muscle proteome. In this context, we suppose that a better understanding of the proteomic changes and the biochemical pathways related to different feeding strategies could be useful for the development of specific strategies aiming at an improvement of the organoleptic quality of lamb meat.

Therefore, the present studies aimed to in-depth investigate post-mortem variation in color and texture induced in lamb meat by feeding supplementation with hazelnut skins (HS) during storage, with a focus on the proteome of *longissimus thoracis et lumborum* muscle. Furthermore, bioinformatics

approaches were used to clarify the relationship between myofibrillar and sarcoplasmic differentially abundant proteins and meat quality characteristics.

CHAPTER 4

Characterization of the sarcoplasmic muscle proteome changes in lambs fed with hazelnut skin by-products: Relationships with meat color

4.1. MATERIALS AND METHODS

4.1.1. Animals, dietary treatments, and meat sampling

All animal procedures were developed in accordance with the European Union guidelines (2010/63/EU Directive) and were conducted under veterinary supervision. The experiment (previously described by Priolo et al., 2021) involved twenty-two Valle del Belice weaned male lambs (initial body weight 15.33 ± SD 1.8 kg) of 2 months of age selected from a local dairy farm. The lambs were transferred from the native farm to the experimental farm of the University of Catania, where animals were randomly divided into two dietary groups. After the adaptation period (5 days), all the lambs were individually fed ad libitum: the control group (C) received a basal diet (maize-barley based concentrate), whereas the other group (H) was given the same diet but 150 g/kg dry matter (DM) of maize was replaced with hazelnut skin (HS) by-products obtained after the roasting phase of hazelnut processing (Dalma Mangimi S.p.a. - Via Sperina Alta, 18 - Marene, Cuneo, Italy). Diet ingredients and chemical composition of the experimental are reported in Table 1.

Table 1.

Ingredients and chemical composition of the experimental diets and hazelnut skin.

	Hazelnut skin	Experimental diet[a]	
		C	H
Ingredient, g/100 g of dry matter (DM)			
maize		26	11
barley		26	26
soybean meal		16	16
alfalfa hay		20	20
wheat bran		7	7
hazelnut skin		0	15
molasses		3	3
vitamins and minerals mix[b]		2	2
Chemical composition, g/100 g DM			
DM, g/100 g as fed	90.4	89.3	89.1
crude fat	30.3	2.53	6.02
crude protein	10.2	19.4	22.2

Ash	2.48	6.28	6.72
NDF[c]	35.5	21.4	25.3
ADF[c]	26.8	11.0	14.4
ADL[c]	14.6	2.68	4.92
NFC[d]	26.3	53.1	43.9
total phenols[e]	13.3	0.32	2.24
total tannins[e]	7.83	0.15	1.60
Protein fractions[f], g/100 g DM			
A	1.19	2.60	2.60
B1	0.36	3.50	1.40
B2	3.85	10.56	14.07
B3	0.99	1.83	2.37
C	3.81	0.91	1.76
Metabolizable Energy, Mcal/kg DM	3.15	2.33	2.47
Individual fatty acid, g/kg DM			
C14:0	0.17	0.08	0.07
C16:0	24.8	4.08	6.56
C18:0	17.5	0.69	3.06
C18:1 *c*9	202	4.63	29.9
C18:2 *c*9 *c*12	35.9	11.2	14.1
C18:3 *c*9 *c*12 *c*15	0.58	1.16	1.26

[a] C: control maize-barley based concentrate diet. H: diet including 15% of hazelnut skin.

[b] Containing: 40% calcium carbonate, 15% sodium bicarbonate, 15% monocalcium phosphate, 12.5% vitamins mix, 10% sodium chloride and 7.5% magnesium oxide.

[c] NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin.

[d] NFC: Non-Fiber Carbohydrate: 100 – [CP + (NDF - NDFIP) + Ether Extract + Ash], where NDFIP representing the protein fraction linked to NDF

[e] Expressed as g tannic acid equivalents/100 g DM.

[f] Protein fractions: A = nonprotein nitrogen; B1 = buffer-soluble true protein; B2 = buffer-insoluble protein–neutral detergent soluble protein; B3 = neutral detergent insoluble protein–acid detergent insoluble protein; C = acid detergent insoluble protein

After 56 days of the experiment, the animals were weighed, transferred to a commercial abattoir, and slaughtered immediately following industrial practices used in Italy and in line with European

guidelines (EU rule n. 1099/2009). Each carcass was weighed and refrigerated for 24 hours at 2-4 °C. After 24 hours post-mortem, carcasses were halved and the whole *longissimus thoracis et lumborum* (LTL) muscle (on both sides) was removed. From the right LTL, three slices (2 cm thick) were cut, placed in polystyrene trays, covered with oxygen-permeable PVC film, and stored in the dark at 4 °C for 1, 4, and 7 days. At the end of the respective storage time, meat color stability and metmyoglobin development of fresh lamb meat were assessed, and samples were kept under vacuum at −80 °C before being transferred on dry ice to the University of Foggia.
Changes in sarcoplasmic proteins were estimated using SDS-PAGE, and Two-Dimensional Gel Electrophoresis coupled with Mass Spectrometry.

4.1.2. Color stability and metmyoglobin formation measurement

Color stability in raw meat over aerobic refrigerated storage was assessed using the method described by Valenti et al. (2019). Three slices (2 cm thickness) from the right LTL were placed in polystyrene trays, covered with PVC film, and stored at 4 °C for 1, 4, and 7 days, respectively. At the end of the respective storage time color parameters were measured by a Minolta CM 2022 spectrophotometer (d/8° geometry; Minolta Co. Ltd. Osaka, Japan) set to operate in the specular components excluded (SCE) mode and to measure with the illuminant A and 10° standard observer. Three measurements were taken on the meat surface and the mean value was calculated. The color descriptors L* (lightness), a* (redness), and b* (yellowness) were measured in the CIE L* a* b* color space. While hue angle (H°) and chroma (C*) were calculated with the following equation:

$$H° = \text{arctg}\,(b^*/a^*)$$

$$C = \sqrt{(a^2+b^2)}$$

Subsequently, the (K/S) ratio between the absorption (K) and the scattering (S) coefficients at the isobestic points 525, 575, and 610 nm wavelength were calculated as follows:

$$(K/S)_\lambda = (1 - R)_\lambda^2 \,/\, 2R_\lambda$$

This ratio was calculated to monitor the accumulation of metmyoglobin (MMb) considering that a decrease in (K/S) was observed with the constant increase of MMb proportion. According to Krzywicki (1979) metmyoglobin percentage (MMb) formation on the meat surface over time of storage was determined.

4.1.3. Proteomics analysis

4.1.3.1. Meat proteins extraction

Meat proteins were extracted and separated based on their solubility, according to the protocol of Marino et al. (2014). Protein extraction was carried out on meat deprived of connective and adipose tissue and finely chopped. 2.5 g of meat were homogenized by means of an Ultra-Turrax homogenizer (IKA T18 basic, Germany) for 3 minutes with 20 mL of cold buffer (0.03 M Phosphate Buffer, pH 7) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and then centrifuged (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany) at 8 000 x g for 20 minutes at 4 °C. After centrifugation the supernatant was aliquoted and frozen at -80 °C and represents sarcoplasmic proteins. Instead, the pellet was washed, and 0.1 g was solubilized in 1 ml of myofibrillar protein extraction buffer (8.3 M Urea; 2M Thiourea; 64mM Dithiothreitol (DTT); cholamidopropyl dimethyl hydroxypropane sulfonate (CHAPS), 2 %; NP-40, 2 %; Glycerol, 10 %; and 20 mM Tris–HCl, pH 8) and kept in contact overnight. The following day, after centrifugation at 15 000 x g for 20 minutes at 10 °C, the supernatant, representing the myofibrillar fraction, was aliquoted and frozen at -80 °C.

Protein quantification of the extracted sarcoplasmic fractions was performed with the 2-D Quant kit (GE-Healthcare) using bovine serum albumin as standard.

4.1.3.2. Gel sample preparation and SDS-PAGE analysis

Sarcoplasmic proteins were resolved using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Samples were treated with a denaturing and reducing sample buffer (0.6 M Tris–HCl, pH 6.8; SDS, 10 %; glycerol, 1 %; DTT; β-Mercaptoethanol, 1%, and Bromophenol blue) and then were resolved with 8-18 % sodium dodecyl sulfate-polyacrylamide gradient gel.

The separating gel was obtained from 2 solutions (8 % and 18 %) at different concentrations and densities of 40 % Acrylamide and 2 % Bisacrylamide (Bio-Rad Laboratories), mixed with a gradient forming apparatus (Hoefer SG 100 Gradient Maker) and a peristaltic pump.

The amount of acrylamide is inversely related to the size of the gel mesh; in the gradient gel, mixing 2 solutions with different densities produces a special mesh that decreases from the top to the bottom along the direction of proteins migration.

After polymerization, stacking gel at 4 % is poured on top of the separating gel to form the wells where the standard (Bio-Rad Precision Plus Protein - All Blue Standards) and the aliquots of each sample (about 40 μg of protein) were placed.

The analysis, here schematically represented in **Figure 7**, was conducted in a Protean II xi electrophoretic cell (Bio-Rad Laboratories) in a continuous running buffer system constitute of Tris–HCl, SDS, and glycine at 24 mA/gel and 11 °C. At the end of the run, gels were stained overnight in Coomassie Silver Blue G-250 solution.

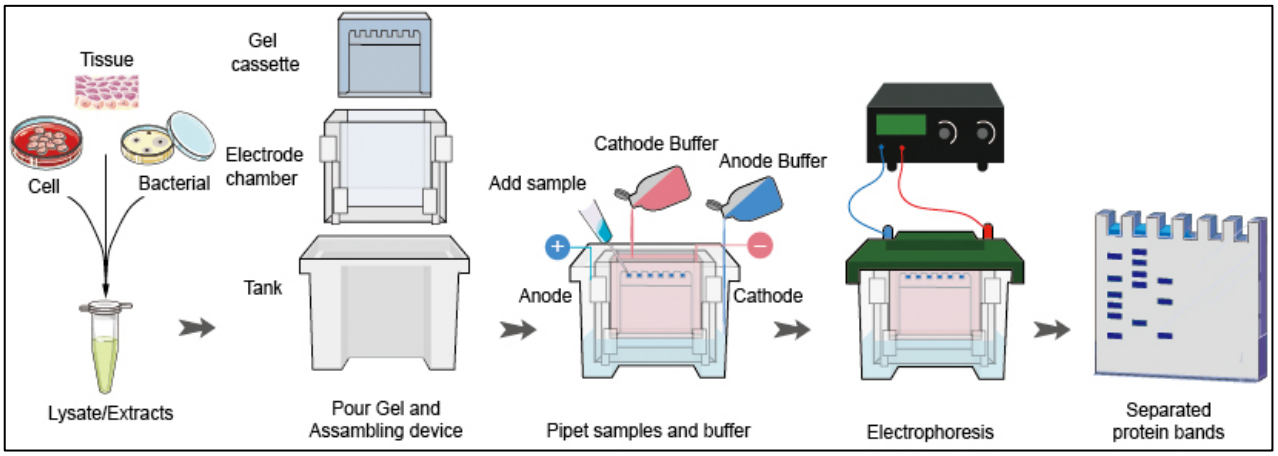


Figure 7. Schematic representation of the SDS-PAGE analysis (Creative Diagnostics, New York)

4.1.3.3. Two-Dimensional Gel Electrophoresis (2DE)

Sarcoplasmic proteins were separated with two-dimensional gel electrophoresis (2DE) conducted according to della Malva et al. (2022a).

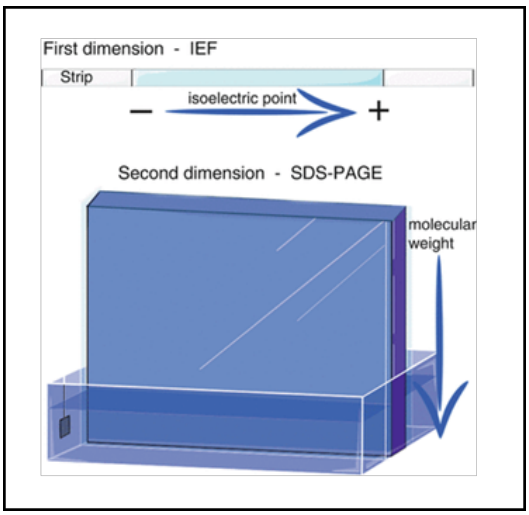


Figure 8. Visual representation of 2D proteins' separation (Saia-Cereda et al., 2019)

The technique involves a separation in the first dimension of the proteins based on their isoelectric point and in the second dimension based on their molecular weight (**Fig. 8**). Extracted proteins were separated in the first dimension on immobilized pH gradient (IPG) dry strips (17 cm IPG strips, Bio Rad, Laboratories). The IGP-strips were selected based on the protein fraction (pH 3-10 for sarcoplasmic proteins) to be analysed and were passively rehydrated with a suspension of protein extract (about 300 µg) and the isoelectrofocusing IPG sample buffer (Ready-Prep Rehydration/Sample Buffer, Bio-Rad Laboratories) for 3 hours.

Then, the dried strips were actively rehydrated using a IPG Protean IEF Cell (Bio-Rad Laboratories) and the following voltage gradient:

	T (°C)	V	h	mA/gel
Step 1	20	653	1:30	50
Step 2	20	1307	1:00	50
Step 3	20	8000	2:30	50
Step 4	20	8000	3:00	50

At the end of the IEF, the strips were equilibrated at room temperature for 15 min in equilibration buffers I and II (ReadyPrep™ 2-D Starter Kit, Bio-Rad Laboratories) containing 50 mM Tris–HCl (pH 8.8); 6 M urea; glycerol, 30 %; SDS, 2 %; and DTT, 1 % (in equilibration buffer I) or iodoacetamide, 2.5 % (in equilibration buffers II). Subsequently, strips were placed horizontally over the polyacrylamide gradient gel 8-18% and blocked with agarose (0.5 %) for the second-dimension separation, performed on a Protean II xi system (Bio-Rad Laboratories, Hercules, CA) at 24 mA/gel and 11 °C. At the end of the run, gels were stained overnight in Coomassie Silver Blue G-250 solution.

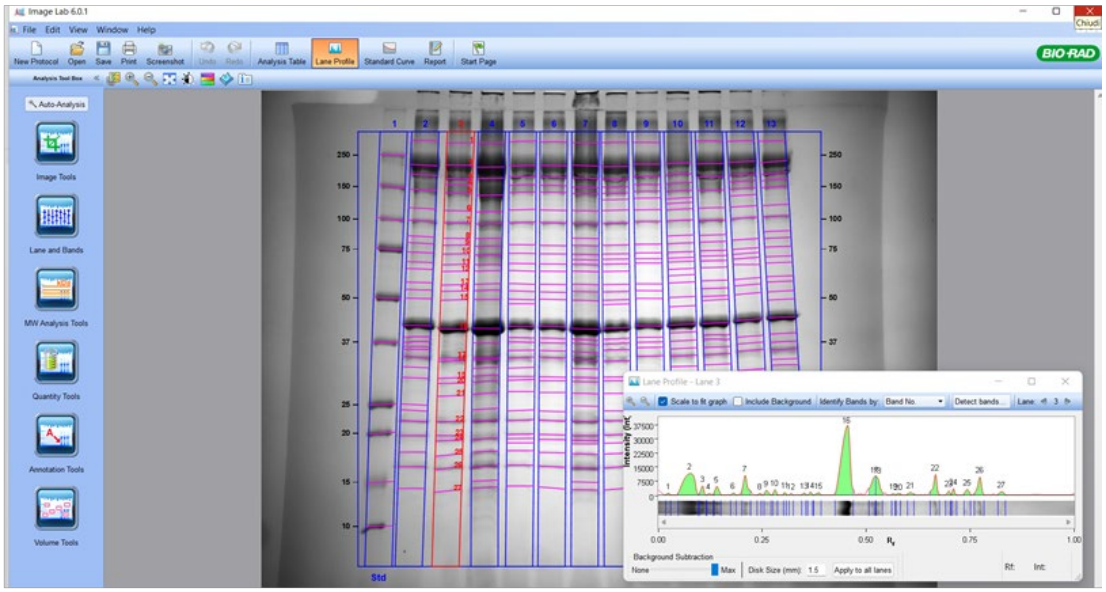
4.1.3.4. SDS-PAGE and 2DE gels image analysis

The destained gels from SDS-PAGE and 2DE were acquired with the Chemi Doc EQ instrument (Bio-Rad, Laboratories, Hercules, CA), and the images obtained were differently analyzed.

Image Lab software (Bio-rad Laboratories) was employed to determine the optical density of the bands of interest in SDS-PAGE gel (**Fig. 9a**); while gels from 2DE were analysed with PD-Quest software (PD-Quest, Bio-rad Laboratories, Hercules, CA) to obtain the total number of spots and information relating to their isoelectric point, volume, area, and intensity (**Fig. 9b**).

The relative volume of each spot in a gel was quantitatively determined after background subtraction and normalization as a percentage of the total volume of all spots detected on the gel.

a



b

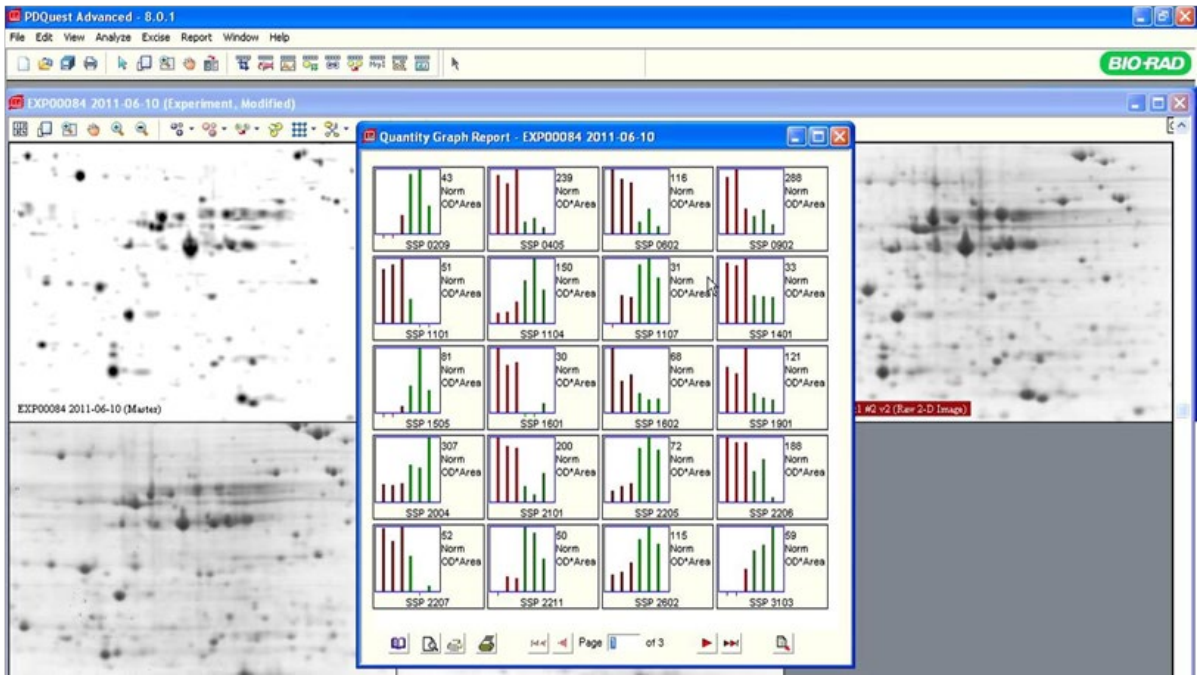


Figure 9. Typical visualization of (a) SDS-PAGE gel analysis with Image Lab and (b) 2DE gel analysis with PD Quest software.

4.1.3.5. Protein identification with Mass Spectrometry

For protein identification, 8-18 % 2DE gels loaded with 300 µg of extracted proteins were run. Differently abundant spots were carried out through a direct extraction of the spots from the preparative gels. Excised spots were destained, washed with 50 mM of NH_4HCO_3, dried under vacuum, and digested overnight at 37 °C with trypsin (12.5 ng/mL, Promega).

Then, peptides were extracted from the gel matrix by three changes of 50 % acetonitrile/0.1 % formic acid and suspended in 15 µL of 0.1 % formic acid to be analysed.

For the separations has been used LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled online with a nano-HPLC Ultimate 3000 (Dionex – Thermo Fisher Scientific) and equipped with a homemade pico-frit column (75 µm I.D., 15 µm Tip, 100 mm, New Objective) packed with C18 material (Aeris peptide 3.6 µm XB-C18, Phenomenex). Peptides were eluted using a linear gradient of ACN/0.1 % FA (from 3 % to 40 % in 19 min), at a flow rate of 250 nL/min.

The instrument operated in a data-dependent mode: a full MS scan at 60000 nominal resolutions in the Orbitrap was followed by the acquisition of MS/MS spectra of the ten most abundant ions in the linear ion trap. The ion source capillary temperature was set at 200 °C and the spray voltage was optimized at 1.3 kV.

Protein identification was achieved with the software package Proteome Discoverer 1.4 (Thermo Fisher Scientific) and confirmation of spectra was searched against the Ovis aries database (version Nov 2021, 70994 entries) with Mascot Search Engine server (version 2.2.4, Matrix Science).

4.1.4 Statistical Analysis

Color parameters, metmyoglobin percentages, SDS-PAGE band intensity, and 2DE spots' volume were analysed using the GLM procedure of the SAS 9.3 statistical software (SAS Institute, 2013) including as fixed effect the dietary treatment, the storage time, and their interaction (diet x storage). All effects were tested for statistical significance set at $P < 0.05$, and when significant effects were found, Fisher's LSD test was used for comparison.

A multivariate regression approach on the standardized data was further conducted on the SDS-PAGE band percentage of differentially abundant proteins to explain variation for each meat color parameter as in (Gagaoua et al. 2015a; Gagaoua et al. 2017a). For the explanatory models, the option "optimal model" was used to achieve regression equations with highest r-squares values. We set the maximum number of proteins to be retained in the regression equations of each color parameter to 4 to meet to the principle of parsimony.

4.2. RESULTS AND DISCUSSIONS

4.2.1. Effects of dietary treatment and storage time on color stability of lamb meat

Consumers' expectations of meat quality and their choice at the point of purchase are strongly correlated with its visual appearance parameters (Font-i-Furnols, & Guerrero, 2014). According to studies (Gracia, & de Magistris, 2013, Ngapo, Martin, & Dransfield, 2007), consumers employ color as a sign of wholesomeness and spoiling, making color one of the most significant fresh meat attributes at the point of purchase (Mancini, & Hunt, 2005). Meat color is related to the different forms of the sarcoplasmic proteins and is affected by both ante-mortem and post-mortem factors. A recent study by Tomasevic et al. (2021) emphasized how animal feeding can control post-mortem phenomena and improve the color of meat.

The effects of dietary supplementation and storage time on color attributes are reported in Table 2.

Table 2
Effect of the dietary treatment (H= hazelnut; C= control) and storage time (1, 4, and 7 days) on color parameters (lightness: L*, redness: a*, yellowness: b*, chroma: C*, hue angle: h°) (means ± SEM).

	Diet	Days			SEM	Effects, *P*	
		1	4	7		Diet	Storage
L*	C	45.10 ab	43.98 b	46.41 a	0.51	***	***
	H	46.31 b	45.80 b	48.50 a			
a*	C	14.42 a	11.26 c	12.31 b	0.30	**	***
	H	14.91 a	11.80 c	13.29 b			
b*	C	11.29 b	10.59 b	12.53 a	0.33	**	***
	H	11.84 b	11.18 b	13.56 a			
C*	C	18.33 a	15.46 b	17.57 a	0.43	**	***
	H	19.05 a	16.27 b	19.00 a			
h°	C	37.94 c	43.13 b	45.49 a	0.40	NS	***
	H	38.34 c	43.37 b	45.55 a			

NS = not significant; * = P < 0.05; ** = P < 0.01; *** = P < 0.001. a, b, c = P < 0.05 in the row (storage effect).

Our results evidenced that the dietary treatment with hazelnut skin affected the lamb meat color characteristics. Particularly, a significant effect of diet was found for lightness (L*; P < 0.001), redness (a*; P < 0.01), yellowness (b*; P < 0.01), and chroma (C*; P < 0.01), with the highest values in meat from lambs fed with hazelnut skin.

Similar results were also observed in *longissimus lumborum* muscle from lambs fed with different tanniniferous diets (carob pulp, acacia leaves or sulla fresh herbage) as reported by Priolo et al., (1998) and Priolo & Vasta, (2007).

Lightness is related to the concentration of myoglobin as well as to light scattering; the increase in the L* value found in the H group could be attributable to the role of tannins in delaying the synthesis of the heme pigment (Liu, et al., 2016). According to Priolo et al. (2000); Bhatta et al., (2002); and Barone et al., (2005), tannins could probably hinder the use of iron and consequently lower the concentration of hemoglobin in the blood, but more recently some studies have attributed this phenomenon to a decrease in the microbial biosynthesis of the vitamin B12 (Priolo, & Vasta, 2007; Liu, et al., 2016).

It is well known that storage time can affect meat color parameters, stability, and metmyoglobin formation (Jeremiah, & Gibson, 2001); thus, monitoring values of a* and chroma (C*) reflect the myoglobin concentration as well as its redox state in meat (Mancini, & Hunt, 2005) (Purslow, et al.

2021). During storage time an increase of L*, b*, and h° parameters ($P < 0.001$) and a decrease of a* values ($P < 0.01$) were observed in both groups. For C* values, a decrease after 4 days of storage was observed in the meat of both groups. Our data evidenced a stable color in terms of a* and C* values after 4 days of storage in the meat of lamb fed with hazelnut by-product, highlighting an increase in the vividness of color and, consequently a delay of the discoloration compared to the control. These findings are in line with the previous knowledge (Jeremiah, & Gibson 2001); more specifically, the decrease in a* and saturation index (h°) values during storage in the control group (15 % vs 11 % for a* in C and H group, respectively; and 4 % vs 0.26 % for C* in C and H group, respectively), highlight the important role of diet supplementation in modulating the post-mortem processes of color variation.

Color stability is usually documented by measuring the change in the predominant myoglobin forms over time. In **Table 3** the myoglobin development's evaluation reported a diet effect only for $(K/S)_{610}/(K/S)_{525}$ that showed lower value in meat for H group, while an effect of storage was found for all attributes.

Particularly, during storage time R_{630}/R_{580} and $(K/S)_{572}/(K/S)_{525}$ values decreased ($P < 0.001$) after 4 days, while $(K/S)_{610}/(K/S)_{525}$ ($P < 0.001$) values increased in both groups.

For metmyoglobin accumulation, no significant differences were found as a consequence of dietary treatment, whereas a significant and progressive increase of MMb % ($P < 0.001$) during storage time was found in lamb meat from both groups, showing the highest values in meat after 7 days of storage.

Table 3

Effect of the dietary treatment (H= hazelnut; C= control) and storage time (1, 4, and 7 days) on meat color stability parameters (reflectance: R_{630}/R_{580}), color stability ($(K/S)_{572}/(K/S)_{525}$, $(K/S)_{610}/(K/S)_{525}$, and metmyoglobin (MMb) percentages) (means ± SEM).

	Diet	Days			SEM	Effects, *P*	
		1	4	7		Diet	Storage
R_{630}/R_{580}	C	1.81 a	1.48 b	1.48 b	0.02	NS	***
	H	1.82 a	1.50 b	1.52 b			
KS_{572}/KS_{525}	C	0.98 a	0.92 b	0.89 c	0.01	NS	***
	H	0.97 a	0.92 b	0.88 c			
KS_{610}/KS_{525}	C	0.42 c	0.50 a	0.47 b	0.01	**	***
	H	0.41 c	0.48 a	0.43 b			
MMb %	C	41.63 c	48.79 b	51.29 a	0.73	NS	***
	H	42.78 c	48.82 b	51.68 a			

$(K/S)_\lambda = (1-R_\lambda)^2/2R_\lambda$

NS = not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. a, b, c = $P < 0.05$ in the row (storage effect).

The accumulation of MMb on the meat surface is responsible for the browning during post-mortem (Wu, et al., 2016). According to the present study, storage time might impact redness and the accumulation of metmyoglobin content in the same manner in meat from both groups, thus suggesting that the dietary treatment had no effect on the trend of biochemical processes, oxidation rates, or the chemistry of myoglobin over time.

4.2.2. Mono-dimensional electrophoresis (SDS-PAGE) of the sarcoplasmic muscle proteome

The electrophoretic profile of sarcoplasmic proteins as affected by diet and storage time is depicted in Figure 10.

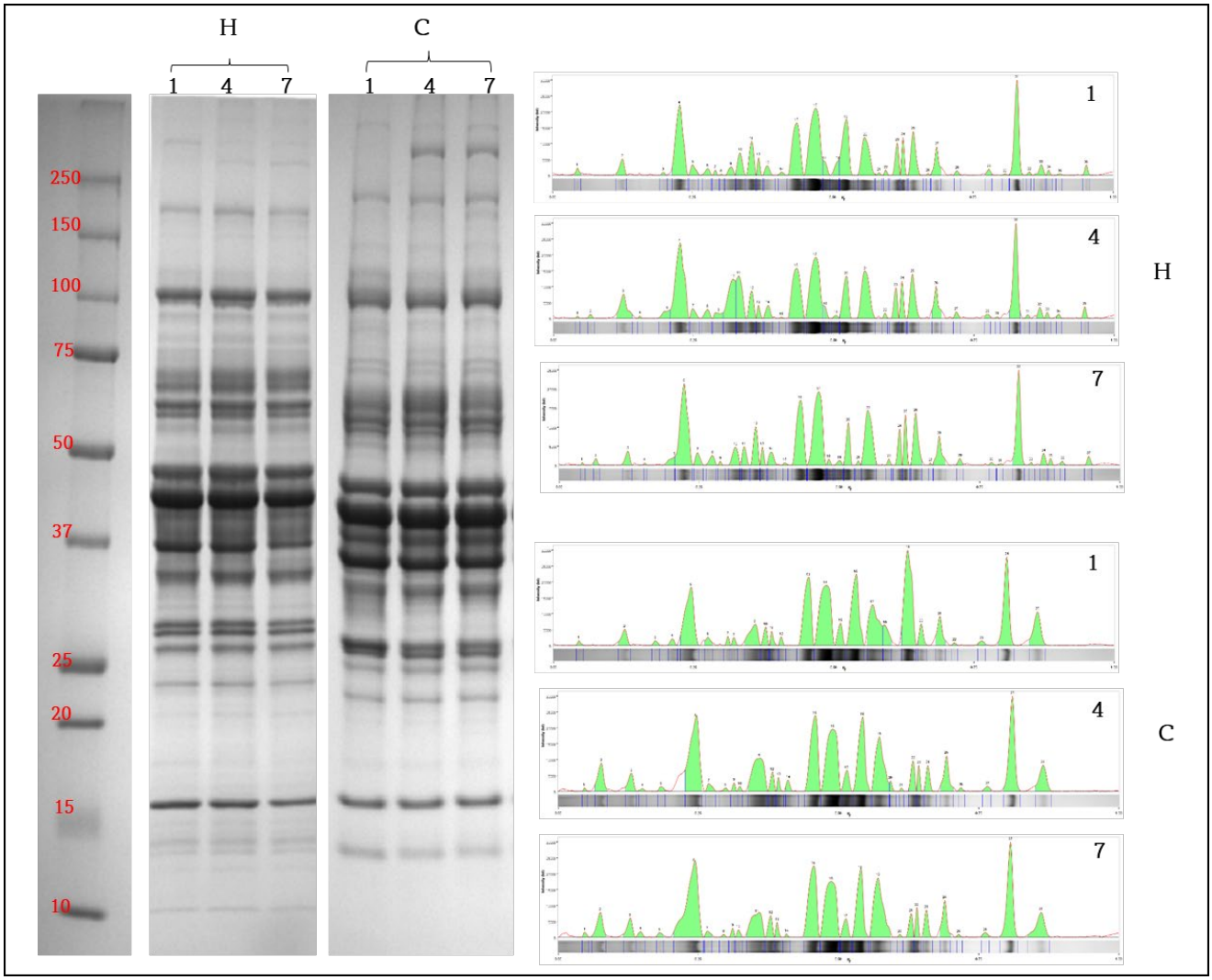


Figure 10. SDS-PAGE of sarcoplasmic fractions and densitometric profile from *longissimus thoracis et lumborum* muscle of lambs fed with different dietary treatment (H= hazelnut; C= control) after 1, 4 and 7 days of storage.

As expected, the results showed that the sarcoplasmic protein profile was significantly affected by dietary treatment and storage time. Particularly, although at 1 day of storage meat from both, C and H groups, was characterized by a similar profile (23 protein bands), after 7 days of storage, only meat from the hazelnut group displayed an increase of protein bands in terms of number and intensity (23 vs 28 for C and H groups, respectively). Bands were identified, starting from the positive electrode, as glycogen phosphorylase b kinase (GPHb), phosphoglucomutase (PGM), pyruvate kinase (PKM), glycogen phosphorylase (PYGM), enolase (ENO1), creatine kinase (CKM), aldolase (ALDOB), glyceraldehyde phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDHA), phosphoglycerate mutase (PGAM), triosephosphate isomerase (TPI1), and myoglobin (MB).

The relative quantity (%) of the major sarcoplasmic proteins extracted from the *longissimus thoracis er lumborum* muscle as affected by the dietary treatment and storage time are presented in **Figure 11**. Meat from the hazelnut group showed higher values of PKM, PYGM ($P < 0.001$, during all storage time), and TPI1 ($P < 0.01$, at days 1 and 4) and lower values of GAPDH ($P < 0.05$, $P < 0.01$ at days 1 and 4, respectively) compared to meat from C group.

During the storage time, a change in the relative quantity of several protein bands involved in different metabolic pathways was detected in H and C groups. In meat from both C and H groups a

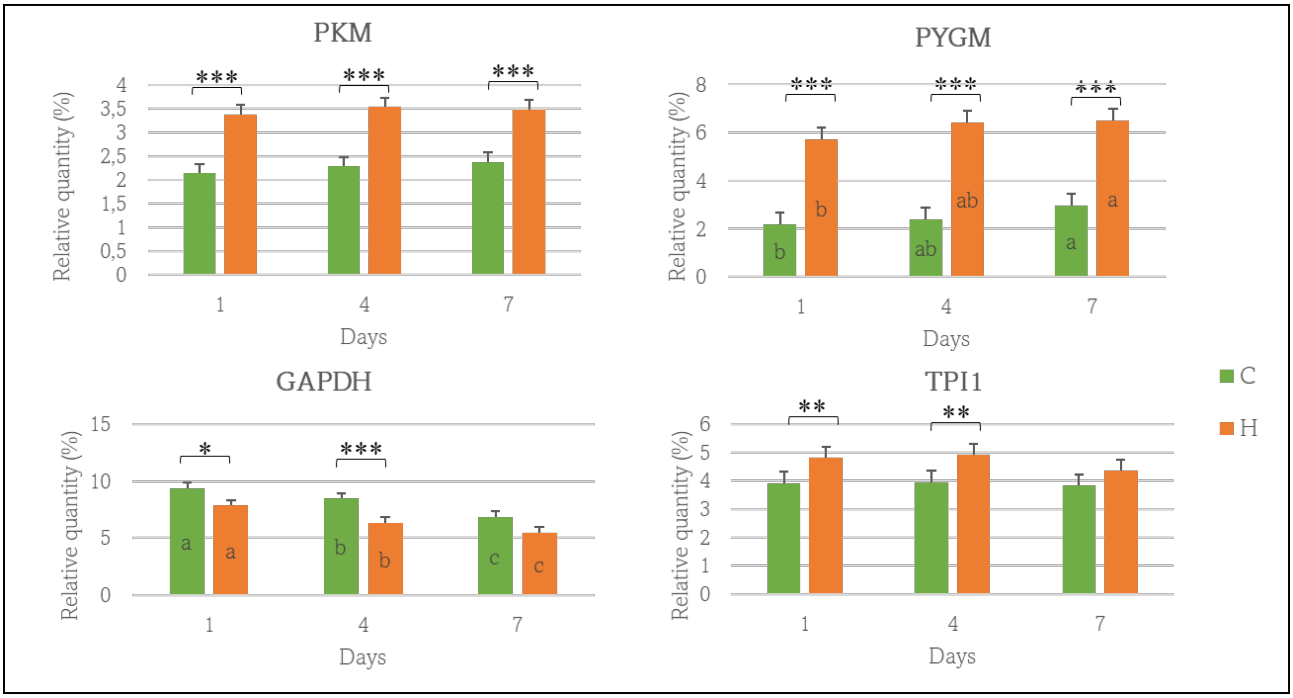


Figure 11. Relative quantity (%) of sarcoplasmic proteins (PKM= Pyruvate kinase; PYGM= Glycogen phosphorylase; GAPDH= Glyceraldehyde-3-phosphate dehydrogenase; PGAM1= Phosphoglycerate mutase 1; TPI1= Triosephosphate isomerase) of *longissimus lumborum* muscle from lambs as affected by different dietary treatment (H= hazelnut; C= control) and storage time. Different letters: a, b, c= $P < 0.05$ (storage effect). *= $P<0.05$; **=$P<0.01$; ***=$P<0.001$

gradual decrease of GAPDH ($P < 0.01$) and an increase of PYGM ($P < 0.05$) was observed starting from day 1, while no variations were detected during storage time in PKM and TPI1 proteins.

The present study highlighted that variation in the sarcoplasmic profile occurred differently during storage time as a result of the dietary strategy. Particularly, proteins involved in the glycolysis (PKM, PYGM, GAPDH, and TPI1) and energy metabolism showed the most changes in band intensity.

These changes are in line with the current knowledge of the importance of glycolysis, energy metabolisms and associated pathways in the determination of meat color (Gagaoua, et al., 2020). It is well known that the activity of metabolic and glycolytic enzymes is crucial for the muscle to meat conversion and the development of meat quality, such as color (Picard, Gagaoua, & Hollung, 2017). Indeed, proteins that play a key role in the post-mortem glycolysis in muscle are important cofactors in the reduction of enzymatic and non-enzymatic metmyoglobin formation (Bekhit, & Faustman, 2005). Previous proteomics studies (Canto, et al., 2015; Wu, et al., 2015; 2016; Gagaoua, Picard, & Monteils, 2018; Gao, et al., 2021) have linked the changes in glycolytic enzymes with color parameters in different muscles, highlighting the involvement of non-myoglobin proteins in the meat color development and stability.

4.2.2.1. Regression analysis of color parameters and sarcoplasmic proteins abundances of lamb meat

The regression models of lamb meat using color parameters as dependent variables and the sarcoplasmic protein abundances as independent variables for each storage time are given in **Figure 12**. Considering all models, thirteen proteins were significantly correlated with color parameters, and they explained between 18 % and 75 % of the variability.

For lightness, at 1 day of storage, the model explained 60 % of the variability ($P < 0.001$) entering PGM1 (negative), CKM and PGAM1 (both positive). At 4 days 53 % of the variability ($P < 0.01$) was explained by PYGM (positive), PGM1 and MB (both negative); and at 7 days 71 % of variability was explained by MB (negative), PGM1 and GAPDH (both positive).

The model of a*, at 1 day, explained 22 % of the variability by LDHA (negative), ALDOA and TPI1 (both positive); at 4 days explained 60 % of the variability ($P < 0.001$) by PKM, ENO3 and TPI1 (all negative); and after 7 days of storage, 74 % of the variability ($P < 0.001$) was explained by GPI, ENO3 and TPI1 (all positive).

The b* model explained 34 % of the variability at 1 day of storage by PYGM, CKM and PGAM1 (all positive); at 4 days 42 % of the variability ($P <0.05$) by PKM, ENO3 and TPI1 (all negative) and

after 7 days of storage 58 % ($P < 0.01$) of the variability was explained by GPI, ENO3 and TPI1 (all positive).

Refers to C*, the model explained, that at 1 day, the 21 % of the variability by PYGM (positive), LDHA (negative) and TPI1 (positive); after 4 days of storage the 45 % ($P < 0.05$) by PKM, ENO3 and TPI1 (all negative) and the 61 % of the variability ($P < 0.001$) by GPI, PGAM1 and TPI1 (all positive) at 7 days.

The hue angle explained, at 1 day of storage, the 61 % of the variability ($P < 0.001$) by PYGM, CKM and PGAM1 (all positive); at 4 days 26 % of the variability was explained by PYGM (positive), PGM1 and MB (both negative); and at 7 days, 37 % ($P < 0.05$) by PGM1 (positive), PKM and LDHA (both negative).

Considering the color stability parameters, the model of R630/R580 explained 21 % and 28 % of the variability at 1 and 4 days of storage, respectively. However, these models were weakly significant. After 7 days of storage, 65 % of the variability ($P < 0.001$) was explained by GPI, PGAM1 and MB (all positive).

The model of KS572/KS525 explained, that at 1 day of storage, 43 % of the variability ($P < 0.05$) by PYGM, GPI (both negative) and LDHA (positive); 22 % after 4 days of storage by LDHA, TPI1 (both positive) and PGAM1 (negative); and at 7 days 32 % of the variability was explained by PGM1 (negative), PKM and LDHA (both positive).

The KS610/KS525 model explained, at 1 day, the 33 % of the variability by PYGM, LDHA and TPI1; at 4 days the 64 % ($P < 0.001$) by PKM, ENO3 and TPI1 (all positive); at 7 days the 75 % ($P < 0.001$) by GPI, ENO3 and TPI1 (all negative).

Finally, the model of MMb explained, that at 1 day of storage, the 54 % of the variability ($P < 0.01$) by GPI, GAPDH and MB (all positive); at 4 days the 18 % by PGAM2 (positive), ALDOA and TPI1 (both negative); while, at 7 days, 39 % ($P < 0.05$) was explained by PGM1 (positive), PKM and LDHA (both negative).

The regression results revealed that most of the proteins are, as expected, involved in the post-mortem glycolysis (energy metabolism).

In the present study, the greater content of PKM protein together with the highest values of a* and C* found in meat from lambs fed with hazelnut skin confirm the greater color stability. It also confirms the potential of using it as a candidate biomarker of lamb meat color as suggested for beef (Gagaoua, et al., 2020). The importance of this enzyme could be explained by its role as a key rate-limiting enzyme in glycolysis (Kraaijenhagen, et al., 1984) and pH decline (England, et al., 2014).

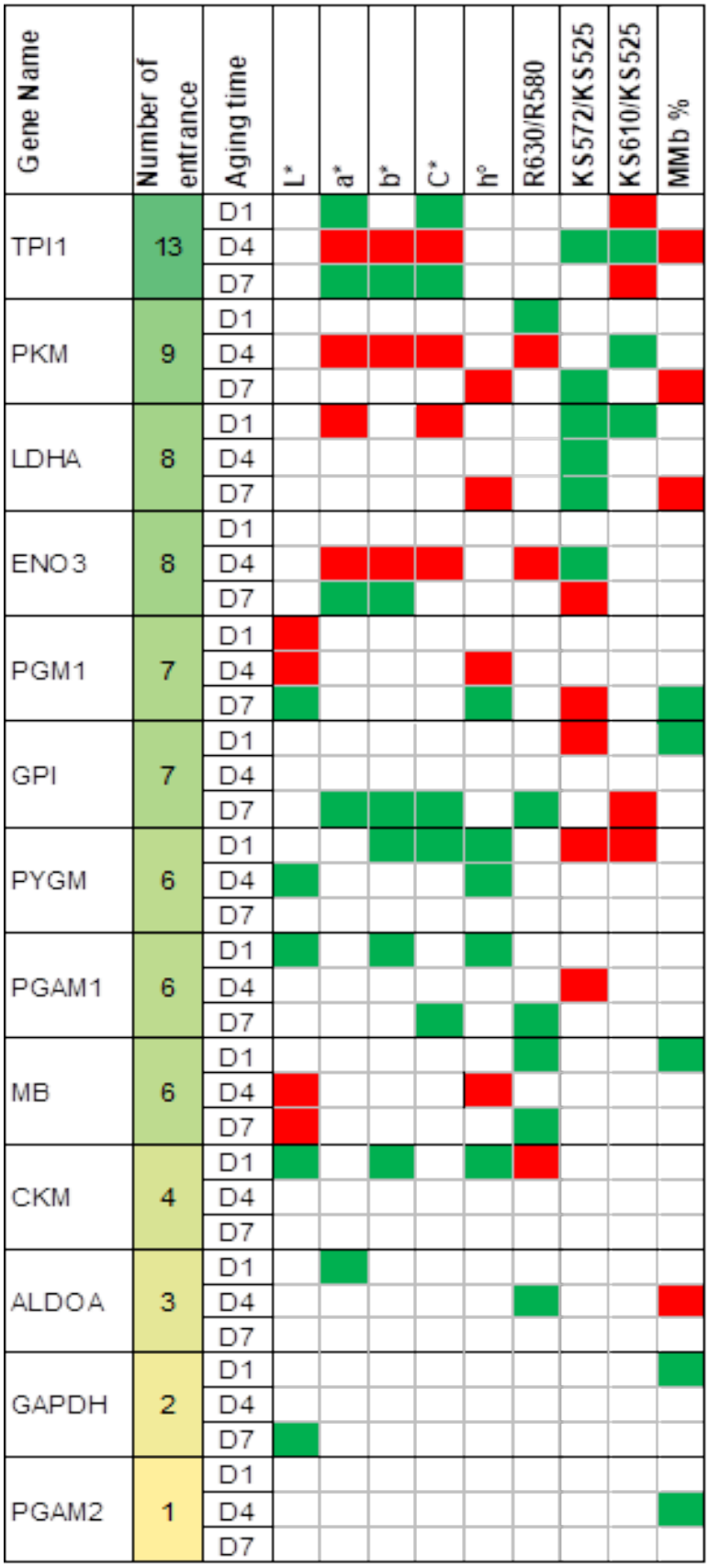


Figure 12. Regression models of meat color parameters and sarcoplasmic proteins abundances of longissimus lumborum muscle of lambs during storage (1, 4 and 7 days). The negative correlations are given in red and the positive in green.

Among the other proteins from the glycolytic and associated pathways, GAPDH has been long recognized as a protein of interest due to its relationship with several meat quality traits (tenderness, WHC, pH, and color) in beef (Marino, et al., 2014; Canto, et al., 2015; Wu, et al., 2016; Gagaoua, et al., 2021a), pork (Lametsch, Roepstorff, & Bendixen, 2002), horse (della Malva, et al., 2022a) and lamb (della Malva et al. 2017; Gao, et al., 2021). GAPDH is another key glycolytic enzyme, which catalyzes the production of NADH via glyceraldehyde 3-phosphate, also known as an important co-factor influencing the stability of meat color in post-mortem muscle though the promotion of metmyoglobin reduction (Purslow, et al., 2021; Ramanathan, Suman, Faustman, 2020). The association of GAPDH with meat color is muscle-dependent. Previous studies on different bovine and ovine muscles showed contrasting results depending on the contractile and metabolic properties of the muscles as well as their color stability (labile versus stable). For example, Gao et al., (2021) reported a positive correlation between GAPDH with a* and C* values in color-stable LTL ovine muscle, while Wu et al., (2016) found a negative correlation in the Psoas major muscle. In our study, meat from the hazelnut group displayed during storage a decrease of GAPDH together with greater a* values suggesting that a tannin-based diet, due to the content of antioxidant compounds, may act on post-mortem mechanisms by reducing the rate and extent of discoloration as well as the formation of metmyoglobin. On another hand and in line with an earlier study from our group on lambs fed with linseed and/or quinoa seed dietary supplementation (della Malva et al. 2017), a significant role of diet in modulating meat tenderization rate supports the impact of pre-harvest feeding strategy on post-mortem biochemical processes and impact on the final outcomes.

4.2.3. Changes in the sarcoplasmic muscle proteome from hazelnut-based diet over storage time

To better understand the impacts of the feeding strategy on post-mortem metabolism on lamb muscles a 2DE analysis was performed. Figure 13 illustrates representative 2DE gel maps of the sarcoplasmic protein fraction from *longissimus thoracis et lumborum* muscle of meat from lambs fed with different dietary treatments at 1, 4, and 7 days of storage. 2DE gel maps showed a different proteome profile in the two experimental groups exhibiting a major number of spots and a lower decrease during storage time in meat from lambs fed with HS compared to meat from control with 306 spots *vs* 246 at day 1 and 252 *vs*164 at day 7, respectively.

Data confirmed the effectiveness of feeding in modulating the post-mortem muscle biochemical processes during storage as evidenced by the major increase of spots number found in meat from lambs fed with hazelnut skin after 7 days of storage. Changes in abundance and number were

observed for proteins of different functions such as glycolytic enzymes, proteins related to energy metabolism, and transport proteins. Most of the glycolytic enzymes were found in the neutral or basic region of the 2DE gels and were identified in multiple spot isoforms.

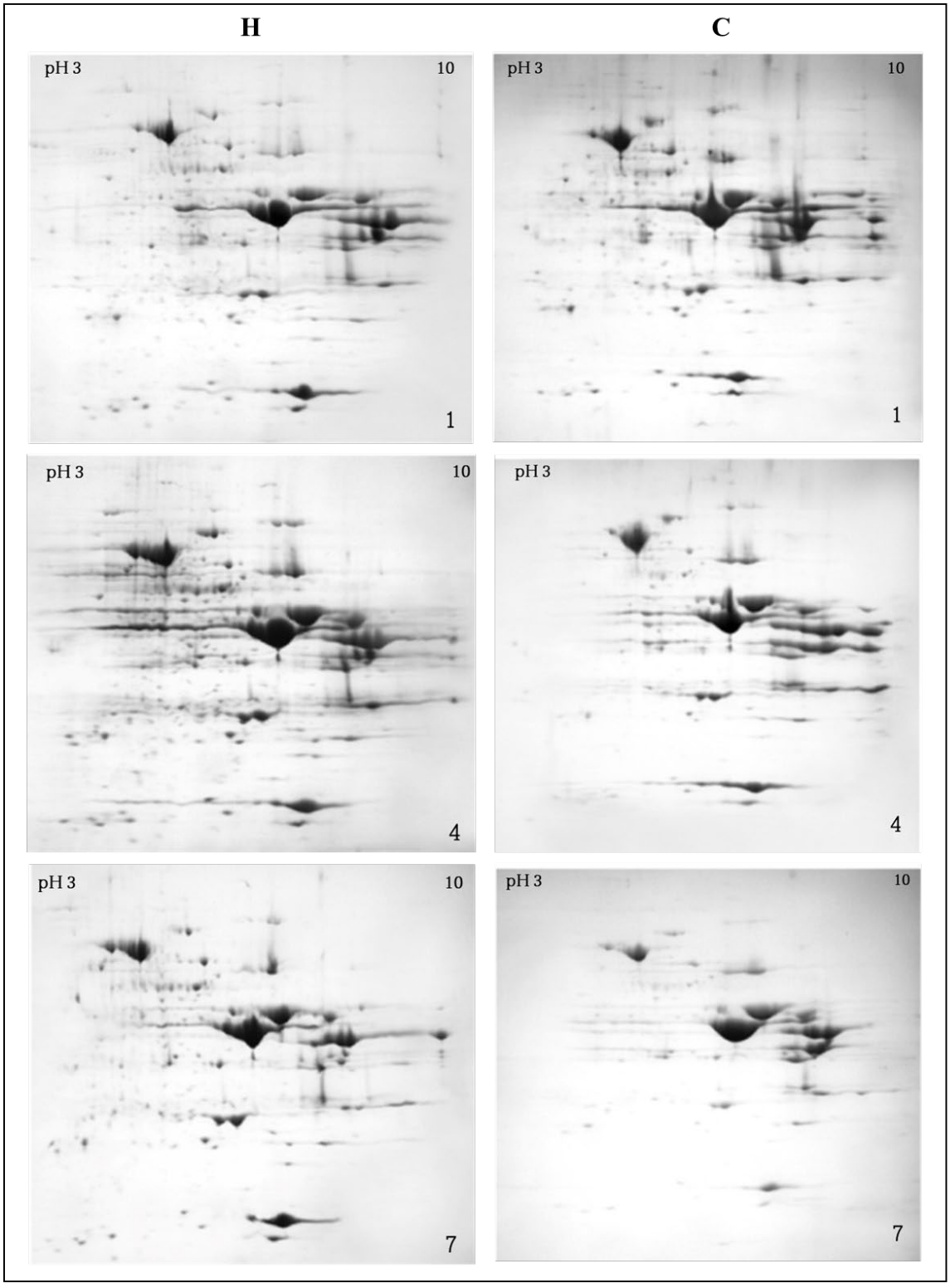


Figure 13. 2DE of sarcoplasmic fractions from *longissimus thoracis et lumborum* muscle of lambs fed with different dietary treatment (H= hazelnut; C= control) after 1, 4 and 7 days of storage.

Spots of fructose-bisphosphate aldolase B (ALDOB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK1), triosephosphate isomerase (TPI), and myoglobin (MB) were found more abundant in meat from the H group.

During storage, in meat from the H group spots of ALDOB, GAPDH, and PGK1 gradually decreased, while intensification of TPI and MB spots was observed starting from day 1. Conversely, in meat from the C group ALDOB, GAPDH, PGK1, TPI, and MB spot intensity decreased but at a lower rate compared to the H group.

Image analysis allowed an accurate evaluation of protein spots significantly different in abundance as a result of the dietary treatment and storage time (7 days). A total of 10 protein spots were found to be differentially expressed due to dietary treatment (**Fig. 14**) and were excised from preparative gels and identified using LC-MS/MS analysis.

From the 10 protein sports 41 proteoforms (gene name), were found to be differentially expressed due to dietary treatment and identified based on homology to Ovis Aries (**Table 4**).

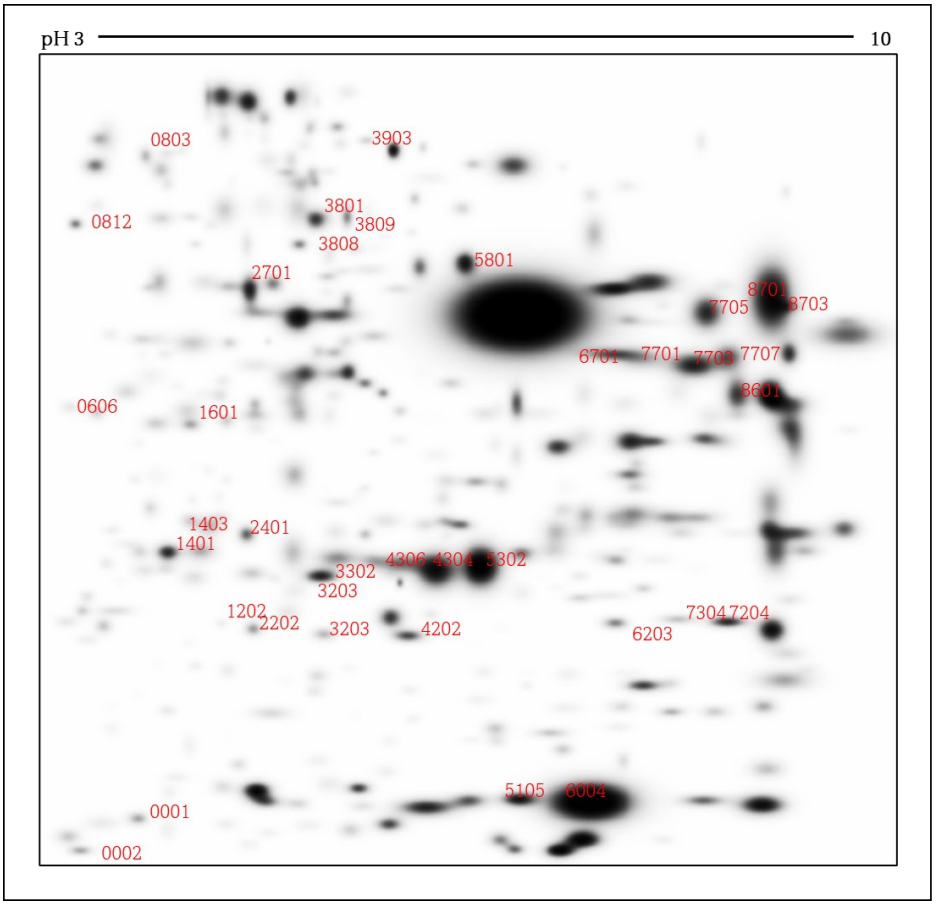


Figure 14. A representative 2DE maps of the sarcoplasmic fraction showing identified proteins.

Table 4

Identified proteins by 2DE gels as different in the sarcoplasmic muscle proteome to be affected by dietary treatment.

Spot number	Identified protein	Gene name	Uniprot ID	MW [kDa]	calc. pI	Score	Seq. Coverage	Matched peptides
0001	14 kDa phosphohistidine phosphatase	PHPT1	A0A6P7DSX4	13,9	5,82	2573,06	52,00	7
	Nuclear transport factor 2	NUTF2	A0A6P3E9M6	14,5	5,38	116,34	48,03	4
	Apolipoprotein A-I	APOA1	A0A6P3TBJ5	30,3	5,97	259,99	46,42	12
	2-iminobutanoate/2-iminopropanoate deaminase	RIDA	A0A6P3ESK7	14,3	8,12	122,06	42,34	5
	Fatty acid-binding protein, heart isoform X1	FABP3	A0A6P3TBK2	14,8	6,57	153,40	33,83	5
	Profilin	PFN1	A0A6P7EI30	15,0	8,28	78,43	22,14	3
0002	Nuclear transport factor 2	NUTF2	A0A6P3E9M6	14,5	5,38	625,07	79,53	7
	Apolipoprotein A-I	APOA1	A0A6P3TBJ5	30,3	5,97	400,30	46,42	13
	LIM domain-binding protein 3 isoform X13	LDB3	A0A6P7DGT7	31,0	9,19	92,38	22,97	5
	Alpha-1-antitrypsin transcript variant 1	SERPINA1	I1WXR3	46,0	6,20	172,21	20,43	7
0803	60 kDa chaperonin	HSPD1	A0A6P7DQC9	60,9	5,87	8436,08	71,55	40
	Fructose-bisphosphate aldolase	ALDOA	A0A6P7DGM3	39,4	8,19	618,84	34,89	13
	Phosphoglucomutase-1 isoform X2	PGM1	A0A6P3DYV7	61,6	6,81	678,67	29,89	15
	15-oxoprostaglandin 13-reductase	PTGR2	W5NRG0	38,4	5,39	196,22	23,93	7
	Prohibitin	PHB	A0A6P3TWZ1	29,8	5,76	127,66	23,53	3
	Pyruvate kinase	PKM	A0A6P3TIA1	58,0	7,53	715,76	23,16	13
	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	A0A6P7DEU0	35,9	8,54	187,59	22,52	5
	Creatine kinase	CKM	W5PJ69	43,0	7,15	196,33	22,05	7
	2-phospho-D-glycerate hydro-lyase	ENO1	A0A6P7EJI2	47,4	6,24	217,99	21,20	4
2202	Maillard deglycase	PARK7	W5PK66	20,1	7,33	2596,94	61,38	15
	ATP synthase subunit d, mitochondrial	ATP5PD	W5PP37	18,7	5,74	486,24	55,90	11
	peroxiredoxin-1	PRDX1	A0A6P3DYP3	22,2	8,40	364,36	52,26	9
	Adenylate kinase isoenzyme 1	AK1	A0A6P9FQJ4	21,6	8,32	411,22	47,42	8
	RAS oncogene family-like 7A	RAB7A	B2LYK6	23,5	6,70	314,54	46,86	9

	Apolipoprotein A1	APOA1	W5NX51	29,5	6,20	167,37	27,41	6
	Triosephosphate isomerase	TPI1	W5P5W9	22,8	5,63	184,22	23,36	4
	Peroxiredoxin 2	PRDX2	C8BKC5	21,8	5,43	174,55	23,23	6
	Phosphatidylethanolamine-binding protein 1	PEBP1	A0A6P3EBI4	21,0	7,49	113,17	22,99	3
	Heat shock 27 kDa protein	HSPB1	A0A6P7DEW2	22,3	6,70	100,53	21,89	3
2301	Triosephosphate isomerase	TPI1	A0A6M6R7Y5	30,6	6,55	2126,90	58,04	13
	Maillard deglycase	PARK7	W5PK66	20,1	7,33	210,60	47,62	8
	3-hydroxyisobutyrate dehydrogenase, mitochondrial	HIBADH	W5PHR0	34,8	7,90	1737,15	46,67	10
	Apolipoprotein A1	APOA1	W5NX51	29,5	6,20	153,30	31,66	7
	ATP synthase subunit d, mitochondrial	ATP5PD	W5PP37	18,7	5,74	79,37	30,43	5
	Carbonic anhydrase	CA3	W5PUC1	29,4	7,84	177,35	25,77	6
	Prohibitin	PHB	A0A6P3TWZ1	29,8	5,76	34,49	23,53	3
2401	peroxiredoxin-6	PRDX6	A0A6P3CY24	25,1	6,38	2363,59	69,20	15
	Triosephosphate isomerase	TPI1	A0A6M6R7Y5	30,6	6,55	1092,14	49,30	11
	Proteasome subunit alpha type	PSMA6	B6EBS6	27,4	6,76	252,05	35,77	8
	Phosphoglycerate mutase	PGAM2	A0A6P3EN06	28,8	8,90	612,51	29,64	9
	Adiponectin (Fragment)	ADIPOQ	A0A0M4KDI9	13,7	8,60	107,25	27,64	3
	Maillard deglycase	PARK7	W5PK66	20,1	7,33	89,66	24,87	4
	3-hydroxyisobutyrate dehydrogenase, mitochondrial	HIBADH	W5PHR0	34,8	7,90	423,20	24,85	6
	Carbonic anhydrase	CA3	W5PUC1	29,4	7,84	216,17	22,31	5
	Apolipoprotein A1	APOA1	W5NX51	29,5	6,20	90,98	21,62	4
3203	Maillard deglycase	PARK7	W5PK66	20,1	7,33	1459,79	64,02	12
	Apolipoprotein A1	APOA1	W5NX51	29,5	6,20	316,52	45,17	12
	Adenylate kinase isoenzyme 1	AK1	A0A6P9FQJ4	21,6	8,32	121,23	30,93	6
	14 kDa phosphohistidine phosphatase	PHPT1	A0A6P7DSX4	13,9	5,82	177,18	21,60	3
	Prefoldin subunit 3	VBP1	W5P0G8	22,5	6,28	127,48	20,30	4
3808	Peroxiredoxin-6	PRDX6	A0A6P3CY24	25,1	6,38	288,65	30,80	7
	Triosephosphate isomerase	TPI1	W5P5W9	22,8	5,63	161,96	23,83	4
	2-phospho-D-glycerate hydro-lyase	ENO1	A0A6P3YP53	47,3	6,58	934,54	21,20	7
4202	Maillard deglycase	PARK7	W5PK66	20,1	7,33	1027,06	80,42	13

	Apolipoprotein A-I	APOA1	A0A6P3TBJ5	30,3	5,97	206,98	40,38	9
	Adenylate kinase isoenzyme 1	AK1	A0A6P9FQJ4	21,6	8,32	110,86	21,13	4
4306	Triosephosphate isomerase	TPI1	A0A6M6R7Y5	30,6	6,55	8219,47	74,48	15
	Maillard deglycase	PARK7	W5PK66	20,1	7,33	557,64	58,73	10
	Carboxymethylenebutenolidase homolog	CMBL	A0A6P3EDS1	28,0	6,79	724,59	49,39	10
	Uncharacterized protein	DHRS11	W5QAX4	28,3	7,40	415,38	34,62	8
	ATP synthase subunit d, mitochondrial	ATP5PD	W5PP37	18,7	5,74	213,52	31,06	8
	High mobility group protein 1	HMGB1	A0A6P7EG22	24,9	5,74	132,47	30,23	6
	Fructose-bisphosphate aldolase	ALDOA	A0A6P7DGM3	39,4	8,19	300,07	29,12	10
	Apolipoprotein A1	APOA1	W5NX51	29,5	6,20	129,28	28,19	6
	Glutathione transferase	GSTM5	A0A6P7EBK2	25,7	7,39	312,83	27,52	5
	Peroxiredoxin-6	PRDX6	A0A6P3CY24	25,1	6,38	97,94	26,79	6
	Glutathione S-transferase	GSTM1	A0A6P3TGT6	25,0	6,02	250,97	25,00	4
	Persulfide dioxygenase ETHE1, mitochondrial isoform X1	ETHE1	A0A6P7ES27	27,8	7,25	341,86	24,41	6
	Peroxiredoxin-1	PRDX1	A0A6P3DYP3	22,2	8,40	87,11	22,61	4
	Proteasome subunit alpha type	PSMA6	B6EBS6	27,4	6,76	149,77	21,95	5
	Phosphoglycerate kinase	PGK1	B7TJ13	44,5	8,27	435,58	20,62	8
	Glutathione S-transferase	GSTM3	A0A6P3TM19	26,8	7,24	169,77	20,00	4

CHAPTER 5

Characterization of the muscle myofibrillar protein changes in lambs fed hazelnut skin by-products: Insights at the proteome level and consequences on meat texture

5.1. MATERIALS AND METHODS

5.1.1. Animals and meat sampling

The experimental design has been previously described in Chapter 4.1.1.

5.1.2. Myofibrillar Fragmentation Index (MFI) measurement

The myofibrillar fragmentation index (MFI) was determined by spectrophotometric assay according to the protocol of Culler, Smith, & Cross (1978) with some modifications. For each sample, four replicates of 2 g of raw meat were homogenized for 30 seconds at 20 500 rpm using an Ultra-Turrax homogenizer (IKA T18 basic, Germany) with 20 ml of cold buffer (100 mM KCl; 20 mM KH_2PO_4, pH 7.0; 1 mM EGTA; 1 mM $MgCl_2$; and 1 mM NaN_3) and centrifuged at 1 000 × g (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany) for 15 minutes at 2 °C to extract myofibrils. Then, the pellet was filtered (1.00 mm mesh strainers) to remove connective tissue, diluted with 5 mL of cold buffer, and used to quantify the protein concentration using the Biuret method (Gornall, Bardawill, & David, 1949) by spectrophotometric assay. After the incubation time, the concentration was adjusted to 0.5 mg/mL and the absorbance was read immediately at 540 nm. The MFI was calculated by multiplying the absorbance at 540 nm by 150 conversion factors.

$$MFI = A_{540} \times 150$$

5.1.3. Total Collagen content of meat

Collagen contents were determined from the hydroxyproline concentration according to della Malva et al. (2017) with slight modifications. 0.1 g of sample, after homogenization with 6 M HCl, was placed in a ventilated oven at 160 °C for 75 minutes to hydrolyse proteins. Subsequently, tubes were cooled to room temperature, filtered with syringe filters (0.45 um i.d.), diluted 1:10 with ultrapure water, and 1 ml of solution was placed into a chromatographic vial for the analysis using the HPLC system Agilent Technologies 1260 Infinity. The analysis was performed in duplicate for each sample, and the mean of all replicates was utilized for statistical analysis.

The retention times of the hydroxyproline peak were compared to those of the standard. Using a conversion factor of 7.25, the quantity of total collagen was determined from the hydroxyproline content and reported as g/100 g of wet meat.

5.1.4. Proteomics analysis

5.1.4.1. Protein extraction and SDS-PAGE analysis

Myofibrillar proteins were extracted as previously described (Chapter 4.1.3.1.) and stored at -80 °C prior to proteomic analysis. The protein concentration of the myofibrillar extracts from each sample was assessed using the 2-D Quant kit (GE Healthcare) using a microplate reader (Biotek PowerWave XS2, Biotek Instruments, Inc. Highland Park, Winooski, Vermont, USA).

SDS-PAGE was employed to separate myofibrillar proteins using an 8–18 % SDS-PAGE gradient gel loaded with 40 µg of protein for each lane. The analysis was carried out using a Protean II xi system (Bio-Rad Laboratories). In the end, gels were stained with Coomassie Blue G 250 and destained to be acquired by the Chemi Doc EQ system (Bio-Rad Laboratories). Image analysis was performed using the Quantity One software.

5.1.4.2. Western blotting

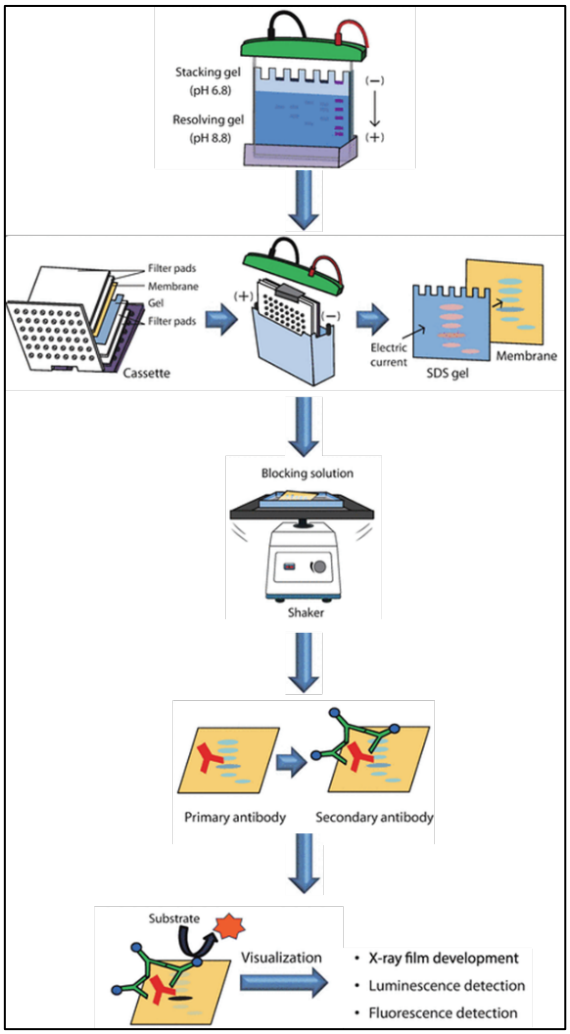


Figure 15. Western Blotting workflow (Jeong, et al., 2018)

Western blotting is a biochemical technique that allows the identification of a specific protein in a mixture of proteins, through the recognition of specific antibodies.

Western blots technique was executed on proteins that have been previously separated by SDS-PAGE and transferred to a membrane (**Fig. 15**). Specifically, a 10 % polyacrylamide separating gel and a 4 % stacking gel were used for Troponin-T and Desmin separation as reported by Marino et al. (2015). Gels loaded with 40 µg of protein were run in a Mini-Protean Tetra (Bio-Rad Laboratories) electrophoretic cell at 100 V for 3 h. Then, gels were transferred to a 0.2 µm mini-sized membrane of nitrocellulose (Bio-Rad Laboratories) by a semi-dry transfer method (Trans-Blot, Bio-Rad Laboratories). Membrane blocking was performed using 5 % BSA in Tris buffered saline containing 0.05 % Tween-20 (tris [TBS]-Tween buffered saline) for 1 hour at room temperature. Then, the membranes were incubated

with the primary antibodies: monoclonal anti-troponin-T (JLT-12; Sigma-Aldrich, St Louis, MO; diluted 1:40 000) and monoclonal anti-desmin (D1033, Sigma-Aldrich; diluted 1:5000) for 1 hour at room temperature. Once the incubation phase was completed, membranes were washed 5 times (10 min/wash) using TBS-Tween at room temperature and were then incubated for 1 hour at room temperature with the secondary antibody goat anti-mouse-HRP (No 2554; Sigma-Aldrich, St Louis, MO) diluted 1:30 000 and 1:5 000 for TnT and desmin analysis respectively, conjugated with a radioisotope, a fluorophore, or an enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). Protein bands were visualized using Clarity Western ECC kit (Bio-Rad Laboratories, Hercules, CA). Images were acquired by the Chemi Doc EQ system (Bio-Rad Laboratories, Hercules, CA) and then analyzed with the Quantity One software (Bio-Rad Laboratories, Hercules, CA) to determine the signal intensity of the defined bands.

5.1.4.3. Two-dimensional electrophoresis (2DE)

Two-dimensional electrophoresis (2DE) was carried out following the protocol described by Marino et al. (2015) using 17 cm-IPG dry strips with a pH range of 4–7 (ReadyStrip IPG strips, Bio-Rad Laboratories) loaded with 300 μg of protein. First-dimension isoelectric focusing (IEF) was performed using the IPG Protean IEF Cell (Bio-Rad Laboratories). After IEF, strips were equilibrated (Ready prep 2D starter kit, Bio-Rad Laboratories) at room temperature for 15 min in equilibration buffers I and II (Bio-Rad Laboratories) and the second dimension was run on 8–18 % SDS-PAGE gradient gel with Protean II xi system (Bio-Rad Laboratories) at 30 mA/gel. The 2DE gel images from gels stained with Coomassie Blue G 250 were captured with the Chemi Doc EQ system (Bio-Rad Laboratories) and analyzed using PD-Quest 7.4.0 software (Bio-Rad Laboratories).
The relative volume of each spot in a gel was quantitatively determined after background subtraction and normalization as a percentage of the total volume of all spots detected on the gel.

5.1.4.4. Identification of different abundant proteins with Mass Spectrometry

Spots displaying statistically significant differences among dietary treatments and storage time were identified following the protocol described in Chapter 4.1.3.5.

5.1.5. Statistical Analysis

Myofibrillar fragmentation index, total collagen content, SDS-PAGE band percentage, western blot band intensity, and 2DE spots' volume were analysed using the GLM procedure of the SAS statistical software (SAS Institute, 2013) including as fixed effect the dietary treatment, the storage time and

their interaction (diet x storage). All effects were tested for statistical significance set at $P < 0.05$, and when significant effects were found, Fisher's LSD test was used for comparison.

The main tendencies in variation between the two dietary groups during storage time were determined with a Principal Component Analysis (PCA) performed using XLSTAT 2021, 1.2.2 software. The PCA was executed on 11 variables matrix (MFI, myosin heavy chain, α-actinin, desmin, actin, troponin T, 30 kDa fragments, troponin I, troponin C, tropomyosin, myosin light chain 1, and myosin light chain 2) to study the relationships among the meat quality traits and the differential proteins along the post-mortem storage within the two dietary treatments.

5.2. RESULTS AND DISCUSSIONS

5.2.1. Myofibrillar fragmentation index (MFI) and total collagen content of lamb meat

After purchase, meat organoleptic quality, mainly tenderness, represents the traits that mostly affect the level of consumer satisfaction. Ultrastructural changes that compromise the integrity of the myofibers in the muscle tissue are mainly responsible for meat tenderization. MFI could be considered a useful assay for assessing changes in muscle tissue (Aroeira, et al. 2020) and the level of myofibril fragmentation.

In our study, MFI values was significantly influenced by dietary treatment ($P < 0.001$) as illustrated in **Figure 16**. Meat from the H group showed, except for day 1, higher MFI values compared to the C group. During storage, MFI values increased significantly in both groups, but with different rates. Particularly, in meat from lambs fed hazelnut skins, myofibrillar fragmentation increased significantly

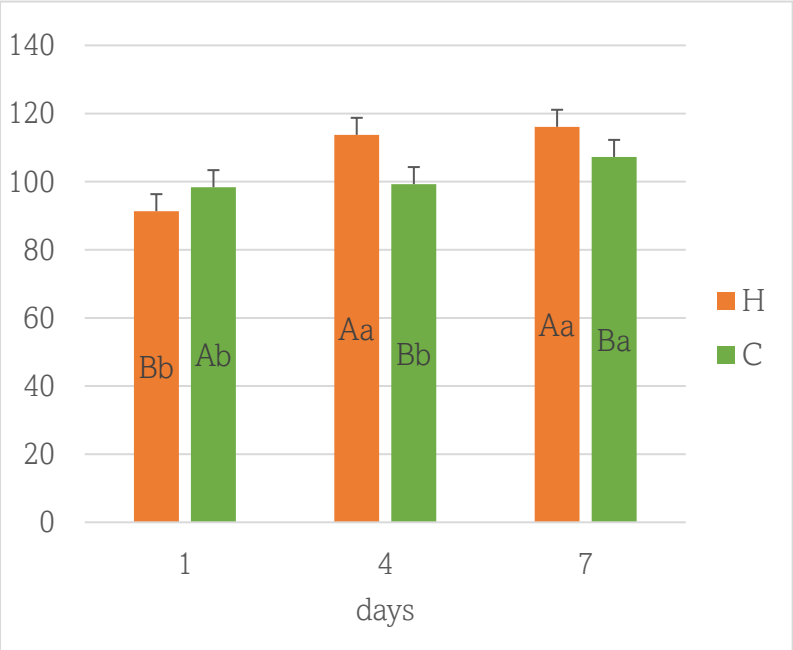


Figure 16. Myofibrillar fragmentation index (MFI) of *longissimus thoracis et lumborum* muscle of lambs as affected by different diet treatments (H= hazelnut; C= control) and storage time. Different letters: A, B= $P < 0.05$ (diet effect), a, b, c= $P < 0.05$ (storage effect). *= $P < 0.05$; **=$P < 0.01$; ***=$P < 0.001$.

by 24 % after 4 days remaining stable afterward, whereas meat from the C group began to exhibit signs of myofibrillar fragmentation (accounted only for 8 %) after 7 days of storage. These changes are in line to the body of knowledge stating that the major changes in the myofibrillar proteins concur in the tenderization period of raw meat (Huff-Lonergan, et al. 2010).

In this study, meat from lambs fed hazelnut skins evidenced a major and more rapid increase of MFI during storage compared to the control, suggesting the functional role of dietary supplementation with natural antioxidants on the endogenous enzymatic activity which in turn led to a major breakdown of the muscle protein structures. Zhao et al. (2018) reported an improvement in meat tenderness of lamb receiving dietary supplementation of wine grape pomace, a rich source of polyphenols. We can suppose that diet rich in polyphenols may have a strong impact on promoting less oxidation of metabolic enzymes involved in protein degradation such as calpains, cathepsins, proteasomes, and caspases (Lana, & Zolla, 2016) thus increasing their functionality and enhancing meat tenderness (Huff-Lonergan, & Lonergan, 2005).

The current study, however, revealed that the dietary treatment with hazelnut skin by-product (at the amount we used) is unable to affect the collagen content of the muscle. No significant effects were observed in the total collagen content values among the two dietary treatments, characterized by values usually observed in stored lamb meat. Values of total collagen content were 3.70±0.24, 4.03±0.24, 4.19±0.24 and 4.62±0.24, 4.12±0.24, 4.17±0.24 mg/g at 1, 4, and 7 d in C and H groups, respectively. Although collagen content varies along with several factors mainly animal age and muscle type, the present data demonstrated that nutrition is unlikely to affect the collagen content of muscle and other body structures in such a short period of time (Weston, et al., 2002). Furthermore, in accordance with Taylor (2004), Listrat et al. (2020a), and Listrat et al. (2020b), our evidence would suggest that collagen has minor significance in determining the texture of the *longissimus* muscle of young animals due to its high solubility after cooking and reduced amounts in this muscle compared for instance to *semitendinosus* muscle.

5.2.2. Changes in myofibrillar profile of lamb meat

SDS-PAGE was employed to investigate the effect of different dietary treatments and storage time on myofibrillar proteins profile from *longissimus thoracis et lumborum* muscle of lambs (**Fig. 17**). Band were identified, starting from the positive electrode, as myosin heavy chain (MYH), α-actinin (ACTN), desmin (DES), actin (ACTA1), troponin T (TNNT), tropomyosin (TPM), myosin light chains 1 (MYL1), troponin I (TNNI), troponin C (TNNC), and myosin light chains 2 (MYL2).

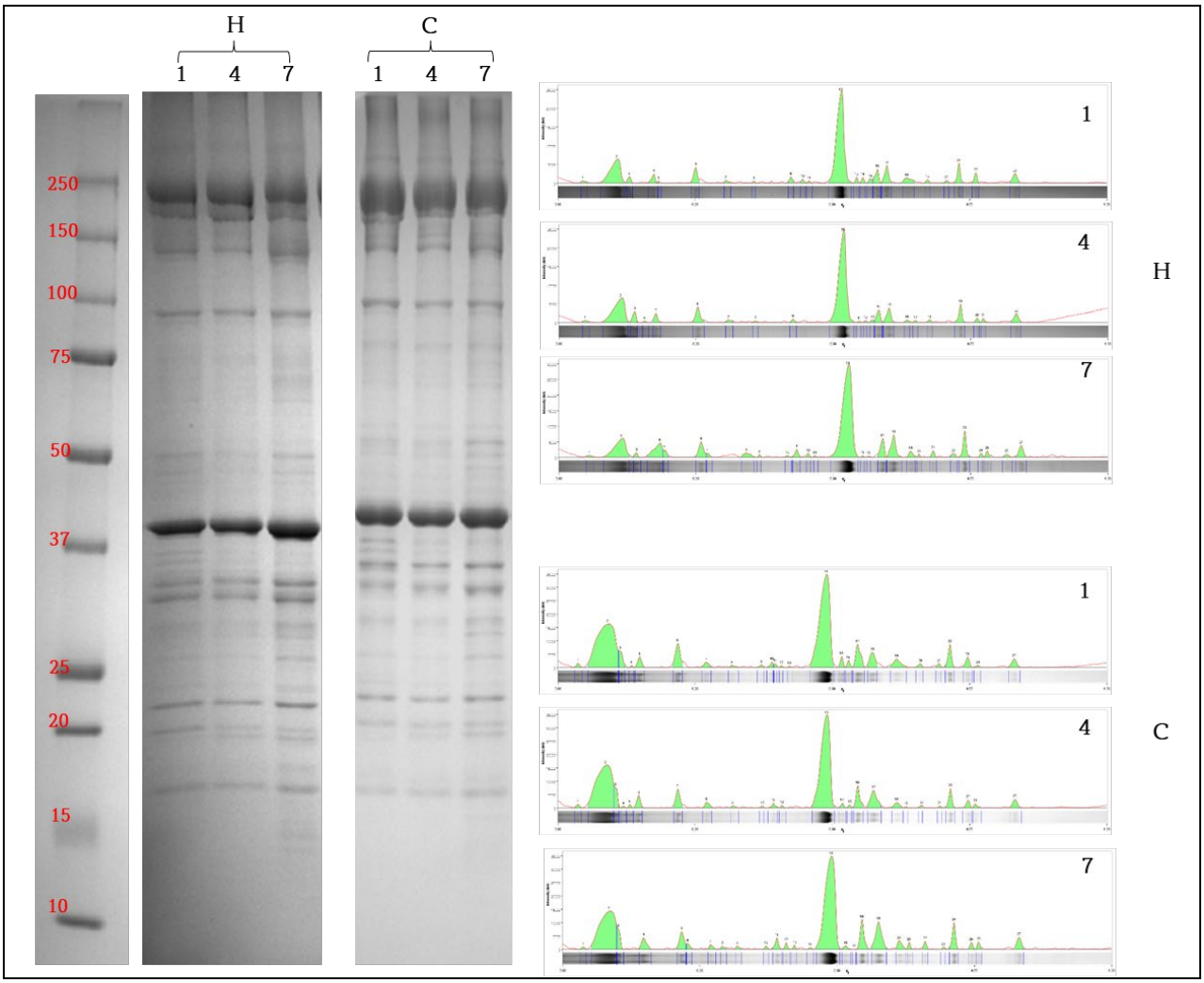


Figure 17. SDS-PAGE of myofibrillar fractions and densitometric profile from *longissimus thoracis et lumborum* muscle of lambs fed with different dietary treatment (H= hazelnut; C= control) after 1, 4 and 7 days of storage.

The relative quantity of the significant myofibrillar bands (**Fig. 18**) revealed that the myofibrillar profile was visibly affected by the dietary treatments and storage time. The meat of lambs fed hazelnut skin showed lower values of several structural proteins. Particularly, meat from the H group showed lower values of myosin heavy chain after 7 days of storage ($P < 0.001$), while desmin ($P < 0.01$) and troponin T ($P < 0.001$) exhibited the lowest intensity after 4 days of storage. In addition, meat from lambs fed hazelnut skin was characterized by the highest intensity of 30 kDa fragment ($P < 0.01$) during all storage time compared to the control group. It is well established that variations in the degree of post-mortem improvement in texture can be reflected by changes in the amounts of the major structural proteins (Gagaoua, et al., 2021a). Particularly, MYH, ACTA1, and DES, as significant components of myofibril thick filaments, were crucial in defining the texture of muscle, especially because they are the first proteins to be degraded by muscle proteases (Ouali, et al., 2013).

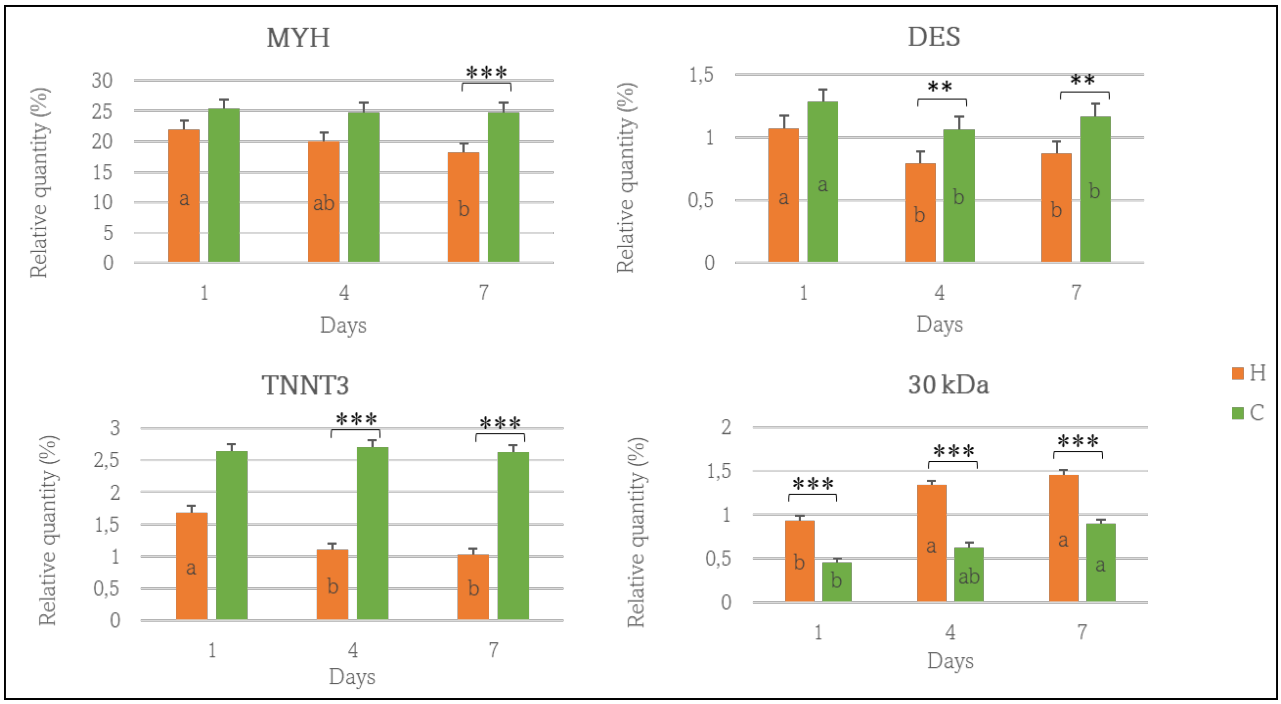


Figure 18. Relative quantity (%) of myofibrillar proteins (MYH= Myosin heavy chain, DES= Desmin, TNNT3= Troponin T, 30 kDa= 30 kDa fragments) of *longissimus thoracis et lumborum* muscle from lambs as affected by different dietary treatment (H= hazelnut; C= control) and storage time. Different letters: a, b, c= P< 0.05 (storage effect). *= P<0.05; **=P<0.01; ***=P<0.001

During storage time, the percentage of band density revealed for MYH, DES, and TNNT3 ($P < 0.001$) a reduction respectively of 17.2 %, 18.9 %, 38.8 % in the H group, and of 2.2 %, 8.9 %, 0.6 % in the control. Particularly, meat from the H group showed a gradual decrease of MYH ($P < 0.05$) during storage time, while a decrease of TNNT3 ($P < 0.01$) was observed after 4 days of storage. Refers to desmin, a significant decrease ($P < 0.05$) was found after 4 days of storage in meat from both, the control and hazelnut groups.

The percentage of 30 kDa fragments was differently influenced by the dietary treatment and storage time in the experimental groups. In both C and H groups, an increase during storage time was observed for the 30 kDa band, but with different rates; the H group showed an increase in the amount of 30 kDa fragments after 4 days of storage remaining constant thereafter ($P < 0.05$) whereas, in the control group an increase was observed only at 7 d of storage. The 30 kDa fragment has long been identified as a meat tenderization marker and it was commonly considered to be a troponin T breakdown product. Recently, Gagaoua, Troy, & Mullen, (2020) demonstrated that the content of fragments from 30-32 kDa may also contain several other protein fragments, such as actin (ACTA1), fast troponin T (TNNT1), and myosin light chain 1 (MYL1) among others. Overall, the greater degradation of myofibril proteins together with the major presence of the 30 kDa band fragments in

meat from the H group evidenced the possible role of diet in modulating post-mortem degradation of myofibrillar proteins.

Western blotting analyses (**Fig. 19a**) confirmed the changes found in the SDS-PAGE profile of DES. Desmin immunoreactive bands were detected at 54 kDa (intact form), while degraded forms were found at 46, 45, 40, and 37 kDa. Image analysis results revealed significant differences due to dietary treatment and storage time. Referring to dietary treatment, meat from lambs fed hazelnut skin showed lower values of 54 kDa desmin intact band ($P < 0.01$) after 4 and 7 days together with the highest values of 46 kDa and 45 kDa ($P < 0.001$, on day 4, and 7) fragments. On the contrary, meat from the control group showed the highest values of 40 kDa ($P < 0.01$, on days 4, and 7).

Due to storage time, a decrease in the 54 kDa intact band ($P < 0.001$) was found for both, the control and hazelnut groups but with different rates. A progressive decrease of intact desmin was observed in meat from lambs fed hazelnut skin throughout storage time, while, in meat from the control group a decrease was found only after 4 days of storage and remained constant thereafter.

Refers to degradation products, an increase of 46 kDa band ($P < 0.05$) after 4 days of storage was found in meat from the hazelnut group, while a progressive increase during storage was found for the 45 kDa fragments ($P < 0.001$). Conversely, only meat from the control group showed an increase

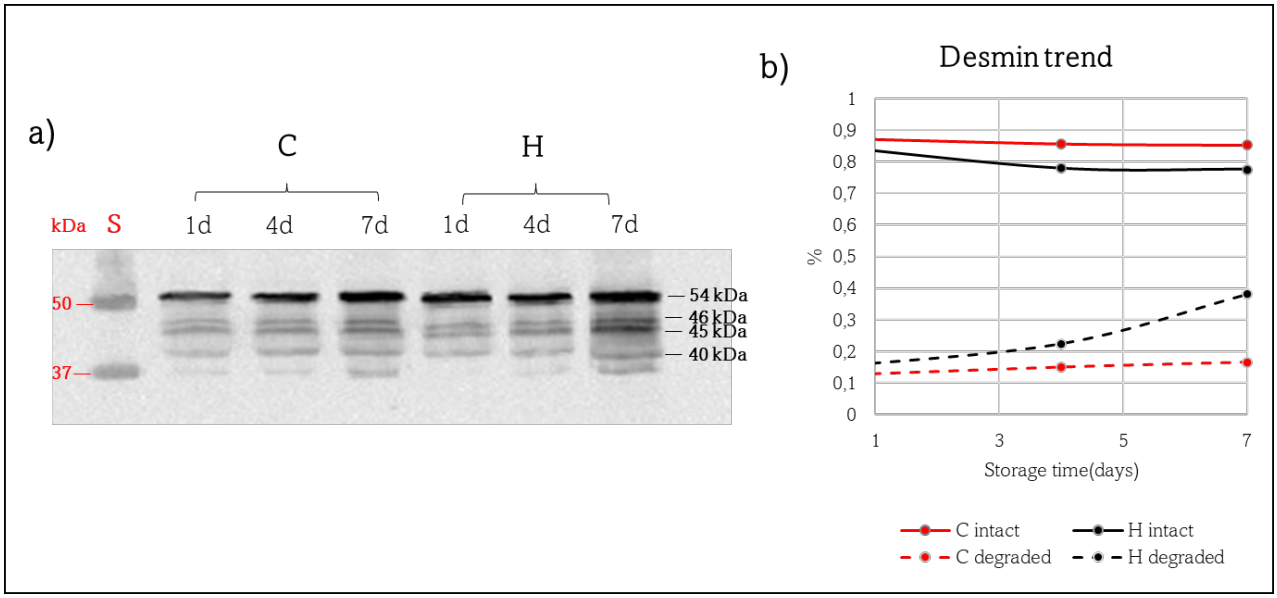


Figure 19. Representative Western blot of (a) Desmin isoforms (54 kDa: intact forms, and 46-37 kDa: degraded forms) and (b) image analysis results representing Desmin degradation trend (formation of degraded forms/total signal) from myofibrillar proteins of *longissimus thoracis et lumborum* muscle of lambs fed with different dietary treatment (H= hazelnut; C= control) after 1, 4 and 7 days of storage.

of the 40 kDa band ($P < 0.05$) after 4 days of storage, while, in meat from both groups an increase of the 37 kDa band was observed at the end of storage time.

Desmin is a major intermediate filament protein essential for muscle structure integrity. It represents the most susceptible substrates of calpains (Huff-Lonergan, et al., 1996) and one of the first proteins to be degraded 24 hours post-mortem (Huff-Lonergan, & Lonergan, 1999; Koohmaraie, 1992). Accordingly, Starkey et al. (2015) observed an improvement in shear force as a result of desmin degradation starting from 1 day in lamb meat. Rowe et al., (2004) found an early production of protein breakdown products in meat from steers receiving Vitamin E dietary supplementation compared to the control demonstrating that post-mortem oxidation could interfere with the tenderization process by reducing calpain activity and delaying the rate of proteolysis in meat. In this study, the rapid decline of the desmin intact form in the meat of the H group confirms that hazelnut skins might have a role in delaying muscle oxidation process thus influencing the rate of autolysis and subsequent activation of μ-calpain.

Western blot analysis was also employed to investigate the differences found during storage time in TNNT3 proteins among the two dietary groups (**Fig. 20a**).

Immunoblot of troponin T showed the presence of 8 immunoreactive bands (37, 36, and 34 kDa, isoforms of the intact protein; 32, 31, 30, 28, and 25 kDa bands, degradation products of TNNT3).

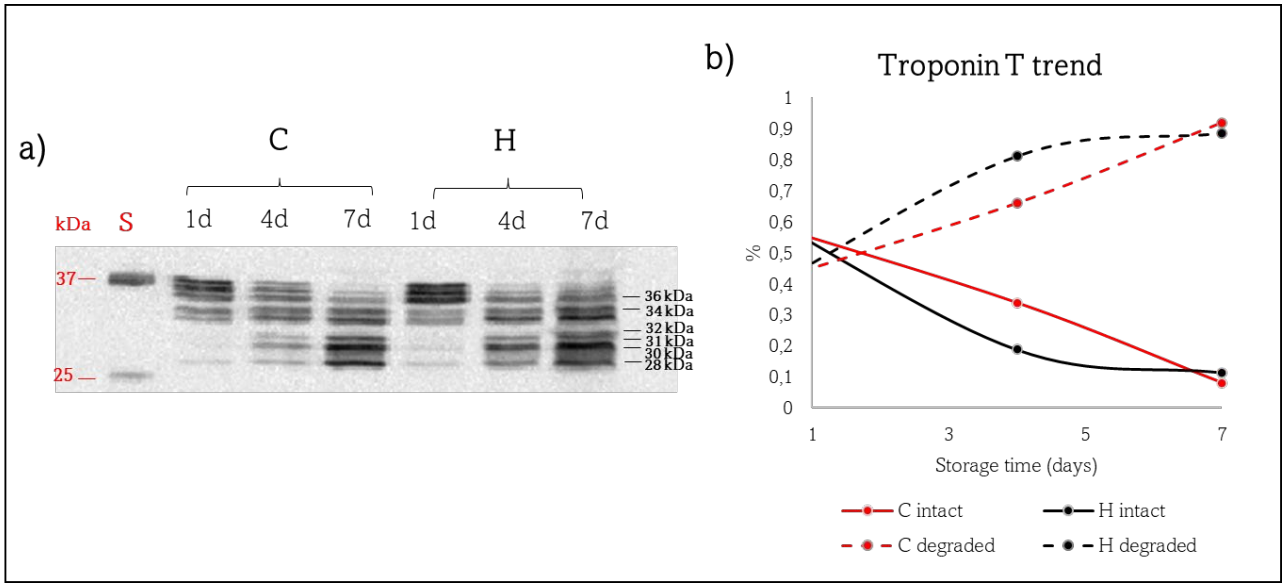


Figure 20. Representative Western blot of (a) Troponin T isoformsm (37-34 kDa: intact forms, and 33-25 kDa: degraded forms) and (b) image analysis results representing Troponin T degradation trend (formation of degraded forms/total signal) from the myofibrillar proteins of *longissimus thoracis et lumborum* muscle of lambs fed with different dietary treatments (H= hazelnut; C= control) after 1, 4 and 7 days of storage.

The differences between the two groups emerged clearly in the image analysis (**Fig. 20b**) with dietary hazelnut supplementation revealing a stronger impact on the rate of TNNT3 breakdown during storage time. Meat from the hazelnut group showed the lowest values of the intact band at 37 kDa ($P < 0.001$, days 1 and 4) and the highest percentage of the bands at 36 ($P < 0.001$) and 34 kDa ($P < 0.001$) only on day 1; at the same time lower values were observed for the 32 ($P < 0.01$, on day 1, and 7) and 31 kDa ($P < 0.01$, on day 1, and 4) degradation forms. In meat from the control instead, the highest values of 37 and 36 kDa ($P < 0.001$) intact band and 30, 28 ($P < 0.001$), and 25 kDa ($P < 0.01$) degraded form were detected on day 4.

Storage time also affected the troponin T degradation; in particular, a decrease in intact isoforms band at 37, 36 ($P < 0.001$), and 34 ($P < 0.01$) kDa occurred in meat from lambs fed hazelnut skin after 4 days of storage and then remained constant. Conversely, in meat from the control group, the percentage of the intact band at 37 kDa progressively decreased during storage time, while a reduction of 36 and 34 kDa band was detected after 4 and 1 day of storage, respectively.

Referring to degradation products, an increase of 32 kDa band ($P < 0.05$) after 4 days of storage was observed only in meat from the hazelnut group; while the degraded band at 31, 30, 28, and 25 kDa increased in both groups but with different rates. A progressive increase of 31 kDa ($P < 0.01$) band was observed in meat from lambs fed hazelnut skin during storage time, while, in meat from the control group an increase was found only after 4 days of storage remaining then constant. Also, in meat from the control group, 30 ($P < 0.001$) and 28 kDa ($P < 0.01$) degraded bands started progressively to increase after 4 days of storage, whereas the band of 25 kDa ($P < 0.01$) increased towards the end of the storage time. Meat from group H, on the other hand, showed an increase in the band of 30, 28, and 25 kDa after 4 days of storage. However, it is important to note that at the end of storage time, the amount of TNNT3 degradation products in the meat from both groups was comparable (92.00 vs 88.60 in C and H, respectively) evidencing that antioxidant molecules contained in hazelnut skin can affect the kinetics of the proteolytic processes.

These findings supported the greater myofibrillar fragmentation observed in the H group at 4 days and confirm that the dietary inclusion of tannins can influence the post-mortem processes through a protective impact on the development of oxidative phenomena. Indeed, the oxidative conditions are responsible for physical and chemical changes in muscle protein properties, including conformation, aggregation, and solubility, that can reduce the protein susceptibility to proteolysis (Huff-Lonergan, et al., 2010; Zhang, et al., 2013). Collectively, data on desmin and TNNT3 fractions together with levels of 30 kDa fragments suggest the role of antioxidant compounds present in hazelnut skin in enhancing tenderization processes in lamb meat during storage.

Finally, for a better understanding of the above results, a PCA was performed using myofibrils fragmentation index and the changes of myofibrillar muscle proteome (**Fig. 21**). The bi-plot accounted for 42.81 % of the total variance, with 27.04 % of the total variability explained by PC1 and 15.77% explained by PC2. MFI and 30 kDa fragment were the factors positively related to the PC1, whereas myosin heavy chain (MYH) and troponin T (TNNT3) were the factors negatively related to the first principal component. Desmin (DES), troponin I (TNNI), and actin (ACTA1) were the main contributing variables along with PC2, with actin negatively related to the principal component. The score plot differentiated the dietary treatment in different clusters that moved along the first principal component across storage time. The PCA and the used protein biomarkers comforted the results discussed above, showing a better improvement in the texture of meat from lambs fed hazelnut skin by-product compared to the controls.

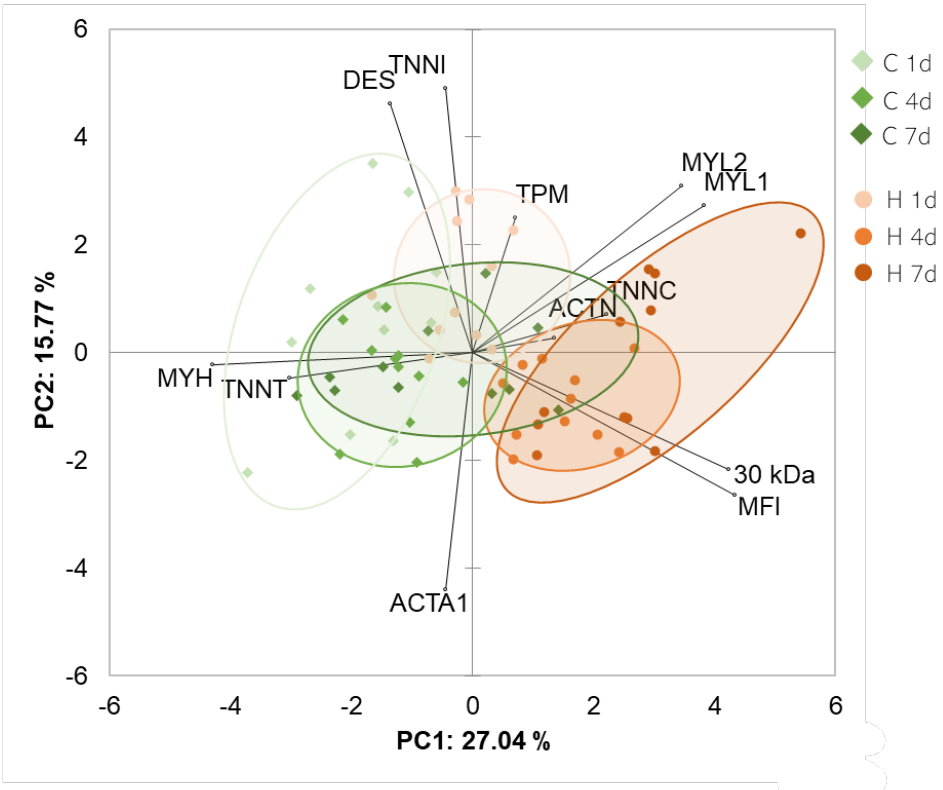


Figure 21. Principal component analysis (PCA) of myofibrillar fragmentation index (MFI) and protein band percentage (MYH = myosin heavy chain, ACTN= α-actinin, DES = desmin ACTA1 = actin, TNNT = troponin T, TPM = tropomyosin, 30 kDa= 30 kDa fragments, MYL1 = myosin light chain 1, TNNI= troponin I, TNNC= troponin C, MYL2 = myosin light chain 2) of *longissimus thoracis et lumborum* lamb muscles as affected by different dietary treatment (H= hazelnut; C= control) and storage time (1, 4 and 7 days).

5.2.3. Two-dimensional electrophoresis (2DE) of myofibrillar proteins

Figure 22 depict the 2DE gel maps of myofibrillar protein fractions from *longissimus thoracis et lumborum* muscle of lambs fed with different dietary treatments after 1, 4, and 7 days of storage.

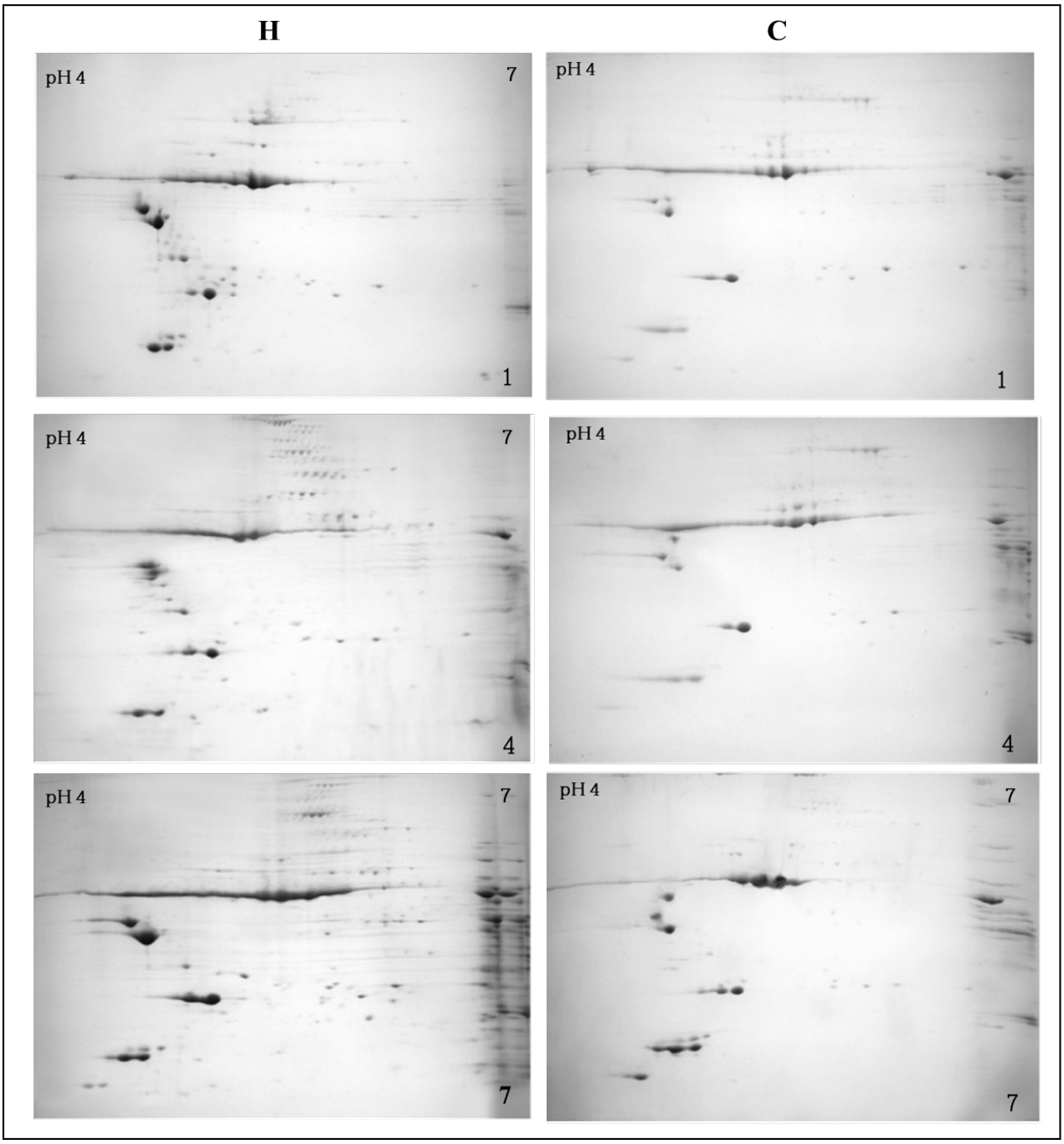


Figure 22. 2DE of myofibrillar fractions from *longissimus thoracis et lumborum* muscle of lambs fed with different dietary treatment (H= hazelnut; C= control) after 1, 4 and 7 days of storage.

The most representative proteins found in all gel maps were ACTA1, TPM1, TPM2, myosin light chain 1 (MYL1), fast skeletal myosin light chain 2 (MYLPF), myosin light chain 1/3 (MYL1), and TNNT3.

On day 1 the number of spots in the C group was 136 compared to 196 spots found in group H; after 7 days of storage, the number of spots increased respectively to 190 and 318, with a percentage change of 36 and 62 % (**Fig. 23**) in C and H group, respectively.

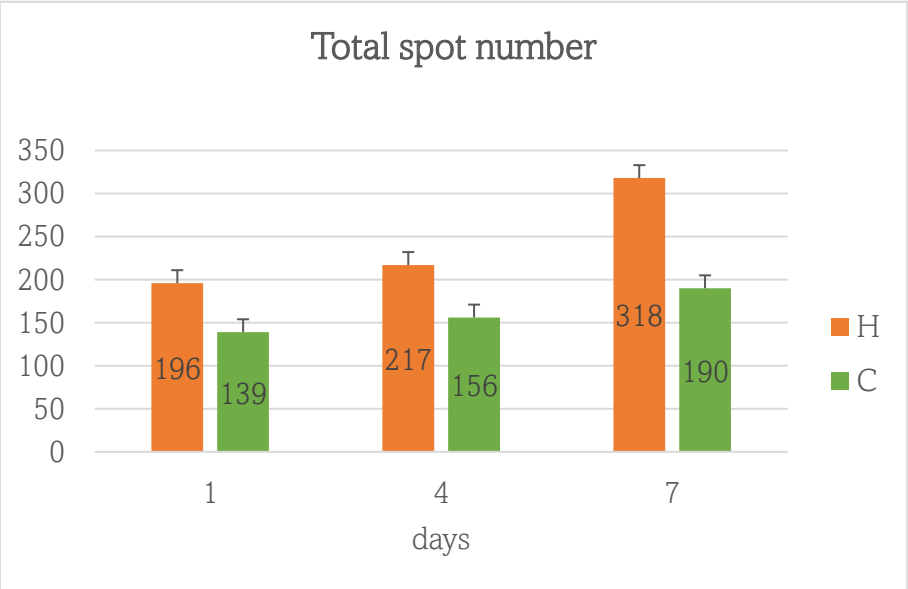


Figure 23. Changes of spot number in myofibrillar fraction of *longissimus thoracis et lumborum* muscle from lambs as affected by different dietary supplementation (H= hazelnut; C= control) after 1, 4 and 7 days of storage.

The meat of lambs of group H showed a major abundance of MYH and TNNT3 degradation fragments in the basic part of the gel (pH 7).

The 2DE image analysis allowed an accurate quantification of the different myosin light chain isoforms (**Fig. 24**).

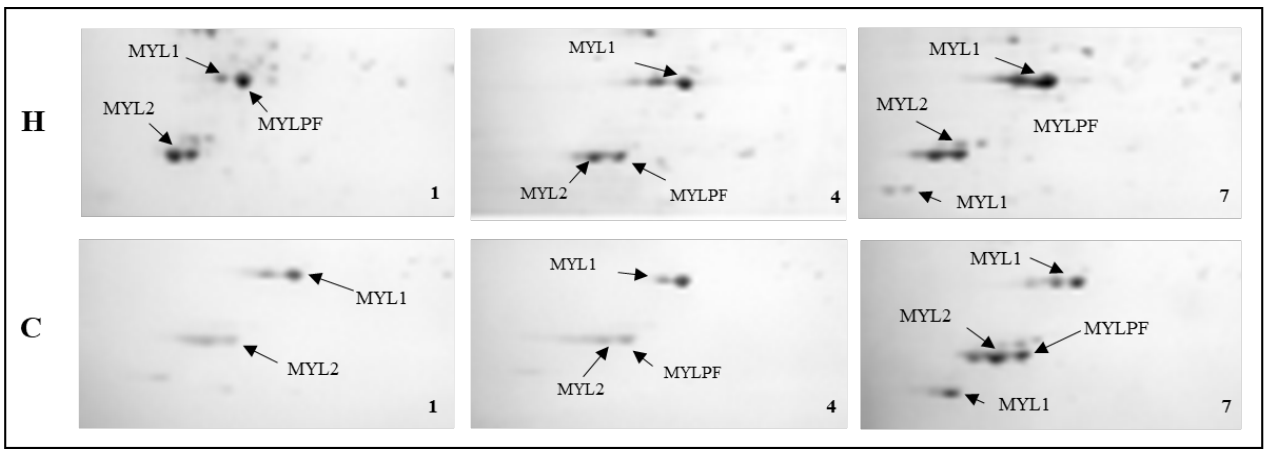


Figure 24. Myosin light chains isoforms (Myosin light chain 1, Fast skeletal myosin light chain 2, Myosin light chain 1/2) of *longissimus thoracis et lumborum* muscle from lambs as affected by different dietary supplementation (H= hazelnut; C= control) after 1, 4 and 7 days of storage.

Meat from lambs fed hazelnut skins displayed more MYL spot isoforms compared to the control at 1 and 4 days of storage, whereas, after 7 days of storage, both groups showed a similar profile. The

presence of several spot isoforms ascribed to MYL starting from 1 day observed in the H group confirmed the greater proteolysis in line with the myofibrillar fragmentation data and degradation of key structural proteins discussed above. In particular, it could be hypothesized that the weakening of the actomyosin complex by muscle proteases may result in greater extractability and release of MYL isoforms.

A total of 13 protein spots significantly changed in intensity and number due to dietary treatment with hazelnut skin after 7 days of storage and were cut out from the preparative gel and identified by LC-MS/MS analysis. The protein subjected to identification is listed in **Table 5** and is annotated in the 2DE gel reference map (**Fig. 25**). From the 13 spots, 44 unique proteins were successfully identified based on the homology to *Ovis Aries*.

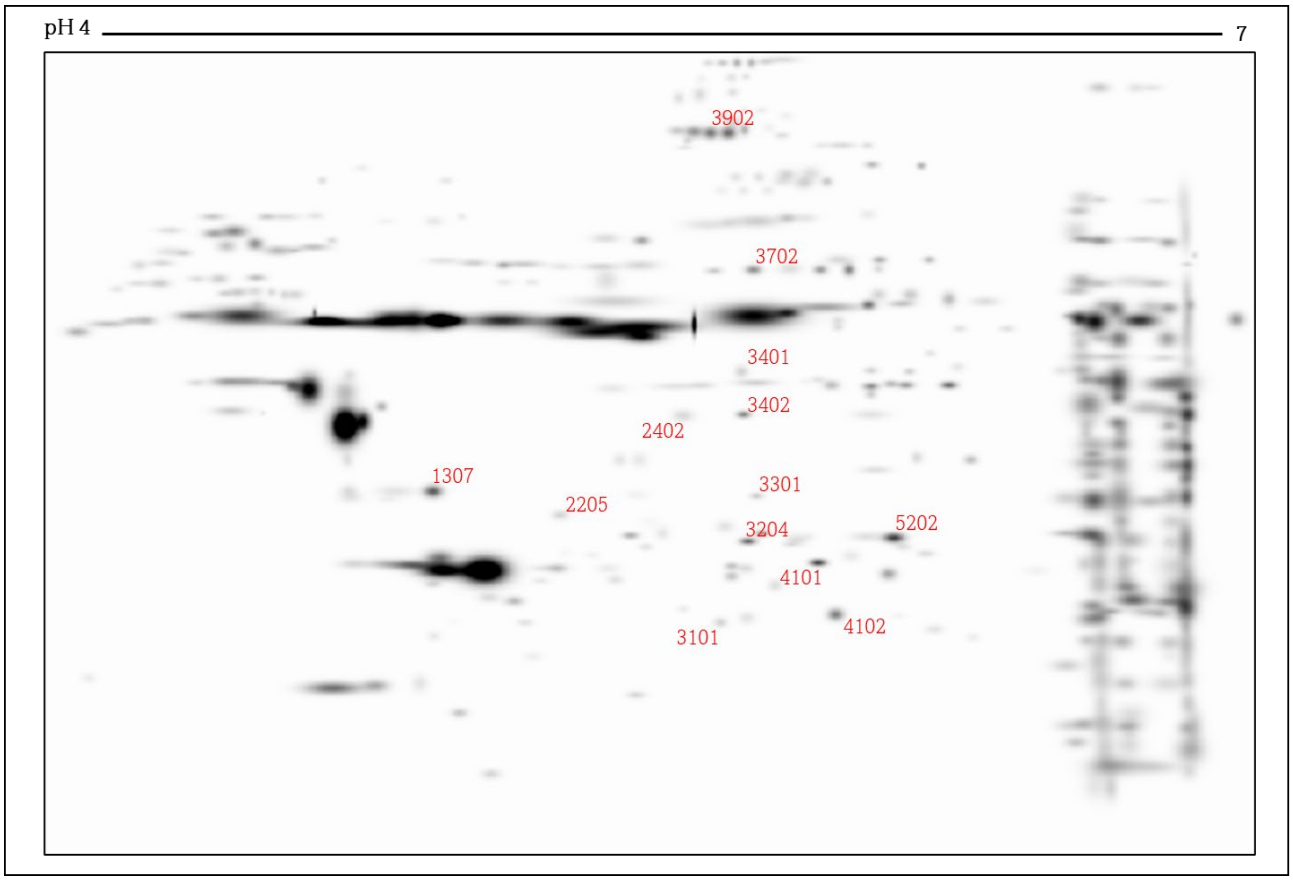


Figure 25. Representative 2DE gel map of the myofibrillar fraction highlighting the identified protein spots in Table 5.

Table 5

Identified proteins by 2DE gels as different in the myofibrillar muscle proteome to be affected by dietary treatment.

Spot number	Identified protein	Gene name	Uniprot ID	MW [kDa]	calc. pI	Score	Seq. Coverage	Matched peptides
1307	Actin, alpha cardiac muscle 1	ACTC1	A0A6P3E6H9	42,0	5,39	2020,96	35,28	11
	Desmin	DES	W5QG29	53,5	5,27	239,71	21,49	9
	Troponin T, fast skeletal muscle	TNNT3	W5NRC7	25,6	10,14	234,85	23,32	5
	Myosin light chain 3	MYL3	A0A0U1Z4T4	21,9	5,07	227,31	26,63	5
	14-3-3 protein gamma	YWHAG	A0A6P7DFS2	28,3	4,89	147,55	20,65	5
	ATP synthase subunit d, mitochondrial	ATP5PD	W5PP37	18,7	6,24	90,06	24,22	3
2205	Heat shock 27 kDa protein	HSPB1	A0A6P7DEW2	22,3	6,70	1075,41	33,83	10
	Actin, alpha skeletal muscle isoform X4	ACTA1	A0A6P3ERW2	42,0	5,39	373,86	32,10	10
	Desmin	DES	W5QG29	53,5	5,27	352,97	20,21	8
	F-actin-capping protein subunit beta	CAPZB	A0A6P7DRJ9	31,3	5,58	282,77	28,52	7
	Myosin light chain 3	MYL3	A0A0U1Z4T4	21,9	5,07	230,00	28,14	6
	Troponin C, skeletal muscle	TNNC2	A0A6P7ELW5	18,1	4,20	139,78	28,13	3
2402	F-actin-capping protein subunit beta	CAPZB	A0A6P7DRJ9	31,3	5,58	813,53	39,35	10
3101	Troponin C, skeletal muscle	TNNC2	A0A6P7ELW5	18,1	4,20	319,90	49,38	6
3204	Heat shock 27 kDa protein	HSPB1	A0A6P7DEW2	22,3	6,70	2138,36	33,83	9
	Actin, alpha skeletal muscle isoform X4	ACTA1	A0A6P3ERW2	42,0	5,39	493,32	25,73	8
	Myosin light chain 3	MYL3	A0A0U1Z4T4	21,9	5,07	226,74	33,17	6
3301	Actin, alpha skeletal muscle isoform X4	ACTA1	A0A6P3ERW2	42,0	5,39	1218,74	61,27	9
	Actg1	ACTG1	A0A3R5SS76	41,8	5,48	496,89	33,33	4
3401	actin, alpha skeletal muscle isoform X4	ACTA1	A0A6P3ERW2	42,0	5,39	2537,57	55,97	10
	Actg1	ACTG1	A0A3R5SS76	41,8	5,48	1143,25	35,20	4
	Eukaryotic translation initiation factor 3 subunit I	EIF3I	A0A6P3E6N2	36,4	5,64	530,79	37,23	12
	Pyruvate dehydrogenase E1 component subunit beta	PDHB	A0A6P7D5G1	39,1	6,44	305,51	20,89	6
	Tropomyosin 2	TPM2	W5PQL7	32,8	4,70	267,68	25,70	7

3402	Pyruvate dehydrogenase E1 component subunit beta	PDHB	A0A6P7D5G1	39,1	6,44	1259,40	21,73	7
	F-actin-capping protein subunit alpha	CAPZA2	A0A6P3EBW2	33,0	5,85	651,52	44,41	8
	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	GNB2	A0A6P7DFT3	37,3	6,00	621,63	32,06	3
	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GNB1	A0A6P7EKA8	37,4	6,00	557,22	32,35	4
	Troponin T, fast skeletal muscle	TNNT3	W5NRC7	25,6	10,14	519,86	32,74	9
	Heat shock 27 kDa protein	HSPB1	A0A6P7DEW2	22,3	6,70	490,75	25,37	6
	Tropomyosin alpha-1 chain	TPM1	A0A6P9FRC8	32,7	4,74	342,44	23,59	8
	Dimethylargininase	DDAH1	A0A6P3E5A7	31,2	6,01	250,79	25,96	6
3702	Cytochrome b-c1 complex subunit 1, mitochondrial	UQCRC1	A0A6P3THK9	52,8	6,32	1660,49	27,29	13
	Actin, alpha skeletal muscle isoform X4	ACTA1	A0A6P3ERW2	42,0	5,39	1166,32	48,54	17
3902	Heat shock 70 kDa protein	HSP70	A0A5A4U680	70,2	5,92	4375,53	53,67	29
	Albumin	ALB	W5PWE9	69,3	6,15	2486,52	58,95	38
4101	ATP synthase subunit d, mitochondrial	ATP5PD	W5PP37	18,7	6,24	314,64	24,22	3
	DNA-directed RNA polymerase II subunit RPB7	POLR2G	A0A6P9FRD6	19,2	5,54	204,98	29,65	4
4102	Heat shock protein beta-7	HSPB7	A0A6P7DS42	18,8	5,96	208,79	22,54	3
	Troponin C2, fast skeletal type	TNNC2	W5P9C1	16,2	4,30	153,03	26,35	3
	Heat shock protein beta-6	HSPB6	A0A6P7ESN6	17,5	6,40	87,08	45,73	4
5202	Heat shock 27 kDa protein	HSPB1	A0A6P7DEW2	22,3	6,70	2261,60	33,83	9
	Metaxin-2 isoform X2	MTX2	A0A6P3T8X3	24,4	7,40	158,20	20,47	4
	Myosin light chain 3	MYL3	A0A0U1Z4T4	21,9	5,07	156,34	24,12	4

CHAPTER 6

In-depth characterization of different abundant spots in the proteome of lambs fed with hazelnut skin using bioinformatics

6.1. Recent advancements in proteomic technologies

In the last years, proteomic-based techniques applied in meat science have greatly increased our knowledge in this field. From the investigation of dynamic changes occurring in post-mortem muscle through new analytical techniques several biological pathways involved in the development of meat quality traits, including energy metabolism, oxidative stress, proteolysis, and apoptosis have been identified. Particularly, bioinformatics tools have allowed to understand the role of proteins on the main biological pathways and to discover and confirm biomarkers correlated with different meat quality parameters (Huang, et al., 2020; Gagaoua, & Picard, 2022; Gagaoua, et al., 2020).

6.2. Bioinformatic analysis of different abundant spots

To further our understanding on the underlying mechanisms related to the impact of supplementation with hazelnut skin by-product on the color stability and texture improvement of lamb meat, we targeted the sarcoplasmic and myofibrillar proteome changes using a classical proteomics approach.

As reported in **Table 4** and **5** (annotation in **Fig. 14** and **Fig. 25**) the LC−MS/MS yielded a total of:

- 41 proteoforms from the 10 different abundant spots of the *sarcoplasmic fraction,*
- 44 proteoforms from the 13 different abundant spots of the *myofibrillar fraction*.

As suggested by Gagaoua et al. (2021b), the ovine gene identifiers were transformed on Uniprot Retrieve/ID Mapping (https://www.uniprot.org/uploadlists) into the human orthologues EntrezGene ID to meet the GO analysis input format. Furthermore, the orthologous human EntrezGeneID generated has the advantage of retrieving a more complete Gene Ontology (GO) annotation compared to the *Ovis Aries* database.

In cells, every biological function is regulated and expressed as a result of protein interactions. Based on the protein molecular function, the web-based search **STRING** database v11.0 (Szklarczyk et al., 2019; https://string-db.org/; accessed on 23 May 2022) was employed to describe the Protein-Protein Interaction (PPI) network among the 41 and 44 proteoforms of the sarcoplasmic and myofibrillar fraction, respectively. In order to reduce the probability of false positive parameters the following default settings were applied: confidence intervals of 0.400 (medium confidence) and false discovery rate (FRD) stringency of 1 (high percent).

Subsequently, the protein human Uniprot IDs were further uploaded on **Metascape®** (https://metascape.org/, accessed on 23 May 2022) web tool to obtain an overview of the pathway and process enrichment analyses based on GO Biological Processes. The terms with the following criteria were taken into consideration: P-value 0.05, minimum count of 3, and enrichment factor >

1.5. Results from sarcoplasmic and myofibrillar fraction were presented in significant clusters hierarchically emphasized and contained the most enriched terms. Also, representative terms were visualized in the enrichment network diagram reporting each enrichment GO term as a circle node and the similarity of the node as the edge. More specifically, each circle node is colored according to the cluster ID, with size proportional to the number of input genes fall under that term, and its color represents its cluster identity (i.e., nodes of the same color belong to the same cluster).
The molecular complex detection (MCODE) method was then used to find highly linked areas in the protein interaction network.

6.3. Functional roles of differentially abundant proteins from sarcoplasmic proteome and their relevance to meat color

Bioinformatics analyses evidenced that several post-mortem pathways impact the quality characteristics of meat, and protein-protein interactions can provide additional information for a better understanding of the complex post-mortem mechanisms in which the proteins are involved and their interconnectedness.
The network of interacting proteins generated using the STRING database (**Fig. 26**) identified 41 nodes (proteins) in biological interactions and 149 edges (interactions) and evidenced that three major biological pathways contribute the biological mechanisms, these being:
i) *central carbon biosynthesis* (n = 11 protein spots; ENO1, PKM, TPI1, GAPDH, HIBADH, CKM, ALDOA, PGAM2, AK1, PGM1, and PGK1);
ii) *biosynthesis* (n=7 protein spots; GSTM1, GSTM3, GSTM5, PARK7, PRDX1, PRDX2, and PRDX6);
iii) *immune and endocrine system* (n= 4 protein spots; FABP3, ADIPOQ, APOA1, SERPINA1).
It is worthy to note that the central carbon biosynthesis and biosynthesis pathways had close interactions with each other and with the heat shock family proteins (HSPB1 and HSPD1). Only the 14 kDa phosphohistidine phosphatase (PHPT1), Carboxymethylenebutenolidase homolog (CMLB), 15-oxoprostaglandin 13-reductase (PTGR2) and Nuclear transport factor 2 (NUTF2) proteins showed no significant interactions with other proteins, but the large majority of proteins displayed interactions.
Interestingly, among these proteins, seven (ENO1, PKM, TPI1, GAPDH, CKM, ALDOA and AK1) were previously evidenced by proteomics to be related to lamb meat color parameters (Gao, et al. 2016; Gao, et al. 2021). Whereas, when we compare our putative biomarkers with the recent beef integromics study of Gagaoua et al. (2020), a total of 16 candidates (ENO1, PKM, TPI1, GAPDH,

HIBADH, CKM, ALDOA, PGAM2, AK1, PGM1, PGK1, PARK7, PRDX1, PRDX2, PRDX6 and FABP3) can be validated. This study, allowed then to confirm previous knowledge and further proposed for the first time 6 new proteins belonging to oxidative stress and cell redox homeostasis (GSTM1, GSTM3, GSTM5), and immune and endocrine system pathways (ADIPOQ, APOA1 and SERPINA1) as candidate biomarkers of meat color in lamb.

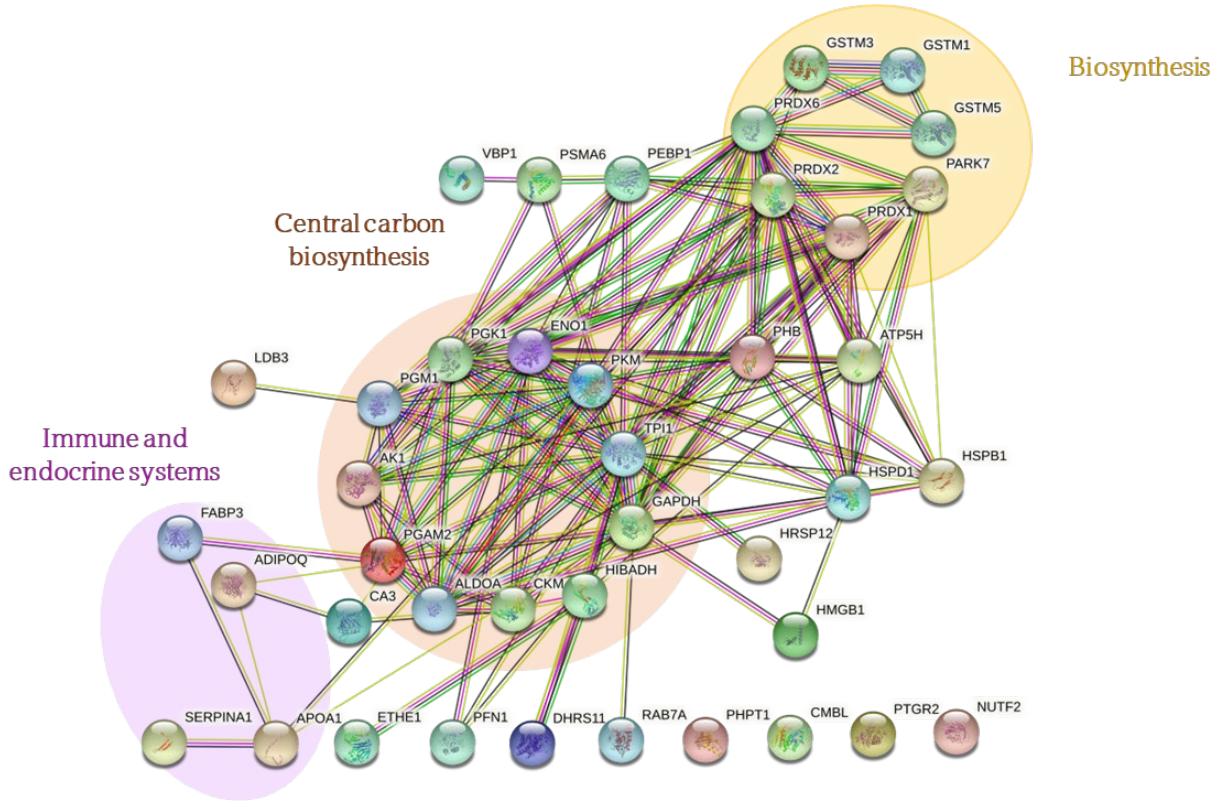


Figure 26. Protein-protein interactions of the differentially expressed proteins identified in the sarcoplasmic proteome using STRING database.

From the Metascape analysis, 15 enriched and significantly GO terms were allowed to construct process enrichment networks and pathways (**Fig. 27a**). The top 4 enriched term clusters were: "ATP metabolic process (GO:0046034)", "cellular detoxification (GO:1990748)", "protein stabilization (GO: 0050821)" and "glutathione metabolic process (GO:0006749). These were followed by "detection of oxidative stress (GO:0070994)", "positive regulation of cytokine production (GO:0001819)", "small molecule catabolic process (GO:0044282), "negative regulation of catalytic activity (GO:0043086), "response to mercury ion (GO: 0046689)", "organophosphate biosynthetic process (GO:0090407), "negative regulation of intracellular signal transduction (GO:1902532), "anatomical structure homeostasis (GO:0060249), "regulation of translation (GO:0006417)", "lipid catabolic process (GO:0016042)". The network association between the representative enrichment terms and their

functional enrichment is given in **Figure 27b**, showing the extent of enrichment of the clusters contributing to each GO term.

Three significant sub-networks of high local network connectivity (MCODE 1-3) were revealed from the protein-protein interaction network (**Fig. 27c**). The analysis generated 3 MCODEs that cluster different groups of proteins based on their "biological meanings":

i) MCODE1 grouped 5 proteins, which are PGK1, PGAM2, ENO1, GAPDH, and ALDOA for glycolytic process, ATP generation from ADP, and ADP metabolic process;

ii) MCODE2 evidence the presence of 4 proteins which are PGM1, HSPD1, DHRS11, and TPI1;

iii) MCODE3 grouped 4 proteins, which are GSTM1, GSTM3, GSTM5, and RIDA for glutathione metabolic process, cellular modified amino acid metabolic process, and sulfur compound metabolic process.

Bioinformatic results evidenced that ***glycolytic processes*** were the most important pathways related to the supplementation of lamb diets by hazelnut skins. Among the proteins identified involved in glycolytic processes, TPI1 was identified as the protein most influenced by dietary treatment due to the presence of 5 protein spots (proteoforms) in the sarcoplasmic proteome of meat from lambs fed hazelnut skin after 7 days of storage. This protein was further the top protein (13 times) retained in the regression equations built in this study to explain the measured color traits at different storage times. Triosephosphate isomerase is a key enzyme involved in energy generation for muscle cells that catalyze the conversion of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate. This study is the first to validate the robustness of TPI1 in explaining lamb meat color variation. In other species, but mainly beef, TPI1 has been suggested as a robust biomarker of several quality traits including color (Wu, et al., 2015; Wu, et al., 2016; Nair, et al., 2016; Gagaoua, Picard, & Monteils, 2018; and Gagaoua, et al., 2020; and Gagaoua, et al, 2021a). In the current research, the greater abundance of TPI1 in the LTL muscle of the hazelnut group could be due to the greater capacity of this muscle to regenerate NADH for subsequent metmyoglobin reduction, hence allowing a better color stability (Nair, et al., 2017). The identification of several TPI1 protein spots in meat from the hazelnut group, together with the lowest decrease in a* and C* values, highlight the important role of this protein in monitoring lamb meat discoloration and point out the reliability of TPI1 as a biomarker of lamb meat color variation or stability.

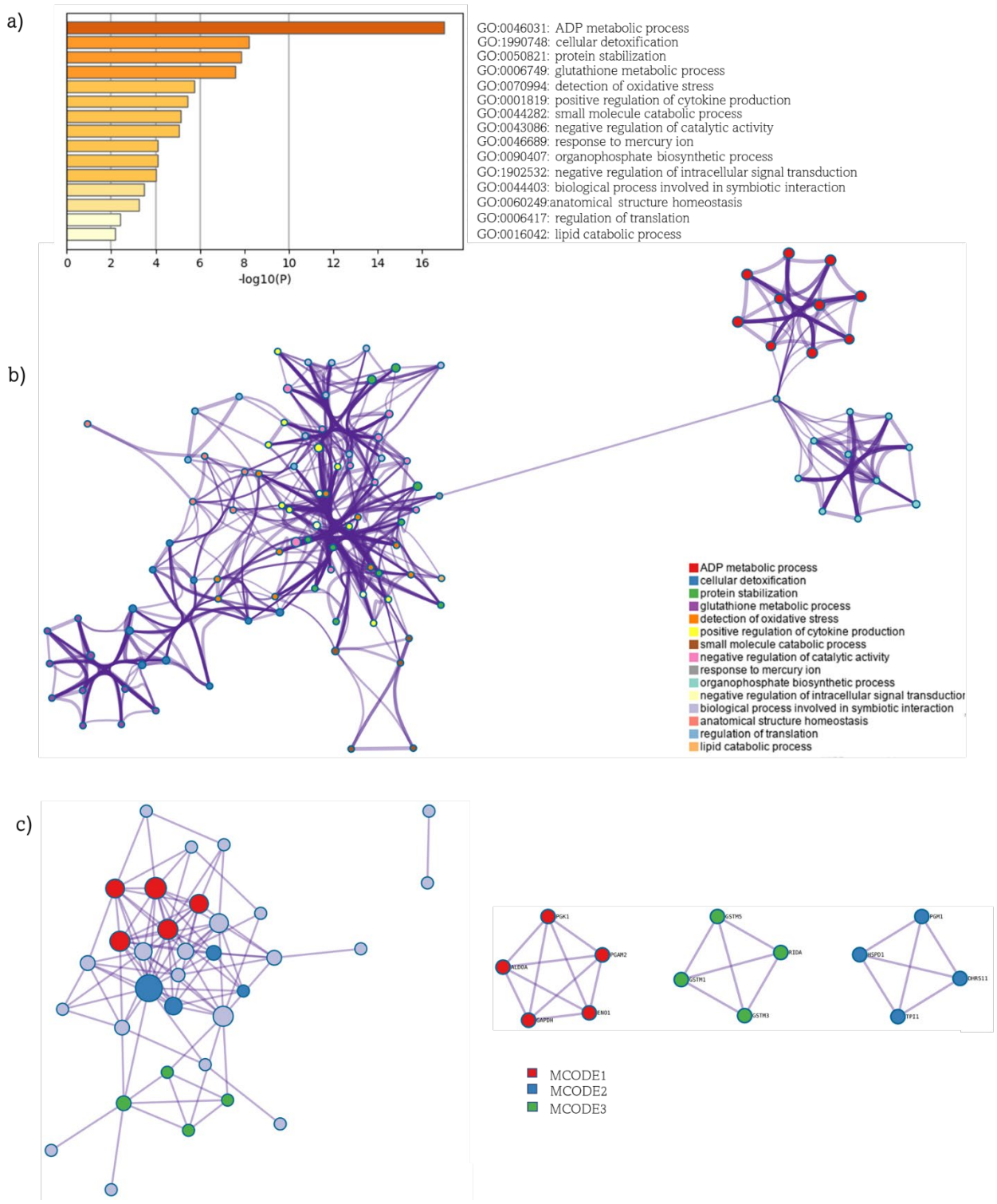


Figure 27. Biological pathway and process enrichment analysis using Metascape® on the proteins identified in the sarcoplasmic proteome extract of lambs to be changing due to hazelnut dietary treatment. a) Functional enrichment analysis based on the list of significant 15 Gene Ontology (GO) terms. The bar graphs highlight the top enriched terms (functional clusters) across the protein lists colored according to P values: terms with a P value <0.01, a minimum count of 3, and an enrichment factor >1.5; b) Networks of pathways and process enrichment cluster analysis; c) Key most significant molecular complex detection (MCODE) components form the network.

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It was interesting to notice that the sarcoplasmic proteome of meat from the hazelnut skin group was also characterized by 3 proteoforms ascribed to AK1, a reversible enzyme, that plays an important role in the adenine synthesis and in maintaining muscle energy homeostasis (Zeleznikar, et al., 1990).

Previous proteomics studies on lamb (Li, et al., 2018; Gao, et al., 2021) and beef (Canto, et al., 2015; Wu, et al., 2016) identified this protein to be positively correlated with a* and C*. Nair et al., (2016) further reported a more abundance of this enzyme in bovine meat with a greater redness surface. In our study, the presence of these spots in meat from the H group confirms that monitoring its changes is a way to monitor muscle color variability, hence allowing us to suggest AK1 for further evaluation following the pipeline of biomarkers discovery (Gagaoua, 2021).

ALDOA, ENO1 and HIBADH proteins (each identified in 2 spots) characterized also the meat of the H group. ALDOA and ENO1 have been very recently identified to be related to lamb meat color stability (Gao, et al., 2021), while HIBADH is identified, for the first time in this study, as a candidate biomarker. However, it should be emphasized that only the proteomic study of Yang et al. (2018) found a positive correlation between the 3-hydroxyisobutyrate dehydrogenase (HIBADH) and C* parameter in the bovine LL muscle packaged under a modified atmosphere. HIBADH is an important mitochondrial protein involved in the tricarboxylic acid cycle (TCA) related to post-mortem muscle metabolism, including valine degradation as reported in the biological mechanisms proposed by Gagaoua et al. (2020). The TCA cycle occurs within mitochondria and requires NAD and NADH (nicotinamide adenine dinucleotide reduced form) and the maintenance of the NAD/NADH ratio is necessary for efficient mitochondrial metabolism as well as for reducing metmyoglobin formation in meat (Ramanathan, Suman, & Faustman 2020; Gagaoua et al., 2021b; Kiyimba, et al. 2022b). The greater abundance of spots ascribed to HIBADH together with the highest values of a* and C* found in the lamb meat of the H group confirm the effectiveness of hazelnut skin dietary treatment in modulating the muscle biology and biochemical processes of color variation. However, further investigations are warranted on this protein to better understand the role of the TCA pathway in the mechanisms of lamb meat color and stability driven by animal feeding strategies.

The generation of reactive oxygen species (ROS) by mitochondria is known as one of the most deleterious causes of oxidative damage in muscle, involving different components of muscle cells (Ouali, et al., 2013; Sierra, & Oliván, 2013). PARK7, known also as DJ-1, is one of the chaperone

proteins localized from the cytosol to mitochondria during oxidative stress (Junn et al., 2009) and plays an essential role in preventing the aggregation and denaturation of proteins. Refers to lamb meat proteome, Ma et al. (2020) is the only study that reported an overabundance of PARK7 in muscles from callipyge mutation relating it to proteolysis and tenderness. Several earlier studies on bovine (Wu, et al., 2015; Gagaoua, et al., 2017a; Yang, et al., 2018; Nair, et al., 2017; and Gagaoua, et al., 2021b) and pork (Sayd, et al., 2006; Kwasiborski, et al., 2008) evidenced major roles of PARK7 in color determination. Taken all together, the current knowledge of evidence highlights the important role of mitochondria in oxygen consumption and their reducing capacity, hence the final impact on meat color (Ramanathan, & Mancini, 2018). In this trial, the presence of six proteins (with a greater abundance) ascribed to PARK7 in the lamb meat from the H group after 7 days of storage support the role that hazelnut skin may play in the anti-oxidative properties of the meat during storage. In support of the above, the identification of peroxiredoxins (PRDX1, PRDX2 and PRDX6) in this trial is in agreement to the major roles ascribed to these proteins in meat color development (Gagaoua, et al., 2020). These proteins are antioxidant enzymes able to reduce the oxides or superoxides produced by the metabolic pathways in the muscle, and consequently reduce the oxidative stress, hence protecting oxymyoglobin from the attack by peroxides (Jia, et al., 2009; and Fisher, 2017). Among peroxiredoxins, PRDX6 was identified in the integromics study of Gagaoua, et al., (2020) as the second top putative biomarker of beef color, while PRDX2 and PRDX1 were proposed as potential biomarkers of beef color stability. In our study, peroxiredoxins were more importantly expressed in the reddest meat from lambs fed with hazelnut skin (3 spots for PRDX6; 2 spots for PRDX1 and 1 spot for PRDX2) thus confirming the antioxidant contribution to protect lamb meat against discoloration, hence leading to the better meat color stability. These findings also suggest that these proteins can be further consider for evaluation as candidate biomarkers for the explanation and/or the monitoring of lamb meat color.

Among the putative protein biomarkers belonging to the *immune and endocrine system* pathway, fatty acid-binding protein (FABP3) was the only previously identified as positively correlated to MRA in the LL muscle of Chinese Luxi yellow cattle (Wu, et al., 2016). ADIPOQ, SERPINA1 and APOA1 were for the first time evidenced in this trial, to drive lamb meat color variation. Particularly, among the putative biomarkers, the APOA1 was identified as the protein mostly influenced by dietary treatment due to the presence of 8 protein spots (proteoforms) in the sarcoplasmic proteome of meat from lambs fed hazelnut skin. Among apolipoproteins, at present only APOA5 and APOC3 genes were associated with pork meat quality (Hui, et al., 2013). Apolipoprotein A1 (APOA1) is the

major protein component of high-density lipoprotein in the plasma membrane that displays anti-inflammatory properties (Mangaraj, Nanda, & Panda, 2016). It is known that as natural polyphenolic substances, tannins have been found to have antioxidants and anti-inflammation properties (Tong, et al., 2021). Therefore, it can be proposed that the presence of several spots ascribed to APOA1 in the sarcoplasmic proteome of H meat could be due to the role of tannins in the production of anti-inflammatory substances, therefore allowing an enhancement of the antioxidant properties of the muscle and consequently improving color stability. Thus, this can partly explain the greater a* and C* values observed in the lamb meat from the animals fed with hazelnut skin by-product. APOA1 should be subjected to further evaluation using appropriate methods before its validation as a biomarker of lamb meat color stability.

Finally, in the sarcoplasmic proteome of lamb meat, further identified spots ascribed to SERPINA1 and ADIPOQ because of dietary treatment with hazelnut skin by-product. SERPINA1, also known as α1-antitrypsin, is a member of the serpins superfamily, the largest protease inhibitor family with multiple roles in intracellular and extracellular activities such as blood coagulation, fibrinolysis, apoptotic regulation and cell migration (Gagaoua, et al., 2015a). Although not yet extensively characterized in lamb meat (Della Malva et al. 2022b) earlier studies discussed the important role of this superfamily of proteins in beef texture (Gagaoua, et al., 2015c; and Zhu, et al., 2021). In pork, a positive correlation between SERPINA members and drip loss was reported (Te Pas, et al., 2013). Conversely, at present, no studies identified serpins proteins, especially SERPINA1, as related to biochemical processes affecting meat color, thus further studies are needed to fully elucidate the role of the members of serpins in the determination of lamb meat color.

6.4. Functional roles of differentially abundant proteins from myofibrillar proteome and their relevance to meat tenderness

Based on bioinformatics results evidenced that hazelnut skins supplementation can induce dynamic changes and modifications in the lamb meat myofibrillar proteome. The protein-protein interaction network (**Fig. 28**) evidenced three major sub-networks:

i) actin filament-based process/cytoskeleton organization (n = 11 protein spots; ACTC1, ACTA1, CAPZA2, CAPZB, ACTG1, TMP2, TMP1, DES, TNNC2, TNNT3 and MYL3);

ii) heat shock proteins (n = 4 protein spots; HSPB1, HSPA4, HSPB6 and HSPB7);

iii) energy metabolism (n = 4 protein spots; ATP5H, PDHB, UQCRC1 and MTX2).

Interestingly, a huge number (n = 18) of these proteins (ACTA1, ACTC1, ACTG1, CAPZA2, CAPZB, DES, TNNC2, TNNT3, TPM1, TPM2, MYL3, HSPB1, HSP70, HSPB6, HSPB7, UQCRC1, ATP5H, and PDHB) have been previously identified as biomarkers of beef tenderness (Gagaoua, et al., 2021a). Refers to small ruminant proteomic studies, 6 proteins were identified as linked with different meat quality traits such as drip loss (Wang, et al. 2016; ACTA1, MYL3, UQCRC1, HSPB1), tenderness (della Malva, et al., 2017, Paim, et al., 2019, Xie, et al., 2021; MYL3, TNNT3) and color stability (Gao, et al., 2016; HSP70, ACTA1).

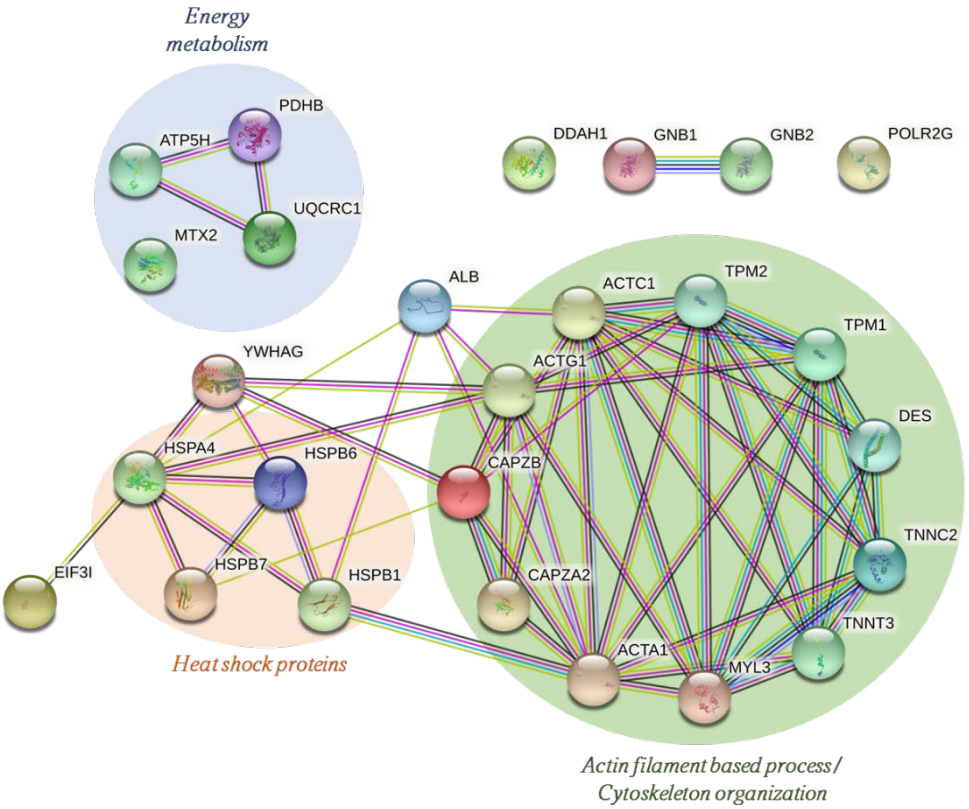

Figure 28. Protein-protein interaction network of the differentially expressed proteins identified in the myofibrillar proteome using STRING database.

The enrichment and cluster process analysis of the identified proteins based on gene ontology (GO) allowed the identification of six significant enriched terms (**Fig. 29a**): Muscle Contraction (GO:0006936), Myofibril assembly (GO:0030239), Tissue homeostasis (GO:0001894), Response to unfolded protein (GO:0006986), Aerobic respiration (GO:0009060), and Positive regulation of angiogenesis (GO:0045766). The interconnectedness of these enriched terms and their functional enrichment was evidenced by three sub-networks given in **Figure 29b**, highlighting the extent of enrichment of the clusters contributing to each GO term.

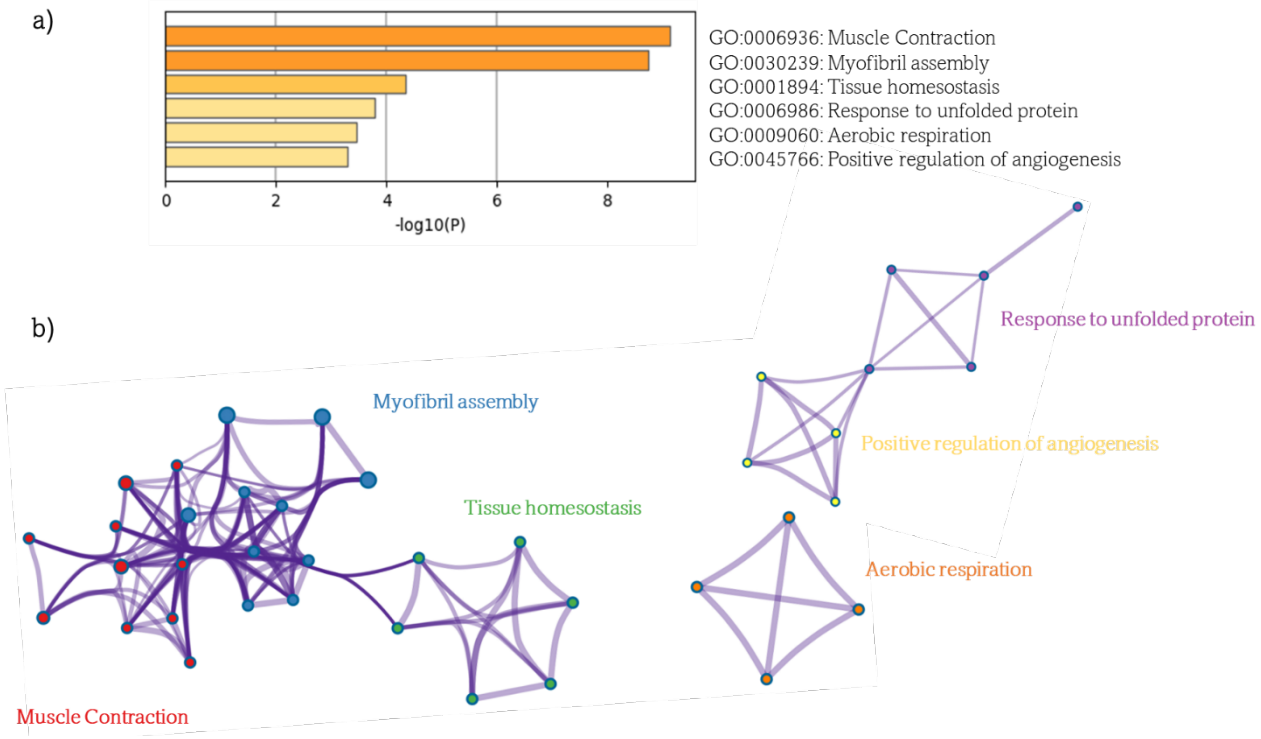

Figure 29. Biological pathway and process enrichment analysis using Metascape® on the proteins identified in the myofibrillar proteome extract of lambs to be changing due to hazelnut dietary treatment. a) Significant enriched ontology clusters and molecular pathways based on Gene Ontology (GO) from the differentially expressed proteins. The bar graphs highlight all the enriched terms across the protein lists coloured according to Log P-values: terms with a P-value <0.01, a minimum count of 3, and an enrichment factor > 1.5; b) Network of pathways and process enrichment cluster analysis.

Overall, the results depicted that ***muscle contraction and myofibril assembly*** were the most important pathways related to the supplementation of lamb diets by hazelnut skins.

Among the dominating proteins, actin (ACTA1) was identified as the most influenced proteins by dietary treatment due to the presence of 5 proteoforms. Actin is the main constituent of the sarcomere thin filaments, and the first protein targeted by caspases in the post-mortem period with the breakdown of the actomyosin complex (Du, et al,. 2004; and Ouali, et al., 2013). Indeed, the integromics study of Gagaoua, et al. (2021a) pointed out that ACTA1 is the most frequently identified protein related to meat tenderization processes. Conversely, among the other proteins of actin filaments, α- actin (ACTG1) has been recently found to be associated with meat tenderization (Malheiros, et al., 2021).

Regarding the contractile and associated proteins, the dietary treatment applied in this study allowed to find the appearance of 5 fragments ascribed to proteins of troponin complex (TNNC2 and

TNNT3) in meat from lambs fed hazelnut skin by-product. The subunits of the troponin complex play a pivotal role in displaying myofilament changes as they are anchored to the tropomyosin-actin filaments and enhanced actomyosin bonds (Gomes, Potter, Szczesna-Cordary, 2002). Consequently, the degradation of troponin proteins highlights that the interacting bonds are impacted and the thin filaments in the sarcomeric I band could be broken. Furthermore, the appearance of the different fragments ascribed to TNNC2 and TNNT3 in meat from the H group, starting from 4 days of storage, confirmed the greater degradation and fragmentation of myofibrils, hence validating the data discussed above using mono-dimensional electrophoresis and immunoblotting. In the context of tenderness, several studies on different species (Laville, et al., 2009; della Malva, et al., 2017; della Malva, et al., 2019; Gagaoua, et al., 2021a) found a strictly link between the degradation of troponin complex proteins and the tenderization rate confirming that troponin-derived fragments are reliable biomarkers of meat tenderization.

The myofibrillar proteome of meat from the hazelnut group was characterized also by the presence of three proteins belonging to the F-actin capping protein family (CAPZB and CAPZA2), involved in the cell signaling and regulation of actin in myofilament contractility. Previous studies on beef (Gullemin, et al., 2011) and pork (Lametsch, et al., 2003; and Wang, et al., 2014) meat, revealed a link between F-actin capping proteins expression and meat tenderness highlighting that these protein patterns could be interesting biomarkers of post-mortem meat tenderization. Among the other myofibrillar proteins that constitute the principal sub-network, 4 proteoforms of MYL3 characterized the myofibrillar proteome of meat from the H group after 7 days of storage time showing a greater abundance than control meat.

Pathways related to ***energy metabolism***, especially those involved in the mitochondrial and ATP metabolic processes are strictly linked with post-mortem underlying pathways of meat texture determination (Ouali, et al., 2013; Wang, et al., 2016; Rosa, et al., 2018; Gagaoua, et al., 2021a; Purslow, Gagaoua, & Warner, 2021). Dietary treatment with hazelnut skin significantly affected the expression of ATP5PD, UQCRC1 and PDHB in lamb meat after 7 days of storage time. Cytochrome b-c1 complex subunit 1 (UQCRC1) is involved in the oxidation/reduction process playing an important role in the cell electron transport and respiratory chain (Kunej, et al., 2007). Mitochondrial apoptosis and release of cytochrome C have been shown to play a fundamental role in the breakdown of myofibrillar protein during post-mortem period (Zhang, et al., 2017). In particular, Wang et al., (2018) demonstrated that the release of cytochrome C induce caspase-9 activation, which may greatly influence meat tenderness. In this study, the greater abundance of spots ascribed

to mitochondrial electron transport chain proteins in the meat from the hazelnut group confirms the high tenderization rate observed in H group. These proteins can be proposed as biomarkers of lamb meat tenderization in line with previous studies on beef (Gagaoua, et al., 2021a).

Among the changing pathways as a consequence of hazelnut skin by-products supplementation, *heat shock proteins* (HSPB1, HSP70, HSPB6, HSPB7), usually associated with the response to stress processes, also impacted the lamb myofibrillar proteome. HSPs as chaperones, play a role in stress resistance by restoring proteins altered by external stimulus thus assuming a fundamental role in controlling the onset of apoptosis and post-mortem proteolytic processes (Ouali, et al., 2013; and Lomiwes, et al., 2014). Additionally, several studies (Balan, Kim, Blijenburg, 2014; and Cramer, et al., 2018) reported that an increase in degradation of small heat shock proteins, like HSPB1 identified in this study, may indicate a loss of its anti-apoptotic function evidencing their important role in the post-mortem processes related to meat quality. The integromics study conducted by Gagaoua, et al. (2021a) evidenced HSPB1 as the top biomarker related to beef tenderness. Several studies (Kim, et al., 2018; and Gagaoua, et al., 2021a) suggested that HSPB1 may delay the activity of endogenous enzymes, and consequently their proteolytic activity. Higher levels of HSPB1 were found to predict tenderness due to the prevention of aggregation processes thus facilitating the action of proteolytic enzymes during the post-mortem period (Morzel, et al., 2008). Recently, an overabundance of HSP70 (Heat shock 70 kDa protein) was observed by Ma, et al. (2020) in callipyge lambs genotypes with tougher meat indicating delayed apoptosis and proteolysis.

Heat shock protein beta-6 (HSPB6), due to the presence of a binding domain for troponin I, play a key role in muscle contraction and metabolic processes (Rembold, et al., 2000). In the meta-proteomics study of Picard and Gagaoua (2020), HSPB6 was identified as putative biomarker of beef tenderness, based on 12 proteomic studies. Additionally, Ma and Kim (2020), in an aging study, found a strong relationship between the greater abundance of HSPB6 fragments and the tenderization rate of different bovine muscles. About HSPB7, Li, and Liu (2022) and Dang, et al. (2022) found an overabundance of this protein in bovine meat after 7 and 16 days of aging, respectively.

The current study also found an overabundance of the above-mentioned HSPs in meat from the H group confirming the role of feeding as an important modulator of post-mortem processes linked with meat tenderness. Our emerging evidence underlines the protective effect of molecules contained in hazelnut skin against oxidative damage thus modulating the trend of post-mortem processes that affect meat tenderness.

CONCLUSIONS

CONCLUSIONS

Hazelnut skin supplementation in the lamb diet was found to be an effective strategy to improve the overall quality of lamb meat during storage time. Data from the present study evidenced the role of antioxidant molecules contained in hazelnut skin in modulating the post-mortem processes linked with meat tenderization and discoloration. Meat from lamb fed hazelnut skins appeared more tender evidenced by a major and more rapid increase of MFI. Furthermore, hazelnut dietary treatment produced meat with better color stability as evidenced by the lowest decrease in redness and saturation index found.

The proteomic approach applied in this study demonstrated that several pathways are involved in the post-mortem processes linked with tenderness and color stability in lamb meat of the hazelnut group. Actin filament-based process, energy metabolism and associated proteins, oxidative stress proteins, followed by heat shock proteins and the immune and endocrine system are the main pathways that could act as potential predictors of lamb meat tenderization and color stability.

The proteomic and bioinformatic analysis proved to be a useful approach to clarify the impact of feeding strategies on lamb muscle proteome related to meat quality characteristics. Data evidenced that ACTA1, HSPB1, TPI1, PARK7, and PRDX6 might be considered reliable biomarkers of color stability and tenderness in lamb meat, as reported in the meat of other species. 11 proteins, these being GSTM1, GSTM3, GSTM5, MTX2, GNB1, GBN2, DDAH1, POLR2G, ADIPOQ, APOA1, and SERPINA1, were not previously detected as predictors of lamb meat quality characteristics, therefore there is a need to validate these novel biomarker candidates using other high-throughput techniques.

In conclusion, this in-depth proteome study provided insight into biochemical pathways related to feeding strategies which might be valuable in developing future strategies to improve the quality of lamb meat.

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