UNIVERSITÀ DEGLI STUDI DI CAMERINO

School of Advanced Studies

Doctoral Course in
Pharmaceutical, Nutraceutical and Food Sciences

XXXV cycle

Quality, shelf-life, and proteomics characterization of organic chicken meat: towards a sustainable poultry supply chain

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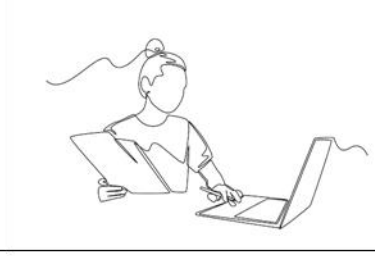

List of publications and communications

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Scientific publications

Related to this thesis work

Alessandroni, L., Caprioli, G., Faiella, F., Fiorini D., Galli R., Huang X., Marinelli G., Nzekoue F., Ricciutelli M., Scortichini S. & Sagratini G. (2022). A shelf-life study for the evaluation of a new biopackaging to preserve the quality of organic chicken meat. *Food Chemistry*, 371, 131134. **IF: 7.51**

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General introduction

General introduction

Our daily food choices have a huge impact on the sustainability dimensions, including the environment. The concerns of consumers including meat eaters about the sustainability of food products they bring to their tables has significantly increased in recent decades (Nguyen *et al.* 2019; Rocchi *et al.* 2019; Rocchi *et al.* 2021). In the production of foods of animal origin, this trend is leading to a rapid and constant growth towards sectors that are more attentive to the extrinsic quality dimensions, i.e., environmental sustainability and animal welfare. Also there is growing concern that non prudent use of antibiotics in animal livestock is linked with the escalating emergence of human infections with antibiotic-resistant pathogens (Moudgil *et al.* 2018).

Chicken meat is the most popular animal protein source in the world. Chicken farming and production methods certainly affects meat sales, in fact not only the price and quality of the product, but also consumer's lifestyle, religious and health concerns, can impact on the choice of food product (Cooreman-Algoed *et al.* 2022). In this perspective, poultry industries have begun to implement strategies considering the sustainability and animal welfare (Nguyen *et al.* 2019). Generally speaking, two strategies are being applied to reach these goals. First, the use of biodegradable and compostable packaging materials for the storage of meat and sale has the purpose of communicating to the consumer the company's commitment to environmental protection (Ivonkovic *et al.* 2017). As researchers, testing the new materials to be used as food packaging for the preservation of quality and appearance of perishable food, such as raw meat, is crucial for consumer’s safety and to provide high quality food products (Chen *et al.* 2019; Bajer *et al.* 2020; Gagaoua *et al.* 2021a; Fernandez

et al. 2022; Gagaoua *et al.* 2022a). Second, the other strategy is to produce high quality-meat by shifting to more extensive farming systems, such as organic production. (Dal Bosco *et al.* 2021; Prache *et al.* 2021). Many factors involved in the farming practices can influence the meat quality, including the animal strain and genetic line, the feeding, the lifespan, the access to an outdoor area etc. The impact of organic production systems on chicken meat quality is not yet clearly understood if we refer the scientific literature as only few studies investigated this aspect with controversial results (Cygan-Szczegielniak & Bogucka 2021; Mancinelli *et al.* 2021; Wurtz *et al.* 2022). Over the past 50 years, fast-growing chickens were selected and widely used in intensive farming standards. Nowadays, the new farming and production practices are becoming more attentive to sustainability and animal welfare, thus leading to the use of new slow-growing chicken strains (Rayner *et al.* 2020; Chodová *et al.* 2021). The use of these new genetic lines and the impact of the farming method on chicken meat quality has not yet been studied in depth by the scientific community.

Classic or traditional approaches used to characterize muscle characteristics were based on a small number of proteins and did not consider the complexity of the underlying biochemical mechanisms and their interactions (Picard *et al.* 2010; Purslow *et al.* 2021). "Omics" techniques, in particular proteomics, have been proposed as a powerful tool to better understand the phenomenon, thanks to the in-depth characterization of the post-mortem muscle proteome, hence in revealing the unknowns and biological mechanisms associated to meat quality determination (Capozzi *et al.* 2017; Munekata *et al.* 2021; Cao *et al.* 2022; Gagaoua & Picard 2022).

Considering the above, the main attempts of my thesis are:

- the evaluation of the possibility of using a new biodegradable and compostable packaging for the storage and sale of chicken meat by analysing its impact on meat quality.
- the comparison of meat quality and shelf-life parameters of meat produced using organic and antibiotic-free production systems
- the application, for the first time, of proteomics approaches to characterize the muscle and meat proteome of two different chicken strains reared within antibiotic-free and organic farming systems.
- discovery of putative protein biomarkers of authenticity and the molecular signatures that contribute to the development of specific meat qualities.

I structured my thesis manuscript in the following section into four sections:

A. An introductive literature review of the state of art about i) the chicken farming systems, ii) chicken meat quality and underlying factors of variation, iii) the impact of packaging on the chicken meat supply chain, and iv) proteomics techniques and applications to research and identify biomarkers of meat quality.

B. Main objectives of the thesis and a brief description of the experimental design.

C. Presentation of all my results acquired during my three years of thesis, in the form of article chapters starting from i) the study of a new biodegradable and compostable packaging in chicken meat preservation; ii) investigation of meat quality and shelf-life parameters among organic and conventional chicken meat; and iii) proteomics techniques and approaches applied to characterize and better understand the differences in chicken muscle/meat proteome between the two production systems

and two chicken strains we selected in my project, including the different the statistical and bioinformatics tools I applied to decipher the unknowns and achieve my goals.

D. An overall conclusion and the future perspectives from this thesis work.

SECTION A
Introduction and literature review

CHAPTER 1

Chicken farming systems with a focus on organic production system

Chicken farming systems with a focus on organic production system

Poultry meat sector

In recent decades, the global poultry industry has seen continuous and steady growth driven by the increase in demand and consumption of poultry meat. The term "poultry" covers a wide range of domesticated birds, from commercial breeds of chickens to ducks, turkeys, guinea fowls, geese, quails, and pigeons. Poultry are raised throughout the world, with chickens by far the leading species everywhere. Over the past 20 years, the number of chickens in the world has increased by about 60 %. Nowadays, poultry, is the world's primary source of animal protein, followed by pork. The United States of America is now the world's largest poultry meat producer, with 18 % of global output (19710 thousands of tons), followed by Brazil, Europe and China (13800, 12470 and 12000 thousands of tons respectively) (Shahbandeh 2021). In coming decades, the population growth, the greater purchasing power, and the urbanization will drive the growth in the global poultry meat production and poultry meat consumption. Accordingly, an increase in production up to 16 % is expected in 2025, most of which will occur in developing countries (FAO 2016).

Chicken is second after pig meat when it comes to European Union (EU) meat consumption where the average consumption is about 24 kg per capita (32.5 kg for pig meat). Poultry meat production in the EU has experienced a cumulative rise of around 30 % over the past 10 years (Figure 1). Today, around 70% of poultry meat production in the EU comes from only six Member States: Poland (16.8 %), United Kingdom (12.9 %), France (11.4 %), Spain (10.7 %), Germany 10.4 %) and Italy (8.5%) (Augère-Granier 2019).

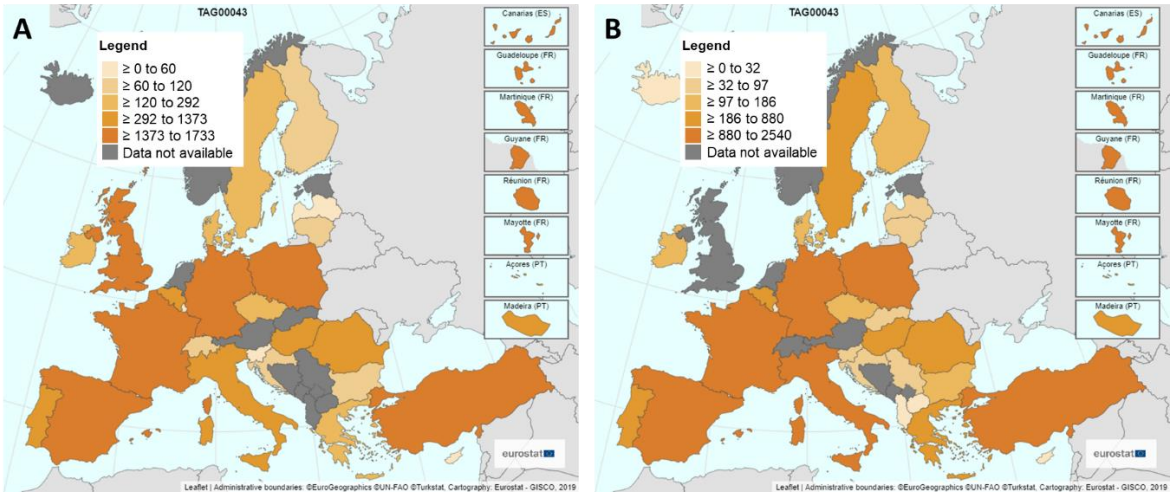

Figure 1 - Poultry production in Europe in 2011 (A) and in 2012 (B) expressed in thousands of tons

The Italian production of poultry meat, in line with the European trend, has experienced a significant increase in the last decade. Most of the national poultry production is represented by chicken meat with about 85%. The Italian poultry sector has excellent levels of self-supplying, resulting overall self-sufficient at 108.4 % (Unaitalia 2022). Furthermore, in recent years, consumers' concern about food products they bring to their tables has significantly increased leading to a rapid and constant growth of the sectors linked to sustainable, local, and respectful of animal welfare productions. According to EUROSTAT data, organic poultry production expanded at an annual pace of 13.5 % between 2013 and 2016 to double its numbers in 2021. In fact, in 2012 the largest exponent of organic poultry farming was France with 11.5 million animals per year, in 2022 it reached 23.8 million heads (Figure 2).

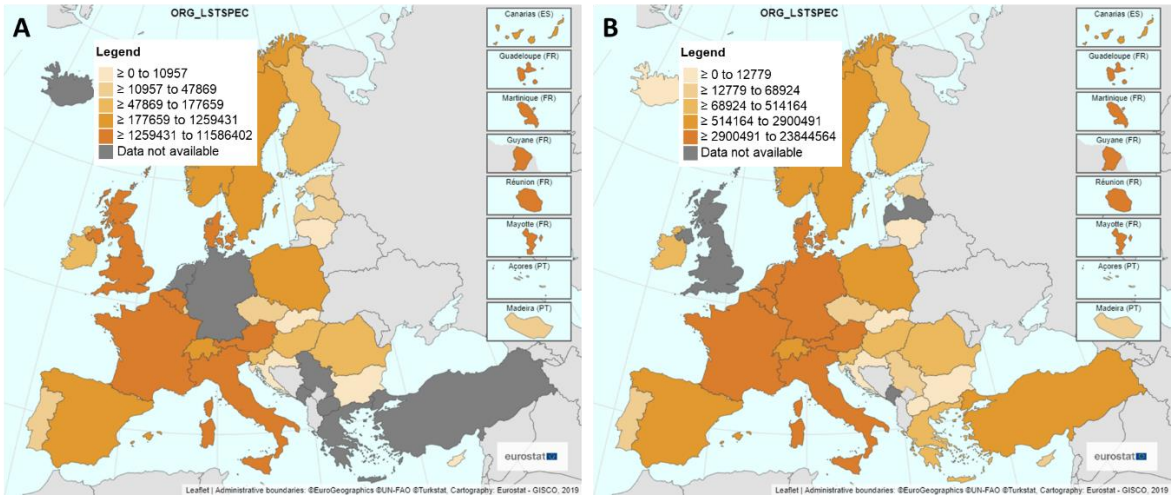

Figure 2 - Organic poultry livestock in Europe in 2012 (A) and in 2020 (B) expressed in number of live animals

Italy was the second largest organic meat producer in Europe in 2012 with 2.8 million heads, and in 2020 it dropped to fourth place despite almost doubling its number to 4.3 million (Eurostat 2022; SINAB 2022).

Organic poultry production

Most poultry meat derives from intensive poultry production systems (95%) and a small portion (5%) from the extensive rearing systems such as organic and free-range. These systems are less standardisable than intensive ones and guidelines are different from one country to another, depending on climate, labour, feed costs, genotypes land resources, and willingness of consumers to pay for this kind of products (Dal Bosco *et al.* 2021). Among the extensive systems, organic animal production has experienced a rapid spread and development since the 1990s driven by the increased demand from consumers for healthier products with higher attention on animal welfare. The International Federation of Organic Agriculture Movements (IFOAM), founded in 1980 to coordinate the international organic agriculture network, developed the first basic standard for organic agriculture, which has been periodically revised and updated. In general, chickens must be allowed to live a life as natural as possible with, among the other points, the access to an outdoor area.

EU regulations on organic farming are designed to provide a clear structure to produce and sell organic goods, to contribute to transparency and consumer confidence as well as to a harmonised perception of the concept of organic production. EU Regulation had an important impact on the harmonization of international organic standards within and outside Europe, as all countries willing to export to EU need to meet the European standards. The goals and principles of organic production throughout Europe are well defined, and the marketing of certified organic products is thoroughly regulated by Council Regulation (EC) No 834/2007 and by two implementing regulations, No 889/2008 and No 1235/2008 (Commission 834/2007, 889/2008, 1235/2008). Table 1 summarizes the key points regulated in EU guidelines for organic livestock and production. In general, these standards are more detailed than standards from the USA, or from Australia. In fact, although many aspects are similar (limits on non-organic feed, prohibition of growth promoters, prohibition of drugs in absence of disease), there are still several areas where harmonization is needed (housing, grazing areas, withholding periods for drugs, conversion time, age at weaning, etc.). In addition, the EU Regulation pays more attention to the animal ethological needs as compared to other international standards (Sanders & Schmid 2013). A new organic legislation has been adopted, the Regulation (EU) 2018/848 entered into force on 1st of January 2022. The aim of this new legislation is to prevent fraud, to improve the competitiveness of European producers with non-EU imports and to ensure consumer confidence in organic certification.

Table 1 - Key points of organic livestock production regulated by Council Regulation (EC) No 834/2007

<table>
<tr><th>Animal origins</th><th>Feed</th><th>Husbandry practices and housing conditions</th><th>Disease prevention and veterinary treatment</th><th>Production</th></tr>
<tr><td rowspan="2">Breeds must be chosen having regard to the capacity of animals to adapt to local conditions, their vitality and their resistance to disease or health problems</td><td>GMO, growth promoters and synthetic amino acids shall not be used</td><td>The use of non-renewable resources and off-farm inputs shall be minimized</td><td rowspan="4">Disease shall be treated immediately to avoid suffering to the animal; chemically synthesised allopathic veterinary medicinal products including antibiotics may be used where necessary and under strict conditions. Restrictions with respect to courses of treatment and withdrawal periods shall be defined</td><td>Site-adapted and land-related livestock production</td></tr>
<tr><td>Livestock shall be fed with organic feed that meets the animal's nutritional requirements at the various stages of its development</td><td>Application of husbandry practices, which enhance the immune system and strengthen the natural defence against diseases, including regular exercise and access to open air areas and pastureland</td><td>The recycling of wastes and by-products of plant and animal origin shall be used as input in plant and livestock production</td></tr>
<tr><td>Disease prevention shall be based also on breed and strain selection</td><td rowspan="2">Feed must be obtained from the farm itself or from other organic farms in the same region</td><td>Observance of a high level of animal welfare respecting species-specific needs</td><td rowspan="2">Organic processed products should be produced using methods which guarantee that the organic integrity and vital qualities of the product are maintained through all stages of the production chain</td></tr>
<tr><td>Reproduction shall use natural methods</td><td>Animals shall be raised on organic holdings since birth or hatching and throughout their life</td></tr>
</table>

Overview of poultry production systems

The production of poultry meat is one of the sectors in which intensive farming is mainly used. Intensive broiler farming is characterised by high stocking densities, fast growth rate breeds, very large holdings, and indoor rearing. This farming model is responsible for around 90 % of broiler production in the EU. Directive 2007/43/EC lays down rules for the protection of broilers for meat production for holdings with more than 500 chickens. This regulation sets a maximum stocking density with the aim to reduce the overcrowding of chicken holdings. Moreover, to ensure better animal welfare it specifies requirements such as feeding, lighting, noise, ventilation, and training for the staff dealing with chickens. The directive 2007/43/EC is the first legislation that includes also 'welfare indicators' that must be followed up at the slaughterhouse (Commission 834/2007). Between intensive broiler production and organic production, there are several intermediate sectors summarized in Table 2. Extensive broiler productions provide lower densities, longer lifespan times and some of them also an access to an outdoor area. In the EU, an estimated 10 % of broiler chickens are raised in extensive systems, around 5 % in indoor systems, up to 4 % in free range systems and 1 % in organic systems. Extensive farming system models are expected to grow in EU in the coming years and slower-growing broilers are starting to be chosen.

Table 2 - Marketing terms and conditions for the production of broilers, according to directive 2007/43/EC and 834/2007 (for the organic).

Production system	Min. age (days)	Max. indoor density	Access to outdoor run
Intensive indoor	35 - 50	33 kg/m^2	No
Extensive indoor	56	15 birds/ m^2	No
Free-range	56	13 birds/ m^2	Yes, 1 m^2 per bird
Traditional free-range	81	12 birds/ m^2	Yes, 2 m^2 per bird
Free-range, total freedom	81	12 birds/ m^2	Yes, 2 m^2 per bird
Organic	70 to 81	10 birds/ m^2	Yes, 2 m^2 per bird

Impact of production systems on chicken genotypic traits

The genetic selection of broilers has been generally carried out to meet the needing of high meat yields in short time, that made the fast-growing chickens the more popular choice as they perfectly fit with intensive rearing systems (El-Deek & El-Sabrout 2019). Fast-growing chickens genotypes can achieve the target live weight of 2-2.5 kg in 35 to 45 days. The main three commercial fast-growing broiler breeds are Cobb, Hubbard, and Ross. Slow-growing broilers, which can reach optimal weight with a life span from 70 to 81 days, have been gaining interest in many European countries in recent years. Slower-growing poultry breeds were reported to be more suitable for alternative productions of broiler chickens as they provide to animals longer lifetimes (Rayner *et al.* 2020). Extensive broiler productions using slower-growing genotypes is increasing in many EU countries. Today, it is estimated that 2 to 5 % of the European broilers are slower-growing birds (Augère-Granier 2019).

Genotype is considered as a combination of specific traits such as: welfare, behaviour, and productive performance (es. daily weight gain). All these parameters are besides influenced by environmental and the management factors (Cartoni Mancinelli *et al.* 2021). In this context, it is essential to define the chicken genotype that better suited for the conditions of each farming method. Meat production performance, together with other traits such as walking activity, exploratory attitude, immune response, thermo-tolerance, and welfare, plays an important role in the adaptability of chickens to the farming treatments. To date, some studies have evaluated the welfare, growth, and meat quality of different chicken breeds in extensive and organic farms, but the knowledge is still lacking in this topic. The first study about the impact of farming system on chicken meat quality was carried out by (Castellini *et al.* 2002b). Conventional (inside ground farming, 0.12 m^2/bird) and organic (indoor pen, 0.12 m^2/bird with access to a grassed paddock, 4 m^2/bird) farming methods were compared using Ross 308 male chickens assigned to the two production systems.

Organic breast and drumstick resulted of higher weight with lower levels of abdominal fat; moreover, they had lower pH and water-holding capacity than the conventional one. Subsequent studies have investigated the impact of organic farming on different chicken genotypes. Three chicken breeds with different growth rate (Robusta maculata, Kabir and Ross, very slow-, slow- and fast-growing respectively) reared under organic system were compared by other three studies from the same research group (Castellini *et al.* 2002a; Castellini *et al.* 2002c; Castellini *et al.* 2006). Behaviour, productive performance, and meat quality were investigated. Results underlined the higher attitude to walking activity and more intense foraging behaviour of the slower-growing breeds while the productive performances (growth rate, carcass weight and feed conversion index) were higher for Ross chickens. However, fast-growing broilers reported higher mortality and culling rate demonstrating that they did not adapt well to organic production (Castellini *et al.* 2002a). Meat quality was affected by the different degree of maturity of the chicken strains at slaughter age due to the different time needed to complete the somatic development. All three genotypes showed good results in meat quality traits. Fast-growing chickens reported a higher amount of fat, lower iron content, lower pH, and paler color, while slower-growing chickens showed excellent results in moisture content. A negative relationship between adaptability and daily weight gain was demonstrated affirming that the slow-growing genotypes appear more adapt to organic systems compared with fast-growing chickens. Subsequently, a multifactorial adaptability score was defined considering simultaneously different variables such as behaviours, plumage conditions, and body lesions. It was applied to six chicken breeds concluding that genotype is the main factor that influenced the adaptability of the animals to the farming system.

SECTION A
Introduction and literature review

CHAPTER 2

Chicken meat and factors impacting its quality

Chicken meat and factors impacting its quality

The ultimate goal of poultry industries is to produce a high quality-meat protein source in an economical and environmentally sustainable manner. To meet this objective the challenge is to balance three interconnected factors that are meat quality, quantity, and cost of production. This chapter will focus on meat quality parameters that need to be monitored to provide high quality products to consumers.

The definition of "quality" reveals the complex character of this topic; according to the standard ISO 8402-94, *the quality is the set of characteristics of an entity that give that entity the ability to satisfy the expressed and implicit needs of its user or consumer*. Food quality concept is used to indicate the overall characteristics of the product including its physical, chemical, morphological, biochemical, microbial, sensory, technological, hygienic, nutritional, and culinary properties (Tougan *et al.* 2013).

Poultry meat quality can be defined from sensorial, nutritional, hygienic, and technological features. The nutritional quality is linked to the ability of the meat to provide proteins, lipids, carbohydrates, and other essential nutrients intake. In addition to this, meat must preserve the consumer's health. The hygienic quality of meat must be monitored in compliance with food safety and security legislations. The technological quality of chicken meat is mainly appreciated from the color, water-holding capacity, and the texture of the meat. Moreover, the ability of meat to tolerate the transformations, preservations and packing processes is another important feature related to the technological quality of poultry meat (Xiong *et al.* 2017; Khaled *et al.* 2021). Consumers can judge meat quality from its sensorial and physic

characteristics such as appearance, texture, juiciness, water holding capacity, firmness, tenderness, odour, and flavour. Furthermore, the quality of poultry meat includes scientifically quantifiable properties of meat which are fundamental for the overall production process, such as post-mortem pH decline, meat pH, shear force, water holding capacity (WHC), drip loss, cooking loss, shelf life, fat and protein content. Intrinsic and extrinsic factors affecting meat quality parameters are resumed in Table 3 and discussed in the following sections.

Table 3 - Main chicken meat quality affecting factors

Affecting factors	**Impact on meat quality**	**References**
Intrinsic factors		
Chicken genotype	Post-mortem pH decline, meat pH, WHC, drip loss, oxidation processes, meat color, fat content	(Fanatico *et al.* 2005; Castellini *et al.* 2006; Fanatico *et al.* 2007; Bihan-Duval *et al.* 2008; Phongpa-Ngan *et al.* 2011; Sirri *et al.* 2011; Chabault *et al.* 2012; Chodová *et al.* 2021; Mancinelli *et al.* 2021)
Type of muscle and fibres	Post-mortem pH decline, meat pH, drip loss, meat color	(Petracci & Fletcher 2002; Berri *et al.* 2007; Abdullah & Matarneh 2010)
Sex	Meat pH, meat color, tenderness, cooking loss, fat and crude protein contents	(Debut *et al.* 2003; Fanatico *et al.* 2005; Abdullah & Matarneh 2010; Peters *et al.* 2010; Lopez *et al.* 2011; Cygan-Szczegielniak & Bogucka 2021)
Slaughter age	Post-mortem pH decline, meat pH, tenderness, WHC, meat color, fat content, meat flavour	(Castellini *et al.* 2002b; Berri *et al.* 2005; Brunel *et al.* 2006; Berri *et al.* 2007; Fanatico *et al.* 2007; Baéza *et al.* 2012)
Live weight	Meat pH, meat color, drip loss, fat content	(Bianchi *et al.* 2006; Berri *et al.* 2007; Abdullah & Matarneh 2010)
Extrinsic factors		
Production system	Meat color, WHC, drip loss, tenderness, fat and protein content	(Castellini *et al.* 2002b; Fanatico *et al.* 2005; Husak *et al.* 2008; Wattanachant 2008; Bogosavljevic-Boskovic *et al.* 2010; Mikulski *et al.* 2011; Michalczuk *et al.* 2017)
Feeding strategy	Tenderness, meat pH, meat color, oxidative processes, fat content	(Garcia *et al.* 2005; Guillevic *et al.* 2009; Baéza *et al.* 2013; Schiavone *et al.* 2017; Cullere *et al.* 2019)
Pre-slaughter conditions	Meat color, meat pH, fat content	(Savenije *et al.* 2002; Bianchi *et al.* 2006; Petracci *et al.* 2015)
Post-mortem aging time	Meat pH, meat color, tenderness	(Fletcher 2002; Fanatico *et al.* 2005; Bianchi *et al.* 2006; Abdullah & Matarneh 2010; Salwani *et al.* 2016)

Meat color and related factors

Transformation of muscle into meat involves a series of chemical and biochemical steps that affect the final color of the meat product. The color is a physical characteristic of the meat, so its disposition is related by dispersion and absorption of light and it refers not only to the raw meat but also to the final ready-to-eat product. This feature relies on the meat pigments concentration and their chemical state. Among them, the main influencing compound is myoglobin, but also haemoglobin, cytochrome C and their derivatives play important role (Bowker 2017). Cooked poultry meat color is dependent on processing such as the irradiation, cooking temperature, storage, processing additives, as well linked to meat biochemical factors as preslaughter conditions, cytochrome C reactions and concentration and chemical state of the myoglobin (Estévez 2015). The color of poultry meat can vary as a direct result of several intrinsic and extrinsic factors (Table 3).

Genotype greatly affect meat color characteristics, (Castellini *et al.* 2006) comparing Ross and Kabir chicken genotypes reared in organic farming systems, reported that different chicken breed led to different values of lightness and yellowness of meat. Strong differences in the meat color were found also when comparing fast- and slow-growing chicken genotypes reporting higher redness values in meat from fast-growing strains (Sirri *et al.* 2011).

Meat color depends also on the type of muscle and fibres. In fact, for example, the *biceps femoris* muscle (thigh part) was reported to contain higher amount of myoglobin than the *pectoralis major* muscle (breast) and this contributes to higher redness and lower lightness values on meat (Petracci & Fletcher 2002).

(Lopez *et al.* 2011), studying broilers sex effect on meat characteristics, reported that females exhibited higher yellowness values than males. These results agreed with the ones previously observed by (Fanatico *et al.* 2005).

The differences in meat color can be also due to chicken slaughter age which can affect the content of myoglobin in muscles. It was found that slow-growing chicken meat was darker and with higher values of redness and yellowness than high-performance strains (Castellini *et al.* 2002b; Fanatico *et al.* 2007; Baéza *et al.* 2012). Moreover, the live weight can affect meat quality composition of chicken carcasses. (Bianchi *et al.* 2006) studied the influence on market live weight, transport time and other processing parameters on chicken meat color. Their results underlined that the birds with live weight higher that 3.3 kg produced darker breast meat. Subsequent investigations by (Bihan-Duval *et al.* 2008) confirmed that heavier chickens present redder breast meat with lower pHu and higher drip loss than the lighter ones.

Among extrinsic factors, production system impact on meat color was investigated by (Fanatico *et al.* 2005) focusing on meat quality evaluation of slow-growing broiler genotypes with and without outdoor access. Their study indicated that meat quality differences exist among breeding strategies. Indeed, the main effect of outdoor access was the higher yellowness values. A similar study was performed by (Mikulski *et al.* 2011) that reported breast and thigh color of chickens with outdoor access as significantly darker compared with broilers raised in confinement.

As the other sensory characteristics of chicken meat, also color depends, also on the raw material used in feed and supplements. Several studies focused their attention on feed modifications and their effect on chicken meat quality traits. (Garcia *et al.* 2005) investigated on the inclusion of sorghum replacing corn in broiler feed and reported that this change led to paler chicken meat. (Schiavone *et al.* 2017; Cullere *et al.* 2019) tried a strategy based on soybean oil replacement by black soldier fly larvae (*Hermetia illucens* L.) fat in broiler diets reporting similar meat color results in control group and in modified diet group.

Pre-slaughter conditions, including holding time and temperature, water and feed withdrawal and other stress parameters, play a fundamental role on chicken meat color. Although no influence of feed withdrawal on meat color was reported by (Savenije *et al.* 2002), subsequent investigations by (Bianchi *et al.* 2006) concluded that chicken breast fillets held at <12 °C were darker than the ones held at > 12 °C reporting a negative correlation between holding time and temperature and L*, a* and b* values. In fact, the shortest holding time (< 6 h) produced the highest L* values. Moreover, post-mortem aging time was reported to negatively affect meat color (Fletcher 2002; Bianchi *et al.* 2006; Abdullah & Matarneh 2010).

Meat pH and related factors

The post-mortem pH decline appears to be a key element for poultry meat quality. It affects the functional properties of muscle proteins and thus many meat quality attributes such as color, WHC, texture, and shelf life (Baéza *et al.* 2022). The initial rate of pH (pH 15 min post-mortem) is essentially determined by a bird's physical activity before death, while ultimate pH (pHu) mainly depends on the quantity of glycogen available in the muscle at death, also called glycolytic potential (Debut *et al.* 2003; Berri *et al.* 2005). Increased in muscle glycogen content is a consequence of muscular activity and results in decreased pHu (Bihan-Duval *et al.* 2008). For poultry meat, colour and pH are strongly correlated features. Light scattering properties of the resulting meat depend on muscle glycogen stores at slaughter and on post-mortem evolution of pH. A low pH is also associated with low WHC values and poor functionality, and a high pH is associated with short shelf life due to the more favourable environment for bacteria proliferation (Fanatico *et al.* 2007). Normal broiler breast meat pHu value is around 5.8 (Petracci *et al.* 2013): the more pHu deviates from this value, the more defects occur (*see section 2.6*).

As others meat quality parameters, also pH is firmly connected with chicken genotype (Table 3). Several studies reported a lower pH of meat from slow-growing genotypes when compared with fast-growing strains (Berri *et al.* 2005; Fanatico *et al.* 2007; Bihan-Duval *et al.* 2008; Chabault *et al.* 2012). Indeed, at the genetic level, the correlation between pH and lightness of chicken fillets was reported to be very strong ranging from −0.65 to −0.91 depending on the strain (Bihan-Duval *et al.* 2008; Chabault *et al.* 2012). Subsequent studies on experimental selection on meat pH of high growth-rate chickens confirmed the relationship between meat pH and quality traits. In fact, such differences in pH resulted in considerable changes in several attributes of breast meat, including lightness, drip loss, cooking loss, shear force after cooking, and cooking yield. Moreover, breast meat pH resulted to be a highly heritable characteristic in poultry (Alnahhas *et al.* 2014; Alnahhas *et al.* 2015).

The impact of chicken sex on meat pH was investigated by (Lopez *et al.* 2011) reporting female broilers to have lower pH values 24 h post-mortem. A study carried out on breast meat cross-sectional area by (Berri *et al.* 2007) concluded that slaughter age is negatively related to post-mortem pH decline rate and final meat pH. Moreover, lower pH values were measured in breasts from heavier chickens, in comparison with lighter ones, by (Bihan-Duval *et al.* 2008).

Several extrinsic factors can also impact the meat pH. For example, the inclusion of sorghum replacing corn in broiler feed was reported to slow down the post-mortem pH decline (Garcia *et al.* 2005). Moreover, the amount of abdominal fat turns out to be negatively related to meat pH (Baéza *et al.* 2013).

Feed is normally withdrawn for several hours before slaughter to allow the digestive system emptying and so reduce the possibility of faecal contamination during slaughter and further processes (Ali *et al.* 2008). The energy stock available at the end of this pre-slaughter fasting

period can influence the kinetics of pH decline during the muscle-to-meat conversion (Tougan *et al.* 2013). Post-mortem aging time is positively correlated with pH values, in fact, meat pH is expected to decline during the first 24 h after slaughter (Bianchi *et al.* 2006).

Meat water content: WHC, drip loss and cooking loss

Water in muscle can be present in three forms: bound, immobilized and free water (Figure 3). The bound water layer consists of water molecules tightly bound to the hydrophilic groups of the proteins. Bound water is resistant to freezing and cooking and generally not influenced by meat processing. The immobilized water layer is made up of water molecules held by steric effects within the muscle structure and by weak attractions to bound water and muscle proteins. Immobilized water represents up to 85% of the total water in meat. Immobilized water is not readily lost in the early post-mortem period, but it is lost in cooking or freezing processes. Intrinsic alterations in the muscle cell structures and post-mortem pH decline strongly affect immobilized water. Free water molecules are only held by weak surface forces. Thus, free water is easily lost during common processing and handling steps such as grinding, cutting, cooking, and storage. In general, during the conversion of muscle to meat, there is a decrease of immobilized water and an increase of free water.

WHC refers to the ability of meat to hold moisture during fabrication, processing, and storage. From a consumers point of view, a low WHC value in fresh poultry meat evolves in less visual appeal due to a liquid excess (drip loss) and inferior juiciness and tenderness. At the same time, from a technological standpoint, a good WHC in poultry meat leads to greater marinade absorbency and retention, better protein functionality, and greater processing yields (Petracci & Cavani 2012). To meet market demands, modern chicken genotypes have been selected for growth rate and carcass yields. Although these remarkable gains have been achieved, chickens seem to be more inclined to stress and myopathies that can have a harmful effect on WHC and other meat quality traits (Bowker 2017).

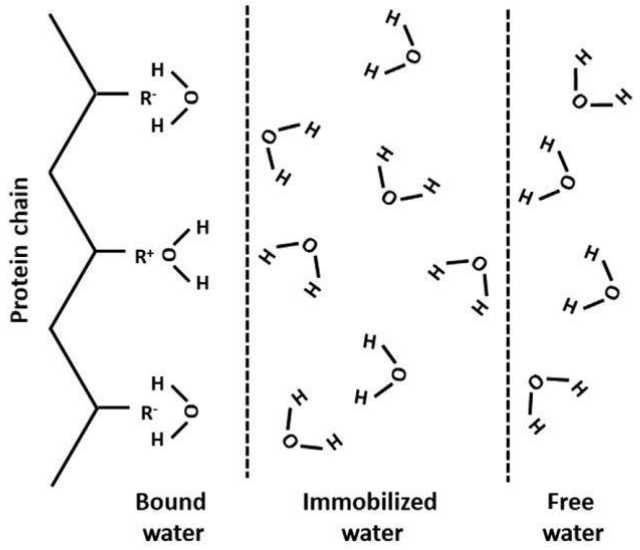

Figure 3 - The three water forms in muscle meat

Poultry meat WHC is a complex parameter related to post-mortem transformation of muscle tissue to meat. Factors connected to the period right pre- and post-slaughter seem to have a primary impact on WHC (Table 3). Drip loss is an ongoing process involving the transfer of water from myofibrils to the extracellular space, this process is affected by structural features of muscle tissue. This definition applies to that fluid that is lost from meat without any added mechanical force other than gravity (Guo & Dalrymple 2022). Meat cooking loss evaluation provides information about muscle protein and meat functionality. This parameter directly influences the yield and the quality of meat products. Therefore, cooking loss is essential to understand factors that influence meat quality and predict processing and postprocessing handling events (Pang *et al.* 2020).

Poor WHC in slow-growing chickens was attributed to a less mature age than fast-growing birds slaughtered at the same age (Castellini *et al.* 2002b). Genotype selection affects meat WHC due to the important differences of post-mortem metabolism between chicken genetic types. In fact, important exudation water loss of raw meat can lead to faster post-mortem pH decline (Debut *et al.* 2003). This fundamental correlation between genotype, pH decline and WHC was assessed also by (Bihan-Duval *et al.* 2008) that concluded that slow-growing

chickens tends to have longer post-mortem pH decline time with lower meat pH and WHC. (Fanatico *et al.* 2007) and (Sirri *et al.* 2011) showed that the higher drip loss in slow-growing chickens was probably owing to smaller size and larger surface area of muscle in those birds than in fast-growing birds. The study of (Abdullah & Matarneh 2010) showed that cooking loss is affected by bird sex with higher values recorded in females. However, in (Chodová *et al.* 2021), the effect of genotype and animal sex on drip loss was not significant. (Phongpa-Ngan *et al.* 2011) elaborated the impact of growth-rate and WHC on chicken meat proteome resulting that these parameters are strongly influent in the expression of specific proteins which can be studied as biomarkers in a predictive perspective.

Among the extrinsic factors, production system can affect meat WHC resulting better values of breast meat from chickens with outdoor access (Mikulski *et al.* 2011). An increased drip loss during post-mortem storage was observed by (Salwani *et al.* 2016). This could be due to the degradation of myofibrillar proteins by endogenous proteases, affecting the ability of muscle to hold water.

Meat tenderness and related factors

Tenderness of broiler breast meat is one of the most important attributes for consumers. Broilers have traditionally been considered a naturally tender product as it is the most used meat for weaning children. However, there are factors that can negatively impact the tenderness of broiler breast meat including technological processes, intrinsic and extrinsic factors (Morey & Owens 2017) (Table 3). The main proven instrumental method in assessing meat tenderness is the Warner-Bratzler shear force that is probably also the oldest one. This method works on the principle of compression, tensile, and shear forces on muscle fibres (Garcia *et al.* 2005).

Among intrinsic factors affecting broiler meat tenderness, bird sex and slaughter age are the most influent ones. In fact, the meat of female chickens was reported to be less exudative

and more tender than that of males (Debut *et al.* 2003; Cygan-Szczegielniak & Bogucka 2021). Moreover, an increase of tenderness and juiciness was demonstrated to correspond with a decrease in the slaughter age of the animals by (Gigaud *et al.* 2011).

The meat tenderness, calculated as shear force, can also vary significantly according to extrinsic parameters such as the production system. (Castellini *et al.* 2002b) studied the effect of organic production on broiler meat quality and showed that the production system affected the shear force value, which was higher in the breast of the organically raised animals, probably as a consequence of their major physical activity. The same tendency was observed by (Husak *et al.* 2008) for the breast meat from chickens reared under a lower stocking density and (Wattanachant 2008) for Thai indigenous chickens bred with or without outdoor access.

It is well documented that tenderness improves (decreasing shear force values) if the post-mortem aging time and the pre-deboning time increases (Cavitt *et al.* 2005; Bianchi *et al.* 2006).

Chicken meat fatty acids

The total lipid and cholesterol content of raw chicken breast meat with skin is reported to be 10.6 g/100 g by IEO - Food Composition Database for Epidemiological Studies in Italy (BDA). The lipids are mostly concentrated in the skin and consequently they can be easily eliminated during industrial and home processing. Chicken genotype has a key role in the fatty acid composition of meat as evidenced by (Lonergan *et al.* 2003) that found that breast meat from fast-growing broilers had higher lipid content than meat from slow-growing ones. About the 98% of them are triglycerides, made up of glycerol linked to three molecules of fatty acids. Fatty acids can be divided into saturated and unsaturated based on their chemical structure. This classification is fundamental as they have completely different effect on human health. In fact, saturated fatty acids (SFA) raise cholesterol level and increase the risk

of cardiovascular disease, while unsaturated fatty acids tend to decrease the levels of cholesterol and LDL (Low Density Lipoproteins) in the blood, disfavouring the atherosclerotic process. Unsaturated fatty acids can be classified as monounsaturated (MUFA) or polyunsaturated fatty acids (PUFA), the sum of which, according to World Health Organization (WHO) guidelines, should be between 7.5% and 10% of daily calories. Standard poultry meat contains low levels of n-3 PUFA, such as eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), but it was investigated that several factors such as sex, feeding strategy, production system and genotype may affect PUFA content in final meat product (Table 3).

In particular, chicken genotype is a key factor as usually a higher percentage of PUFA in slow-growing chickens than in the fast-growing ones was reported (Sirri *et al.* 2011). A similar study, published by (Mancinelli *et al.* 2021), compares meat fatty acid profile from different growth-rate chicken genotype. It comes out that the highest total fat content was obtained in meat from fast-growing chicken samples, the same for SFA and MUFA percentages, while a better fatty acid profile was reported by medium- and slow- growing chicken genotypes with higher amounts of n-3 PUFA, n-6 PUFA, EPA and DHA. Moreover, meat lipid content was showed to be higher in female chickens, whereas crude protein content was significantly higher in males (Peters *et al.* 2010; Cygan-Szczegielniak & Bogucka 2021). These evidences underlined the strong relationship between genotype and sex and crude protein and lipid contents.

Chicken meat fatty acid profile can be also influenced by the slaughter age and live weight of chicken (Brunel *et al.* 2006; Bihan-Duval *et al.* 2008).

Fat content variation can be linked also to the production system since the organic farming was reported to reduce by three times the lipid content of chicken breast meat (Brunel *et al.* 2006; Michalczuk *et al.* 2017). Indeed, it comes out that a fat content of 2.37% and of 0.74%

was observed for conventional and for the organic production systems respectively (Castellini *et al.* 2002c). Thus, the organic production system is then very interesting in term of nutritional quality of meat; it allows not only to obtain less fatty meat but also rich in heme iron and protein (Bogosavljevic-Boskovic *et al.* 2010). Furthermore, a study conducted by (Guillevic *et al.* 2009) demonstrated that the MUFA and PUFA proportions in chicken meat are strongly related to the animal feeding strategy.

Chicken abnormalities and causing factors

The genetic selection criteria, addressed to increase the size and the yield of *pectoralis major* muscle, have disclosed huge advancements in broiler productivity, but it has also coincided with the development and expansion of muscular defects along with an increased susceptibility to stress-induced myopathy that can affect the breast muscles of fast-growing broilers (Baldi *et al.* 2020). Such muscle abnormalities have several implications for the quality of meat products. Chicken meat defects can be grouped in growth-related and pH-related. The first group, composed by white striping (WS), wooden breast (WB), and spaghetti meat (SM) defects, have raised the attention of the scientific community due to the noteworthy incidence levels and its detrimental implications for meat quality and marketability (Figure 4).

Although occurrence levels can vary depending on country, animal age, and live weight, it is assumed that these muscular abnormalities can appear in all countries where fast-growing strains are used for meat production (commercial chicken strains slaughtered at the young age of 30 to 55 days, at weights ranging from 1.5 to 5 kg) (Petracci *et al.* 2013). The WS condition was described by (Kuttappan *et al.* 2012) as the occurrence of white or greyish striations parallel to muscle fiber direction on the surface of *pectoralis major* muscle of heavy birds. The striations have been mainly identified as accumulations of lipids (lipidosis) and connective tissue (fibrosis).

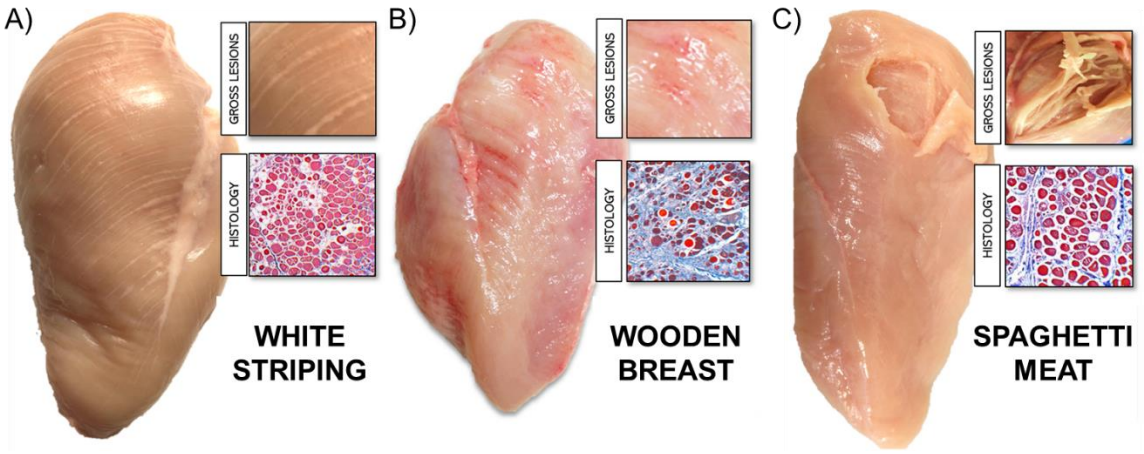

Figure 4 - Scheme reporting macroscopic and microscopic features of the main growth-related muscular abnormalities affecting the pectoralis major of fast-growing broiler chickens. A) White striping breast, B) Wooden breast, C) Spaghetti meat (Soglia et al. 2016).

WB myopathy occurs as a focally or diffusely hardened consistency of breast muscle, which appears pale, rigid, swollen and may have viscous exudate and haemorrhages on its surface (Sihvo *et al.* 2014). WB and WS often take place concurrently within the same muscle and share common histological features. On the contrary, SM condition gets its name from the disconnected muscle fiber bundles composing the meat, which appears mushy, sparsely tight and shaped by very soft and crumbly muscle fibres. Microscopically, SM fillets show inflammatory cells infiltrations and a rarefaction of connective tissue, thus leading to the muscle fiber detachment (Baldi *et al.* 2018).

Changes in the kinetics of post-mortem pH decline mainly affect the pectoral muscle meat because of its high levels of glycogen and glycolytic activity (Debut *et al.* 2003). The normal pH value of broiler breast meat is around 5.8; pH deviations lead to defect occurrence (Figure 5). Chicken breast meat with pH values lower than 5.7 is often associated with PSE-like (pale, soft, and exudative) syndrome, while chicken meat with pH value higher than 6.1 exhibits physicochemical properties that result in DFD (dark, firm, and dry) syndrome (Barbut *et al.* 2005). PSE and DFD meats look unattractive and discriminated against by consumers. They have poor processing characteristics, reduce yield and high potential of

spoilage compared to normal meat. There can also be the danger that consumers will begin to associate poor quality meat to food safety issues (Adzitey & Nurul 2011).

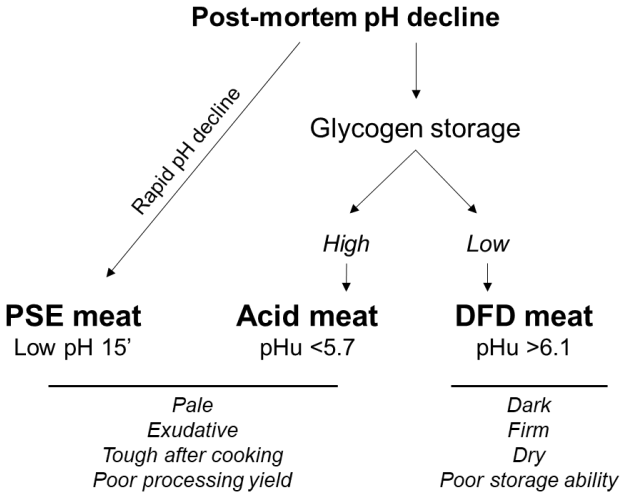

Figure 5 - Schematic summary of Variation in muscle pH decline during post-mortem time and meat quality defect occurrence

The most frequent factor responsible of both PSE and DFD meat is pre-slaughter stress. Exposing animals to intense stress just before slaughtering leads to PSE meat. Acidification occurs in post-mortem muscles due to the conversion of glycogen in lactic acid. In PSE meats, the post-mortem pH decline rate is faster than in normal meat and lower pH values in the final product are reached. These conditions can cause the denaturation of several muscle proteins leading to a reduction in meat WHC. When PSE meat is cut, a high amount of exudate is released, and the great cooking loss values lead to a hard and dry cooked product (Petracci *et al.* 2017). When animals are exposed to chronic or long-term stress before slaughtering, DFD meats can occur. Examples of chronic stress are long distances transportations, long fasting period, and long-time animal overcrowding. Chronic pre-slaughter stress leads to the consumption of quite the total amount of the stored glycogen; thus, the limited quantity of residue glycogen cannot act in the normal process of post-mortem pH decline leading to a high pH of the final product. DFD meat has lost a lot of water and muscle fibres are very close together; it will be tough and stringy after cooking.

Impact of housing system on chicken meat quality

Consumers are increasingly interested in purchasing more healthy and sustainable food products; both free-range and organic chickens are in line with these principles. Housing systems have been recognised to have a large impact on meat quality (Bogosavljevic-Boskovic *et al.* 2010). Meat products obtained through extensive rearing systems are reported to have higher nutritive quality and better taste because of an increased physical activity, a slightly higher amount of feed intake that led also to a significantly lower abdominal fat content compared with birds that are reared in indoor systems (Horsted *et al.* 2010; Bogosavljević-Bošković *et al.* 2012). The notable differences in results obtained with respect to the effects of extensive production systems on poultry meat quality probably came out from the different ages and genetic origins of the studied chickens (El-Deek & El-Sabrout 2019). There is very few scientific evidence about the impact of organic production system on chicken meat quality. (Castellini *et al.* 2002b) reported that the carcasses of organic chickens showed heavier breast and drumstick with lower percentages of abdominal fat. Organic muscles showed lower pH and WHC values which led to lower cooking losses than conventional chicken. Moreover, lightness, shear force, and n-3 PUFA values were higher in organic chicken meat while it presented an increased level of thiobarbituric acid reactive substance (TBARS), related to a greater muscles oxidative status, probably due to greater physical activity. Another study performed by (Grashorn & Serini 2006), compared meat quality parameters of breast meat from chicken reared under organic and conventional systems. Cooking losses, flavour, juiciness, and texture were better in organic meat, though it was less tender than the conventional one. In conclusion, organic production system was assessed to be a good alternative method to provide better animal welfare conditions and good quality of the meat product even though the choice of the chicken genotype remains a key parameter.

SECTION A
Introduction and literature review

CHAPTER 3

The impact of packaging on chicken meat quality

The impact of packaging on chicken meat quality

Food packaging is used to allow for easy transport of goods, protect the integrity of food products, and ensure separation from harmful chemicals, particles, bacteria, and pests. It also comprehends food labelling processing which is crucial to provide information for consumers. As shown in figure 6, food packaging plays a fundamental role across the overall meat supply chain, from farm, processing, cutting, distribution, retail, consumer, and end-of-life food. In this perspective, the food packaging system can influence the amounts of food waste generated both in industries and in households. For example, packaging that is too large or too difficult to empty may hinder the entire consumption of a food item or may lead to more food waste next to higher environmental burdens (Molina-Besch *et al.* 2019). This aspect is described in scientific literature as indirect environmental impact effect while the direct impacts are caused by the production and disposal of packaging materials.

In the last decades, the efforts of scientists and industries have been directed towards sustainable strategies in the field of packaging materials and packaging methods. This led to an increased interest in new environmentally friendly materials to replace non-biodegradable plastics with biodegradable plastics (Kumar *et al.* 2021; Gil & Rudy 2023). The sustainable food packaging area is an ever-growing sector in terms of innovation (Bajer *et al.* 2020).

Regarding poultry supply chain, different types of packaging are used during the production process but the final one, present in supermarkets, is the one that has a direct impact on the consumer's choice. The perishable nature of fresh meat products might also challenge households to store them safely and therefore minimise food waste. Various technological

approaches are being attempted in the current historical period to achieve these objectives of material sustainability and good meat preservation (Gagaoua *et al.* 2022a).

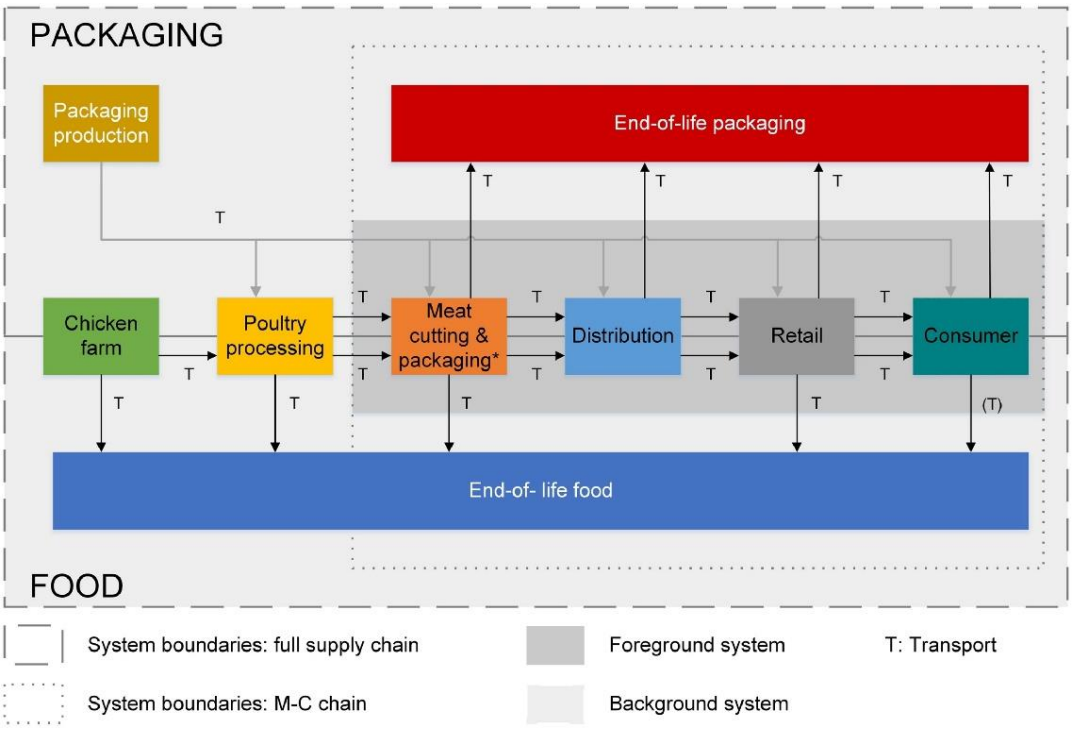

Figure 6 - Schematic representation of chicken supply chain (Cooreman-Algoed et al. 2022)

Currently, a series of new technologies and materials have been developed in food packaging sector (Arvanitoyannis & Stratakos 2012; Lee *et al.* 2015; Ghaani *et al.* 2016; Alessandroni *et al.* 2022; Fernandez *et al.* 2022). Many novel materials are gaining huge interest as they may inhibit microorganism proliferation, enhance sensorial properties of the food product, increase its shelf-life and decrease the food waste and the environmental impact of the packaging.

Active packaging is a novel method that utilizes various active compounds that can interact with the food product or the surrounding environment to extend its shelf life by maintaining food quality, safety, and integrity (Nanda *et al.* 2022). The modified atmosphere packaging (MAP) can be included in this class. MAP packaging is a system that involves a specific

internal gaseous atmosphere in which the food is better and longer preserved (Lee *et al.* 2015). The main task of intelligent packaging is to capture and provide information about the quality and/or safety conditions of the food product. An intelligent packaging system contains smart devices such as labels or tags that can acquire and transfer information about the properties of the packaged food. The most used smart devices in intelligent packaging of meat and meat products are barcodes, time-temperature, gas, freshness, and pathogen indicators (Fang *et al.* 2017).

Petroleum-based plastics are the most used packaging materials due to their stiffness, flexibility, desirable barrier properties, inexpensiveness, and ease of processing. However, recently packaging industries are considering substituting petroleum-based plastics with more sustainable compostable/biodegradable or biobased materials. Several technologies were applied to reach more eco-friendly food packaging solutions.

- Edible coatings are defined as "*soluble formulations applied on food surfaces such that a thin layer of edible film is formed directly on the food surface or between different layers of components to prevent the migration of moisture, oxygen, and solute into the food*" (de Azeredo 2012). Edible films are classified based on their structural material, namely hydrocolloids (polysaccharides and proteins), lipids, and composites. The use of specific coatings can prevent surface dehydration, reduces lipid oxidative reactions, and favors the maintenance of the meat color (El-Obeid *et al.* 2018; Mauricio *et al.* 2022). However, if on the one hand this type of packaging obviates the environmental problem of packaging disposal, on the other hand the food acceptance of these products is a crucial point as consumers seems to prefer recyclable, biodegradable, or compostable materials, which can help in reducing municipal solid waste, CO_2 emissions, as well as reliance on petroleum-based resource (Cox & Evans 2008; Ma *et al.* 2022).

- Recycling is the process of converting used materials into something new. But there are limits to how many times some materials can be recycled. Standard plastics and paper, for example, can usually be recycled only a few times before they become unusable, whereas others, such as glass, metal, and aluminum, can be recycled endlessly (Post *et al.* 2020).
- Biodegradable polymers consist of a chain network of macromolecules, which are acquired from natural sources through enzymatic or chemical polymerization. The European Environment Agency defines a biodegradable material as "*capable of decomposing rapidly by microorganisms under natural conditions (aerobic and/or anaerobic)*" but length of time needed for products to decompose is not fixed. Biodegradable plastics decompose much faster than their traditional counterparts which may take hundreds of years. They are considered, therefore, a more environmentally friendly solution (EEA glossary).
- Compostable products are made from natural materials such as starch and cellulose. Composting is a process that involves biological decomposition of organic matter, under controlled conditions, into soil conditioner (*compost*) without producing toxic residue. The minimum requirements that a packaging must meet in order to be processed by industrial composting are defined in European standard EN 13432 (Standards 2000).

Indeed, biodegradable polymers offer a possible alternative to traditional non-biodegradable plastics when recycling is impractical or not economical (Magni *et al.* 2020). Compostable food packaging can solve the disposal problems of difficult-to-recycle plastic packaging, avoiding their disposal in landfills or incineration. At the same time, compostable food packaging allows the enhancement of organic waste, which, if properly treated, can generate quality compost to restore soil nutrition (Flury & Narayan 2021).

Among the biopolymeric materials used for biopackaging applications, polysaccharides such as cellulose, alginate, gelatin have been proposed. More recently, chitosan has been the most explored polysaccharide material for the development of biodegradable packaging (Díaz-Montes & Castro-Muñoz 2021). Beyond polysaccharide-based materials, various studies proposed the use of proteins-based biopolymers (gluten, whey proteins, or casein) and lipids biopolymers (waxes, oils, free fatty acids). Moreover, biopackaging can be formulated with biopolymers synthesized from bioderived monomers (polylactic acid, polyesters) or produced directly from microorganisms (polyhydroxyalkanoate, polyhydroxybutyrate) (Chen *et al.* 2019; Díaz-Montes & Castro-Muñoz 2021). However, from all the proposed materials, starch is the most used biopolymer for biopackaging formulation due to its mechanical properties close to those of traditional plastics like polyethylene and polystyrene (Jiang *et al.* 2020). Nowadays, studying biopackaging and monitoring their quality to reduce economic loss and food waste is more important than ever. Therefore, a key challenge is to assess the performance of biopackaging like starch-based packaging in preserving food quality during storage, especially easily perishable products.

Brief overview of the Abriopack project

Food packaging is one of the largest and most important market for the development of plastic films able to protect, preserve and prolong the duration of food products. At the same time, the constant increase in the production of plastics means high environmental impact and high management costs for the correct waste disposal. The aim of the ABRIOPACK project is therefore to replace the traditional materials used in agroindustrial packaging with a biodegradable packaging suitable for the preservation of antibiotic-free chicken meat. Furthermore, the project aims to consider the effects of the scrap materials of this process (poultry manure and composted bioplastics) on the soils destined to the production of farming raw materials, in a circular economy perspective. The innovation is to apply a model

of circular economy with low environmental impact to an agroindustrial system. The ABRIOPACK project will lead to the achievement of a pack (bowl, label and film) for antibiotic-free white meat entirely made from biodegradable and compostable material. This innovation will enable the reduction of mixed waste and the recycling of organic material through a virtuous and economically sustainable end of life (composting).

This project, founded by Marche Region, involves several partners divided in farming companies (Carnj Cooperativa Societa' Agricola (Leader), Società Agricola Sorriso SRL and Società Agricola Biologica Fileni SRL), research institutions (University of Camerino, Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati" and Novamont SPA) and communication and information company (ARCA SRL Benefit).

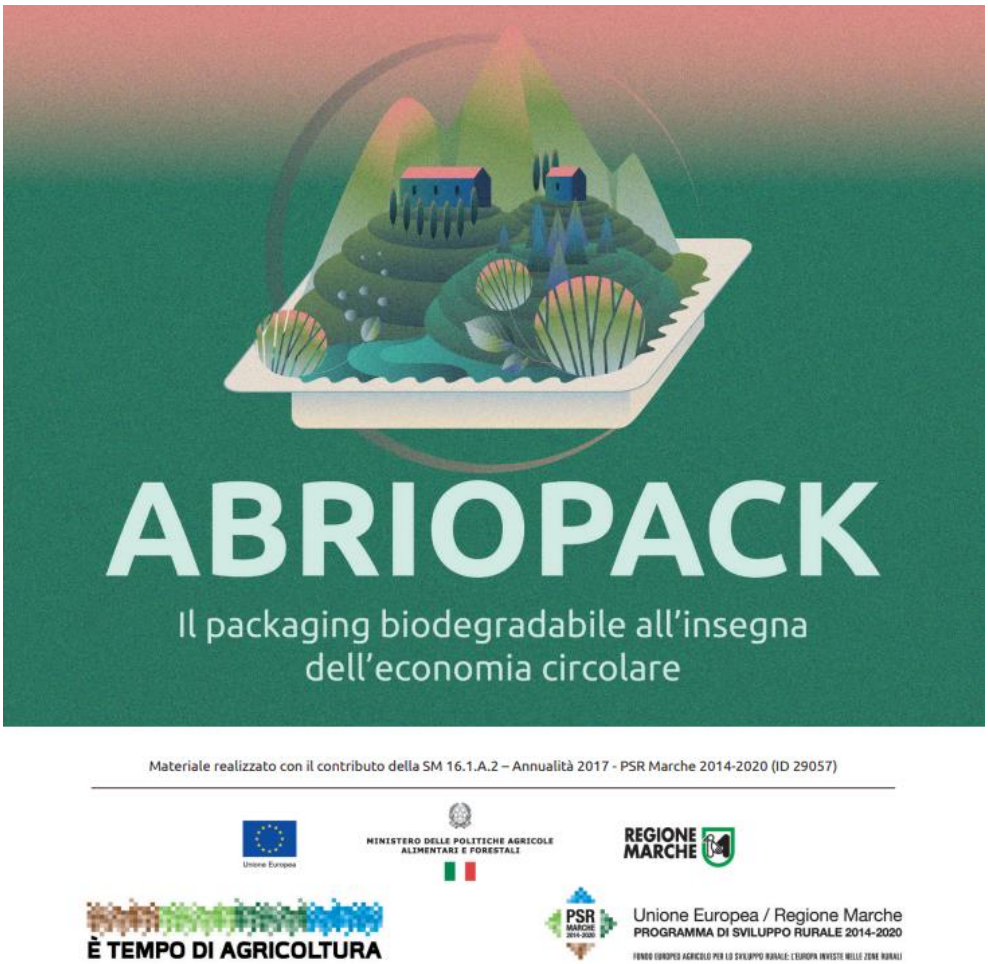

Figure 7 - Abriopack project poster

Mater-Bi: from starch to biopackaging

Starch is one of the most important biodegradable polymers because of its abundance, low cost, biodegradability, and renewability. Starch-based films show excellent film-forming ability and unique gelatinization properties, along with their odorless, tasteless, and colorless nature (Zhu *et al.* 2022). Starch-based films have been widely used in the packaging of different types of food products (such as meat, fruit, oil, and cheese) as they manage not to impact sensorial characteristics of the food and present gas barrier properties (Pelissari *et al.* 2019). However, starch-based materials are easily breakable and hydrophilic, which limits their processing and use. To increases the strength of the processing properties of the materials, starch can be mixed with various natural polymers to improve its properties (Gil & Rudy 2023). In nature, starch is present as crystalline beads of approximately 15 -100 μm in diameter, it can be found in three crystalline modifications designated A (cereal), B (tuber), and C (various beans), all characterized by almost perfect double helices (Bastioli 1998). Starch can be made thermoplastic according to a technology very similar to extrusion cooking. Extrusion cooking and forming is characterized by compression and heat applied to a cereal-based product to completely gelatinize all the ingredients. A thermoplastic starch can be solubilized without any formation of maltodextrins, and its solubility depends on the used extrusion temperature, on the moisture content of the starch before extrusion and on the amylose/amylopectin ratio. Thermoplastic starch can be processed as a traditional plastic but its sensitivity to humidity makes it unsuitable for most applications (Puglia *et al.* 2003). Nowadays, there are four classes of Mater-Bi products available on the market discriminated in terms of processability, physico-chemical and mechanical properties and biodegradation behavior (Bastioli 1998).

- Class Z: Biodegradable and compostable, mainly for films and sheets. They contain thermoplastic starch and polycaprolactone and were introduced into the market at the beginning of 1992. This grade has the "OK COMPOST" certificate.
- Class V: Biodegradable, compostable and soluble, used as a replacement for polystyrene packaging foams. They have a starch content > 85% and are defined as thermoplastic plasticized starch.
- Class Y: Biodegradable and compostable, for rigid and stable shaped items; they contain raw materials from natural origin, such as thermoplastic starch in a dispersed form and cellulose derivatives. Their mechanical properties and moldability are very close to those of polystyrene.
- Class A: Biodegradable, non-compostable materials They contain thermoplastic starch complexed with ethylene-vinyl alcohol copolymers. Their main uses are in sectors where compostability is not required.

Mater-Bi materials are designed for films and layers. At industrial level, the films and bags can be produced using traditional equipment for low density polyethylene reaching similar productivities. An important example of a successful application of these products is represented by the compostable bags for organic waste collection already used by millions of European citizens. Besides composting and municipal organic waste bags, other uses of Mater-Bi products are in the field of short-life applications, such as shopping bags, wrapping film, make-up remover pads, biodegradable cotton swabs, disposable cutlery etc. Currently, the Italian company Novamont owns a wide patent portfolio and is international leader in the bioplastics sector. Today it is the main European producer of Mater-Bi materials as the result of a significant investment in research and development from early nineties.

SECTION A
Introduction and literature review

CHAPTER 4

Proteomics in poultry meat research

Proteomics in poultry meat research

Introduction to Proteomics

The “omics” sciences emerged during the last two decades and have been greatly applied in many research fields. From the new findings in genetics genomics and transcriptomics have stemmed, which focus on the analysis of DNA and gene expression modulation (Tyers & Mann 2003). However, due to the presence of mutations in the coding sequence, variations in the starting site for translation of mRNA, and post-translational modifications, several millions of protein species are present in tissues. Proteins are the functional units that constitute the cell engine, while the proteome was defined as the complete set of proteins characterized according to their localization, interactions, post-translational modifications, and turnover at a given time point and for a given condition. Proteomics supplements the other “omics” technologies to expound proteins identity, structure, functions, and interactions with other proteins in the control cellular activity (Aslam *et al.* 2017). The discipline that combines “omics” techniques to improve quality and safety of food product for consumer’s health and well-being, and to increase our knowledge on the underlying pathways and mechanisms behind technological and sensorial qualities of foods, is called “foodomics”. It comprehends all the OMICs-based approaches such as genomics, transcriptomics, proteomics, metabolomics, lipidomics, etc. that can be applied in food science (Gagaoua *et al.* 2020; Munekata *et al.* 2021; Gagaoua & Picard 2022). In recent years, among the foodomics approaches used in meat science, proteomics has been chosen by the majority of researchers for several reasons. Firstly, the analysis of the complex proteome has been enhanced by the improvement of high-resolving separation systems,

along with the even more accurate high-resolution tandem mass spectrometers. Such analytical potential is extensively used for quantitative analysis (Zhu *et al.* 2021). Secondly, the proteome is the main constituent of muscle tissue, and it has crucial roles in the biochemical changes occurring during the muscle-to-meat transformation. On this line, proteomics allows a better understanding of the mechanisms related to this phenomenon. Thirdly, proteomics was successfully applied to investigate mechanisms at the origin of the variability of several meat quality traits and defects (Purslow *et al.* 2021; Gagaoua & Zhu 2022).

In poultry meat science, understanding the full proteome provides information about the cellular activities, mechanisms for signalling between cells and tissues, and the causes of diseases and defects such as microbial infections and muscle myopathies (Zhang *et al.* 2022). High throughput and large-scale proteomics approaches are important means to analyse not only meat quality but also to understand how food processing is affected by the physiology of the product and through this, to enable the optimization of the overall food production process (Pedreschi *et al.* 2010). The main used methodology to investigate proteome develops in two steps: protein extraction and separation from the cell or tissue to reduce sample complexity, and identification and quantification using mass-spectrometry (MS) (Toldrá & Nollet 2012; Mora *et al.* 2018). Obtained data are then processed through statistical tests to list differential protein, bioinformatics analysis and verification of proteins functions and related molecular mechanisms. In food samples, the most employed chemical and biochemical techniques for sample purification and analysis are one-dimensional (1DE) or two-dimensional (2DE) electrophoresis, gas-chromatography (GC) and high-performance liquid chromatography (HPLC). Even though these methods can be extremely helpful for routine separation analyses, they can merely be descriptive, as they always need a reference value (standard) to give reliable results. The combination with spectroscopic methods turned

out to be very helpful to overcome these limitations. The MS is widely used in proteomics due to its high versatility, sensitivity, specificity, and capability of high-throughput screening. In MS-based proteomics, for instance, it is possible to explore the whole range of proteins and peptides in a sample coupling MS with a prior separation step (electrophoresis and/or chromatography). MS technology can provide the molecular mass and the fragmentation of the molecules, which is a key feature to identify the proteins and even characterize unknown ones. Moreover, chemometric and bioinformatic tools, are the basis of data analysis of proteomic approaches (Ferranti *et al.* 2015; Capozzi *et al.* 2017).

The two main ways to perform MS-based proteomic are called the bottom-up and the top-down approach and differ for the type of MS instruments used and the separation technique. The separation by 2DE followed by an in-gel proteolytic digestion and the analysis of the produced peptides by matrix assisted laser desorption ionization-time of flight (MALDI-TOFMS) or HPLC-MS/MS and subsequent database search are characteristic steps of the bottom-up approach, which is the most widely used workflow. The 2DE allows the resolution of complex protein matrices, but it presents some disadvantages as it is very laborious and has a limited dynamic range (Ferranti 2018). Moreover, the in-gel digestion is a crucial step, which can provide some problems: a peptide or several peptides may be related to more than one protein or protein form, large regions of the protein may not be identified, and modifications or sequence variations may occur on disparate peptides following digestion (Chait 2006). Top-down approach can eliminate these problems by injecting the intact protein into the mass spectrometer so both its intact and fragment ions masses are measured. This approach allows to have a 100% sequence coverage so a full protein characterization, the protein form resulting from combinations of genetic variation, alternative splicing, and post-translational modifications. The potential ability to obtain full protein characterization has led this approach highly helpful for single proteins or simple

mixtures analyses. However, the technical difficulty of full proteome investigation, in terms of coverage, sensitivity, and throughput, has caused Top-down proteomics to be less diffuse than the Bottom Up in complex matrices analysis such as food (Catherman *et al.* 2014).

Objectives of using proteomics in meat research

Proteomics has been used in meat research to overcome the drawbacks of the traditional methods and to explore meat quality in ever greater detail (Purslow *et al.* 2021), hence improving both the accuracy and sensitivity thanks to the large quantities of data that can be obtained. Considering the different purposes for which proteomics approaches/methodologies have been used as innovative tools to study meat quality variation/determination and the origin/reasons of the appearance of certain quality defects (Gagaoua & Picard 2022), three major objectives can be identified (Figure 8).

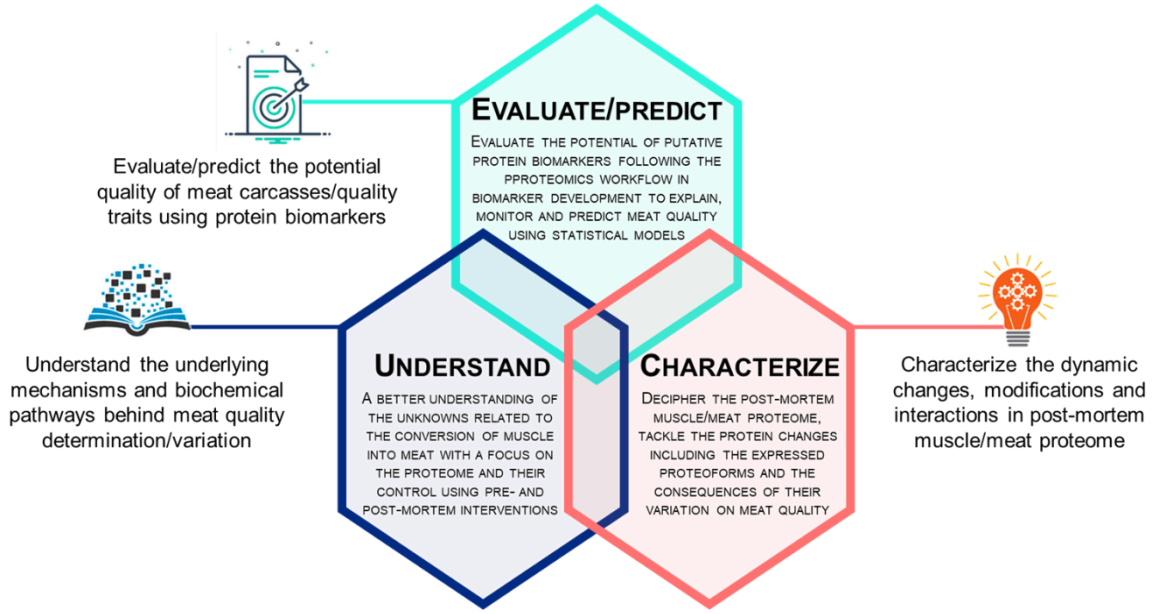

Figure 8 - The major three objectives of proteomics as a tool enabling quantitative analysis and systematic characterization/identification of the post-mortem muscle and meat proteome (whole proteins) present in tissue or cell rather than focusing on a single protein to study meat quality variation and its defects (Gagaoua et al. 2022b).

First, proteomics is used to characterize and decipher the dynamic changes, modifications and interactions in post-mortem muscle/meat proteome. In this sense, mono-dimensional SDS-PAGE gel electrophoresis can be a valuable tool to quickly investigate the proteolytic and physicochemical mechanisms involved in meat texture development, more specifically to target the proteolytic fragments appearing during storage and tenderization of meat, to be used as indicators of the rate and extent of tenderization (Gagaoua *et al.* 2021b) or to reveal defective quality (Hughes *et al.* 2019; Gagaoua *et al.* 2020).

Second, proteomics is used to better understand the underlying mechanisms and biochemical pathways underpinning the determination and variation of the phenome (phenotypes of interest in animal production including meat quality traits), especially because the proteome is the main constituent of muscle tissue, and it has important roles in the biochemical changes occurring during the transformation of muscle into meat and structural/metabolic changes in post-mortem muscle.

Third, the main and challenging outcome for which proteomics is used is to evaluate, predict and monitor the potential quality of carcasses and meat quality traits near line or at line based on protein biomarkers, and therefore to propose (i) explanatory mechanisms at the origin of the variability and (ii) predictive equations of the major eating quality traits. Therefore, proteomics workflows provide a complementary analysis tool to other OMICs approaches to uncover novel biomarker candidates.

From the above, proteomics can be considered as a sophisticated data-driven tool rather than hypothesis-driven research, allowing insights into the metabolic state of the post-mortem muscle and meat matrix, therefore responding to the question about the metabolic and biochemical reactions occurring in the post-mortem muscle and during storage (Gagaoua *et al.* 2021b; Della Malva *et al.* 2022). Proteomics was further used to decipher, thanks to

comparative proteomics, the differences that exist as a consequence of different treatments likely the pre-slaughter conditions or management and production systems (Sierra *et al.* 2021; Gagaoua & Zhu 2022).

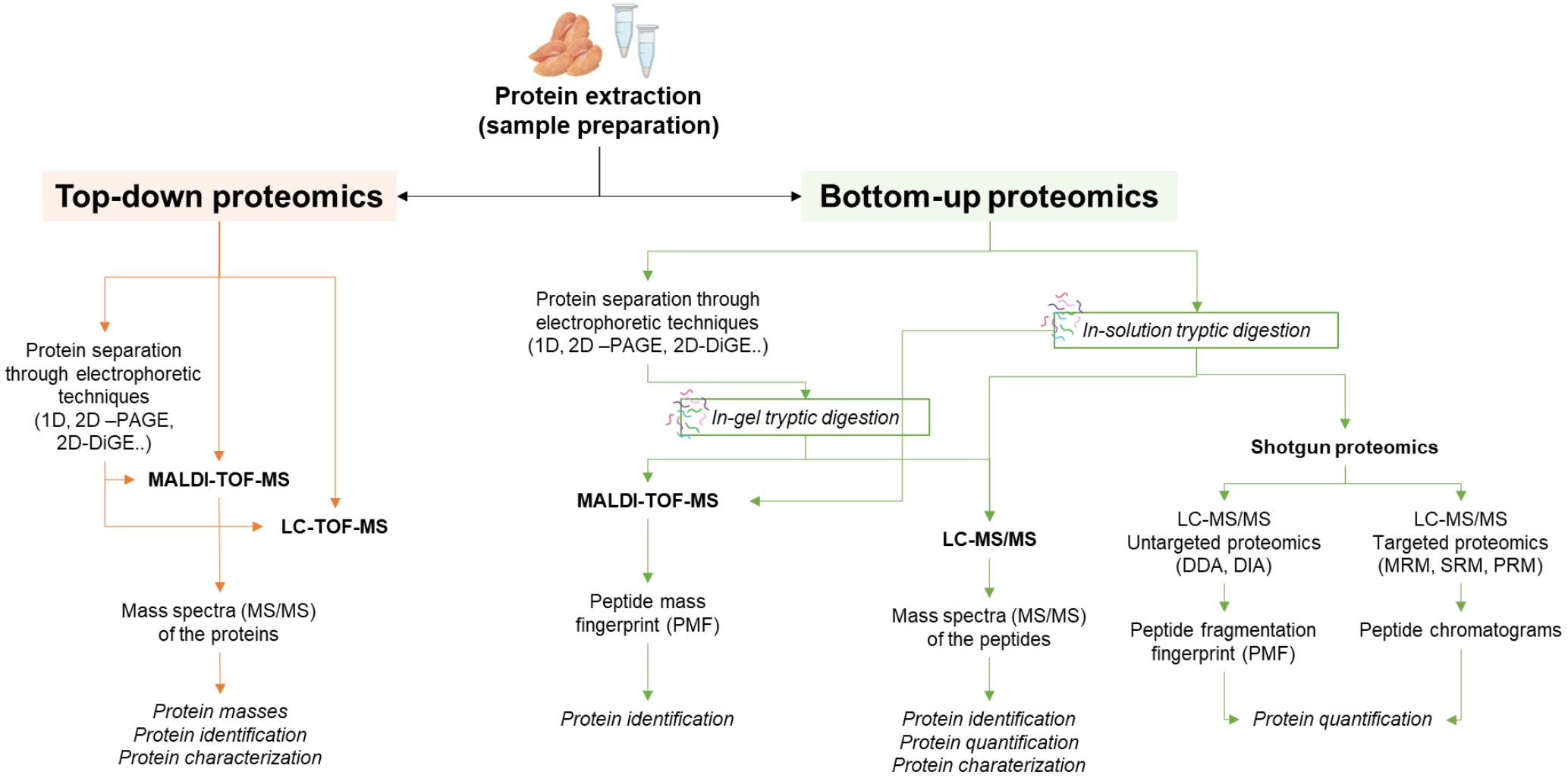

Figure 9 - General schematic representation of proteomic analytical techniques. Figure adapted from Gagaoua et al. 2022b.

Targeted Proteomics

Protein identification has been dominated by data dependent acquisition methods (DDA), where the MS instruments scan the precursor ions (peptides) separated from a chromatographic system, followed by the selection of a limited set of ions to be fragmented, usually the most intense ones (Figure 10). The obtained fragmentation spectra are characteristic of a specific peptide and will then be used for its identification. This method is particularly effective, but it presents some disadvantages that has limited its use in protein quantification in multiple samples such as the tendency to be biased towards the most abundant proteins, which makes it particularly inaccurate when working with a high sample complexity and/or dynamic range. Its widespread diffusion was because the quantitative MS in proteomics was initially limited to untargeted proteomic screenings of a limited set of defined targets (peptides), or to the Multiple Reaction Monitoring (MRM) method, the gold standard method for MS quantification (Anjo *et al.* 2017). However, label-based approaches resulted to have a highly challenging sample preparation and a high cost of analysis that can limit the number of analysed samples, moreover, targeted analysis requires a very hardworking acquisition method development needing the selection of the peptides and respective transitions. These methods can characterize only a limited number of proteins in a single LC-MS run and outcoming in a partial coverage of the sample proteome (Bruderer *et al.* 2015; Guo & Huan 2020). To address the limitation of DDA in acquiring MS/MS spectra, the recent implementation of DIA can generate MS/MS spectra for all precursor ions, therefore capable of detecting and identifying a larger number of compounds. In DIA mode, the MS instrument cycles through the precursor ion m/z range with a large precursor ion mass width to fragment more than one precursor ion simultaneously (Figure 10) (Zhu *et al.* 2014). Commonly used DIA methods include all-ion fragmentation, where all precursors are fragmented, and sequential window acquisition of all theoretical spectra (SWATH),

where a medium window (20-25 Da) is used. Although DIA is attractive for theoretically furnishing MS/MS spectra for all *m/z* acquired in full-scan, the link between the intact peptide and its fragments is lost leading to complex MS/MS spectra. Thus, for the protein identification step, a great informatics challenge to deconvolute the raw MS/MS spectra is needed. Several DIA acquisition methods were developed thanks to novel high resolution mass analysers (HRMS) and/or using different fragmentation methods. Generically, DIA methods can be classified in two groups, those that can simultaneously acquire the fragmentation spectra of the mass range, and those that divide the m/z range in sequential isolated windows and scan all the fragmentation spectra in a specific window, resulting in a reduction of the complexity of the MS data (Chapman *et al.* 2014; Guo & Huan 2020). In both acquisition methods, quantitative information is obtained from the precursor ion signal, while the fragmentation spectra are used for peptide identification. SWATH-MS can be considered as the untargeted fitting of targeted methods such as high-resolution MRM (MRM-HR) and parallel reaction monitoring (PRM) using high speed and HRMS analysers that allow an accurate quantification even of complex samples. Contrary to the other targeted methods, any individual preselection of the precursors is automatically performed in SWATH-MS which reduce the method development time not limiting the number of analysed peptides. Usual SWATH-MS method combines a parallel analysis of samples with an optimized DDA method for peptide identification followed by a DIA acquisition to be used to extract the quantitative information of the previously identified peptides.

At the same time, the innovation of SWATH-MS method is also related to its targeted data extraction from the acquired data file, in fact instead of taking quantitative data from the precursor intensity (as performed by the other DIA methods), it works with the quantification of the intensity of the fragment ion (MS2 intensity) (Gillet *et al.* 2012; Midha *et al.* 2020).

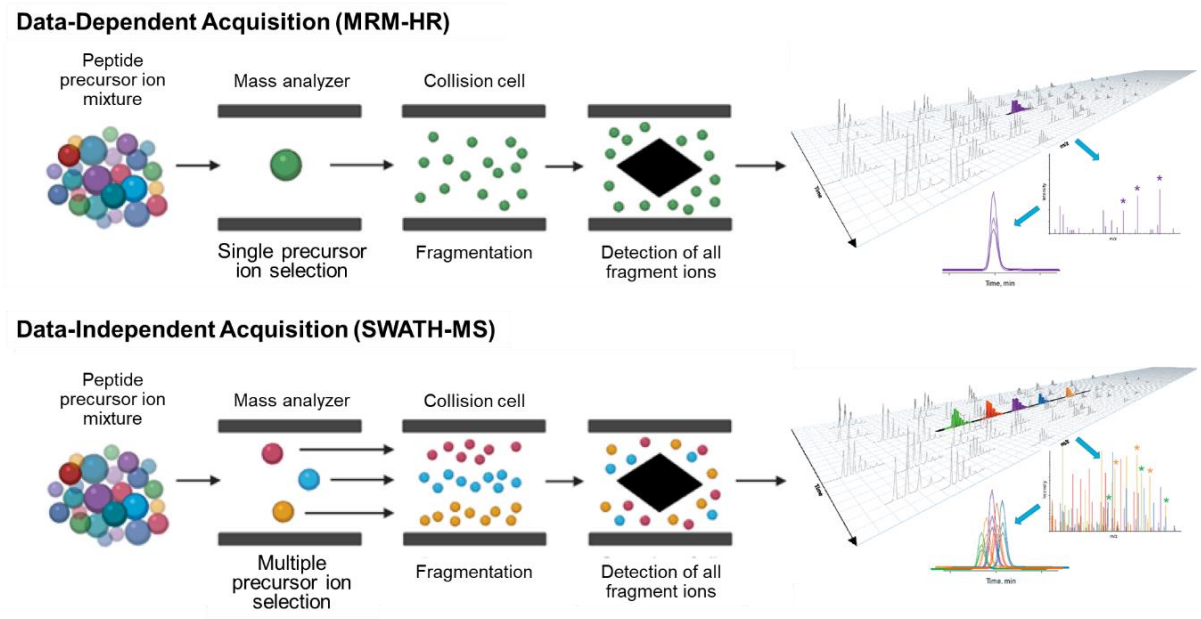

Figure 10 - Comparison between MS^2-based quantification methods: schematic representation of DDA acquisition method using high resolution MRM and of DIA acquisition using the SWATH-MS technique (Neagu et al. 2022)

In this perspective, SWATH-MS a promising strategy for biomarker discovery from large-scale screenings (Marquioni *et al.* 2021). In conclusion, the choice between DDA and DIA is dependent both on the experimental design and on the availability of the analytical platform. Next-generation hybrid instruments have gained not merely in terms of resolution, but also in their acquisition frequency which allows easier DIA analysis.

Application of proteomics in the evaluation of chicken meat quality

Differences in protein abundance reflect differences in gene expression. Specific proteomic signatures that are differentially expressed provide clues on biochemical processes behind the poultry meat quality and allow the determination of biological markers that are indicators of quality defects. Meat quality is determined by several complex factors previously described in *chapter 2*. In general, the most common factors used to evaluate the meat quality are pH, WHC, color, tenderness, and flavours. The only application of traditional methods such as texture test, color and drip loss measurements, and flavour composition are not able

to fully achieve the aim of control and predict meat quality. Indeed, the meat characteristics are controlled by multiple genes, breeding management, processing techniques, and conditions, so adulteration detection and protein function in meat quality description are a big challenge today. The proteomics approach not only increase our understanding of the cellular and biochemical mechanisms involved in muscle-to-meat processes but also helps meat industries to develop new technologies to improve the quality of meat products (Cao *et al.* 2022).

Therefore, using proteomics techniques to find the molecular pathways and biomarkers related to meat quality traits has become an important topic of research. Although proteomics research has been successfully applied to find biomarkers and study molecular mechanisms related to the quality of different kind of livestock meat such as beef (Gagaoua *et al.* 2021b; Gagaoua *et al.* 2021c), pigs (Marrocco *et al.* 2011), sheep (Zhao *et al.* 2022), donkey (Della Malva *et al.* 2022), etc., there are limited reports on similar studies on poultry. Proteomic studies in poultry mainly include proteomics in muscle development that is related to growth performance, infectious diseases, and breast meat defects (PSE, DFD, WB, WS, SM). However, some applicative challenges are still present in this field. The main complication is the understanding of the post-translational modifications, in fact, protein structures can be modified after their synthesis, by the addition of chemical groups, polypeptides, complex molecules, directly modification of amino acids, or proteolysis (Spoel 2018).

The achievements of proteomics in chicken meat quality traits and studied biomarkers are resumed in Table 4. The technique of choice for this kind of analysis is the 2DE-MALDI-TOFMS. (Teltathum & Mekchay 2009) isolated the differentially abundant proteins (DEPs) from the breast meat of Thai local chickens previously discriminated according to their tenderness (high and low shear force) and characterized and quantified their expressions. Three proteins were identified as DEPs of chicken breast meat tenderness: pyruvate kinase

(PKM), phosphoglycerate mutase 1 (PGAM1), and triosephosphate isomerase1 (TPI1). All these proteins are related to the glycolytic pathway. They showed a positive correlation with the meat shear force values as they were significantly up-regulated in lower shear force group. Obtained results were confirmed by (Mekchay *et al.* 2010), that used the same approach to compare the Thai local chicken and commercial broiler. Other studies have confirmed that glycolytic proteins have a high impact on meat tenderness and toughness from different animal origin (Picard 2020; Gagaoua *et al.* 2021b). This indicate that these three proteins can be used as the chicken tenderness putative biomarker proteins. (Phongpa-Ngan *et al.* 2011) investigated DEPs of broiler breast in high and low-WHC groups 2DE-MALDI-TOFMS technique. Three DEPs were identified, namely heat shock protein 70 (HSPA2), TPI1, and PKM. HSPA2 was proved to be closely related to oxidative stress as previously published by (Stagsted *et al.* 2004). TPI1 and PKM are related to cell glycolysis and energy supply. These pathways were proven to be linked to shear forces as previously described. These results could provide an important basis for biomarkers research in chicken production and meat quality improvement. Unfortunately, due to the presence of limited scientific evidence on the use of proteomics to study chicken meat, specific biomarkers for chicken breast meat quality traits have not yet been defined.

Table 4 – Summary of proteomic approaches in the study of chicken meat quality traits

Studied factors	Animal protein source	Age at slaughter	Sampling time	Proteomics method	Database/ platform	Main findings	Reference
Meat tenderness	Mixed-sex Thai native chickens and commercial broilers breast meat	16 weeks	early post-mortem	2DE-MALDI-TOF	NCBI, Swiss-Prot and Mascot	Expression of proteins related to the glycolytic pathway showed a positive correlation with the meat tenderness	(Teltathum & Mekchay 2009)
Meat tenderness	Mixed-sex Thai native chickens and commercial broilers breast meat	16 weeks for Thai native and 6 weeks for commercial chickens	early post-mortem	2DE-MALDI-TOF	NCBI, Mascot	Glycolytic protein expression has a high impact on meat tenderness in both chicken strains	(Mekchay *et al.* 2010)
Growth rate and WHC	Breast meat from randomly bred genetic line segregated into high and low growth rates and high and low WHC	6 weeks	2 days post-mortem	2DE-MALDI-TOF	NCBI, Mascot	TPI1 and PKM as cell glycolysis and energy metabolism related proteins were proven to impact both on tenderness and WHC values	(Phongpa-Ngan *et al.* 2011)

Proteomics in chicken breed characterization

Different chicken breed implies differences at the gene level, therefore in protein expression. Chicken genotypes affect the ability to adapt to farming conditions, gene expression, proteomic profile, and meat quality. There is a paucity of papers about proteomics for the investigation of biomarkers to identify chicken strains, five studies were found in literature and are reported in Table 5. A first proteomic approach in the differentiation of broiler chicken breeds was published in 2007 (Jung *et al.* 2007). Muscle samples from Cornish and Large White breeds were subjected to 2DE-MALDI-TOFMS analysis resulting in 17 identified protein spots. Among them 4 DEPs were reported between the two chicken breeds, PGAM1 in pectoralis major muscle, and nuclear RNA export factor 2 (NXF2), superoxide dismutase (SOD1) and troponin I (TNNI2) in peroneus longus muscle.

A similar 2DE-MALDI-TOFMS technique was used to characterize DEPs from the chicken breast of three Italian chicken breeds at 190 days of age (Padovana, Ermellinata di Rovigo, and Pe`poi). A total of 10 DEPs were characterized. Padovana had four marker proteins: cofilin-2 (CFL2), annexin A5 (ANXA5), apolipoprotein A-I (APOA1) and myosin light chain (MYL1). Ermellinata chickens had four marker proteins: bromodomain-containing protein 4 (BRD4), protein LZIC, glycerol-3-phosphate phosphatase (PGP) and growth factor receptor-bound protein 2 (GRB2). The Pe`poi chicken had three marker proteins: heat shock protein beta-1 (HSPB1), lactoylglutathione lyase (GLO1), and MYL1 (Zanetti *et al.* 2011). These biomarkers are related to interconnected cell pathways such as muscle contraction, energy metabolism, regulatory processes, and other binding and transport processes.

The contributions of muscle proteins expression to meat flavour from Korean native chickens and commercial broilers were investigated by (De Liu *et al.* 2012) using 2DE-MALDI-TOFMS approach. MS identification showed that phosphoglucomutase 1 (PGM1), PGAM1, ATP synthase subunit beta (ATP5F1B), myosin heavy chain (MYH1), cytochrome

c reductase (UQCRC1), and DNA methyltransferase 3B (DNMT3B) were overexpressed in Korean native chickens. The authors assessed that these proteins could contribute to meat quality traits, especially the flavour. In fact, low molecular weight peptides, that can be produced in chicken meat during processing served as precursors for Maillard reaction and might contribute to the formation of key volatile organic compounds (VOCs) during heating (Zhang *et al.* 2022).

A novel approach which coupled 1DE separation with nano-LC-MS/MS performed on a linear ion trap mass spectrometer (LTQMS) was proposed by (Zapata *et al.* 2012). A proteomic characterization of the sarcoplasmic proteins in the *pectoralis major* and *supracoracoideus* breast muscles was conducted for two different chicken genotypes, Ross 708 as commercial broilers and Leghorn chicken. Results suggested that glycogen phosphorylase (PYGL), CFL2, elongation factor 1 (EEF1A), creatine kinase (CKM), fructose-bisphosphate aldolase C (ALDOC), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were DEPs in the breast meat of the two studied chicken breeds. In conclusion, these differences indicate a shift in the energetic metabolism in muscle growth between the strains as the proteins identified were mainly related to glycolytic and ATP-related pathways.

Table 5 – Summary of proteomic approaches in the study of chicken strains

Chicken strains and protein source	Age at slaughter	Sampling time	Proteomics method	Database/ platform	Main findings	Reference
Cornish and Large White breast meat		early post-mortem	2DE-MALDI-TOF	Mascot	Four DEPs related to muscle structure, energy metabolism and oxidative stress were reported comparing the strains	(Jung *et al.* 2007)
Low diffusion Italian male breast meat (Padovana, Ermellinata di Rovigo and Pe`poi)	190 days	15 min post-mortem	2DE-MALDI-TOF	Mascot	Four marker proteins were reported for Padovana, four for Ermellinata and three for Pe`poi chickens	(Zanetti *et al.* 2011)
Korean native chicken and Ross broilers breast meat	35 days for commercial chickens and 77 days for Korean native	24 h post-mortem	2DE-MALDI-TOF	NCBI and Swiss-prot	In strain comparison, seven DEPs spots were reported, mainly related to meat flavour traits	(De Liu *et al.* 2012)
Ross 708 and Leghorn broilers breast meat	28 days	early post-mortem	1DE-LC-MS/MS	NCBI and Mascot	Different energetic metabolism in muscle growth between the strains with DEPs mainly related to glycolytic and ATP-related pathways.	(Zapata *et al.* 2012)
Five genetic strain broilers breast meat	8 weeks	24 h	2DE-LC-MS/MS	NCBI and Mascot	Broiler strain affect the protein expression and the meat quality (WB and normal). One particular strain was reported to be genetically more inclined to develop WB.	(Zhang *et al.* 2021)

Proteomics applications to study chicken meat abnormalities

The application of proteomics in chicken myopathies and meat quality defect mainly involves comparison between normal and PSE or WB breast meat as shown in Table 6.

PSE meat is a major quality defect of broiler breast (*see section 2.6*). It results from the combination of elevated muscle stress and acidic pH leads to rapid proteins breakdown. The denaturation of myoglobin increases the reflectance of light on the meat surface which lead to meat paleness. PSE meat tissue is dry and tough after cooking. All these factors negatively impact the meat quality and consumer acceptance. (Desai *et al.* 2016) studied the whole muscle proteome of normal and PSE broiler breast meat and identified 13 DEPs. PGAM1, alpha-enolase (ENO2), ATP-dependent 6-phosphofructokinase (PFKM), and fructose-bisphosphatase (FBP1) were overabundant in normal meat, whereas PKM, CKM, MYH1, β-enolase (ENO3), malate dehydrogenase (MDH2), phosphoglycerate kinase (PGK1), actin alpha (ACTA1), carbonic anhydrase 2 (CA2), and proteasome subunit alpha (PSMA4) were overabundant in PSE broiler breast. These results denoted that the differences in expression of proteins involved in glycolytic, muscle contraction, proteolysis, and energy metabolism pathways could be related to the meat quality differences between normal and PSE breasts.

(Xing *et al.* 2017) aimed to identify the changes in protein profiles in PSE-like and normal breast meat of broiler subjected to pre-slaughter stress. Proteomic analysis, based on isobaric tags for relative and absolute analysis quantitation (iTRAQ), allowed the identification of 113 proteins and comparative analysis revealed 31 DEPs between the two stressed groups (normal and PSE-like). Identified proteins were involved in interconnected pathways. In particular, an overexpression of proteins related to energy metabolism, calcium signalling, and regulation of cellular processes showed to have a negative impact on the meat quality.

(Kuttappan *et al.* 2017) compared the proteomic profiles of normal and myopathic chicken breast muscle tissue using a 2DE-LC-MS/MS method. Over 800 proteins were identified

among which 62 were demonstrated to be DEPs between normal and myopathic breast meat. A down-regulation of glycolysis and gluconeogenesis pathways and up-regulation of eukaryotic initiation factor-2 (eIF-2) signalling, mechanistic target of rapamycin (mTOR) signalling, and eIF4 and p70S6K signalling were reported in myopathic muscle tissues. These changes in protein metabolism and translation could be related to higher growth rate and degenerative changes in myopathic muscle tissues. A similar study investigated the changes in protein expression between normal and woody breast meat in relation to meat quality traits (Cai *et al.* 2018). Nine DEPs were identified, six proteins overabundant in WB meat samples and three in normal breast meat. An increased oxidative stress and decreased glycolytic activity was observed in WB meat according to overabundant proteins related pathways.

Early post-mortem changes in the whole muscle proteome from normal and WB breasts at 0 min, 15 min, 4 h, and 24 h after slaughter were analysed using 2DE-LC−MS/MS by (Zhang *et al.* 2020b). A total of 19 proteins were identified among four post-mortem time points in either NB or WB muscles. Elongation factor 2 (EEF2), EH domain-containing protein 2 (EHD2), PGAM1, and T-complex protein 1 subunit gamma (CCT3) were differentially abundant in both NB and WB muscles during the early post-mortem storage. Changes in protein degradation processes were supposed in WB samples, because of the detection of desmin (DES), ovotransferrin (TF), and TNNI2 in form of fragments while all the other protein spots were full-length proteins. Moreover, in WB, some glycolytic proteins were overexpressed, including ENO3, PGM1, PGAM1, and PKM. The same research group applied this technique to study the proteome differences between normal and WB of different genetic broiler strains at different post-mortem time points (Zhang *et al.* 2020a). A total of 24 DEPs were identified, 21 of these were overexpressed in WB meat and three in normal meat. The WB overexpressed proteins are mainly involved in carbohydrate metabolism,

oxidative stress, cytoskeleton structure, transport and signalling. Two of the five analysed chicken strains had more protein profile differences between WB and normal meat than the others, which potentially indicates a stronger genetic component for these strains to WB formation.

These results underlined how broiler genetics have a primary impact on the animal growth rate, feed conversion ratios, carcass weight, and composition. Genetic variation was studied on five commercially available broiler strains and related to the formation of WB meat (Zhang *et al.* 2021). It was demonstrated that proteomes of broiler breast muscle act as a molecular link between the genome and phenotypic characteristics. Breast proteomes of a specific strain (called strain 5) evidenced an increased apoptosis, protein synthesis, muscle contraction, and high oxidative stress in comparison to other strains. This suggested that these birds are genetically more disposed to develop WB. In summary, proteomic analyses indicated that the chicken breast abnormalities are related to different factors as genetics, nutrition, pre-slaughter stress and rigor mortis. In the future, he genome sequencing of broiler strains will facilitate the application of proteomics to detect biomarkers for meat defects and myopathies.

Table 6 -Summary of proteomic approaches in the study of chicken abnormalities

Meat defect	Studied factors	Animal protein source	Age at slaughter	Sampling time	Proteomics method	Database/ platform	Main findings	Reference
PSE	Meat quality	Male Hubbard × Cobb 500 breast samples	40, 47, and 54 days	24 h post-mortem	2DE-LC-MS/MS	Mascot and UniProtKB	Meat quality differences between normal and PSE breasts are related to differences in expression of proteins involved in muscle contraction, glycolysis, proteolysis, and energy metabolism pathways.	(Desai *et al.* 2016)
PSE	pre-slaughter stress	Arbor Acres chicken breast meat	42 days	early post-mortem	iTRAQ-LC-MS/MS	UniprotKB	Overexpression of proteins mainly related to energy metabolism, calcium signalling, and regulation of cellular processes have a negative impact on the meat quality.	(Xing *et al.* 2017)
WB/WS	meat myopathies	Male broiler breast meat samples	52 days	early post-mortem	2DE-LC-MS/MS	MaxQuant	Overexpression of proteins related to protein biosynthesis pathways were found in breast muscle with severe myopathies	(Kuttappan *et al.* 2017)
WB	meat quality	Ross 708 broiler breast meat samples		24 h post-mortem	2DE-LC-MS/MS	Mascot and UniProtKB	Overexpression of proteins related to oxidative stress and under-expression of glycolytic enzymes in WB meat.	(Cai *et al.* 2018)
WB	post-mortem events	Male broiler breast meat samples	8 weeks	0 min, 15 min, 4 h, and 24 h post-mortem	2DE-LC-MS/MS	Mascot and NCBI	WB meat showed a faster and more oxidative muscle-to-meat conversion process compared to normal breast.	(Zhang *et al.* 2020b)
WB	meat myopathy	Five genetic strain broilers breast meat	8 weeks	0 min, 15 min, 4 h, and 24 h post-mortem	2DE-LC-MS/MS	Mascot	Genetic strain, diet, and post-mortem time strongly influence the proteome profiles of WB and normal breast meat.	(Zhang *et al.* 2020a)
WB	genetics	Five commercial broiler strains breast samples	8 weeks	24 h post-mortem	2DE-LC-MS/MS	MASCOT and NCBI	Broiler strain affect the protein expression and the meat quality (WB and normal). One strain was reported to be genetically more inclined to develop WB.	(Zhang *et al.* 2021)

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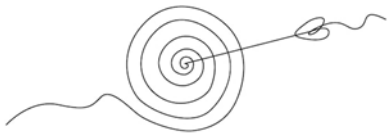

SECTION B

Thesis objectives and experimental design

THESIS OBJECTIVES

Thesis objectives

The bibliographic data presented in the previous section clearly show that the organic market represent a big slice of the food market and the quality of the food product is essential for the consumers. At the time of purchase, the choice of an organic meat product is closely linked to ethical reasons, known as extrinsic cues. The paucity of scientific literature about organics, in particular meat products, does not help the consumers to take their decisions. In the last two decades, a small number of studies revealed that species, genetic type, age, sex, muscle type and the stress state of the animal at slaughter, influence post-mortem muscle metabolism and meat quality. Equally few studies have focused on protein expression in different meat products obtained through different processes and conditions but an exhaustive answer on the actual added value of the organic method on the final product has not been clarified yet. To solve this problem, it is imminent to set up all the analytical and predictive tools, including authenticity, necessary to increase our knowledge about qualitative characteristics of organic meat products.

In this context, the support of a leading company in the Italian poultry sector such as Fileni (Cingoli, Italy) was essential to achieve the goal of better characterizing the organic meat qualities with innovative techniques. The union between the experience and facilities of university research and the corporate standpoint was essential for the success of this thesis work. Moreover, the collaboration and supervision of Dr. Mohammed Gagaoua during my research internship of 6 months in Teagasc Agriculture and Food Development Authority (Dublin, Ireland) was fundamental to carry on the second part of my thesis focused on

proteomics applications to better decipher the unknowns behind organic meat quality determination.

Thus, my thesis project, aims to better evaluate the potential quality chicken meat by a multidisciplinary approach. It was possible by working through different steps, ranging from meat quality assessment to the biochemical mechanisms involved in muscle to meat conversion. Therefore, the main objectives of my thesis were:

1. Evaluate and test the possibility of using a new biodegradable and compostable biopackaging for the market distribution of organic chicken meat;
2. Study the qualitative differences between organic and conventional chicken meat through the evaluation of specific intrinsic meat quality and shelf-life traits;
3. Explore for the first time chicken breast meat proteome in two main chicken strains according raised under organic and antibiotic-free production systems with the aim of identifying biomarkers;
4. Apply new "omics" strategies in the frame of shotgun proteomics combined to bioinformatics to better understand the underlying biological mechanisms in chicken meat as a consequence of farming system and chicken strain (genotype).
5. Combine proteomics and advanced multivariate statistical tools (chemometrics) to discriminate meat from different chicken strains and farming systems, with the goal of proposing for the first time putative biomarkers of authenticity.

SECTION B

Thesis objectives and experimental design

EXPERIMENTAL DESIGN

Experimental design

The analysis carried out involved a total of four groups of samples taken for each experiment at Fileni industry (Cingoli, Italy). Organic chickens were reared according to European Commission Regulation No 848/2018 for organic systems for poultry and livestock production, using organic feed, controlled housing, having access to an outdoor area with the presence of pasture for at least one third of their life. Antibiotic-free (conventional) chicken meat featured a standard broiler inside-ground farming system, using concentrated feed and controlled housing (artificial light and climate control, automatic water, and feed supply) according to the European Directive 2007/43/EC.

All the animals were reared and slaughtered in standardized conditions by the company and post-mortem *Pectoralis major* muscle (breast) biopsies were taken within 3 h after slaughter with randomisation between left and right sides. The analyses were performed on the final raw meat product, the same one that can reach consumer's kitchen. Main characteristics of the samples are reported in Table 7.

Table 7 - Main characteristics of chicken meat samples analysed in this thesis.

	Antibiotic-free (conventional)		**Organic**	
				
Chicken strain	Ross 308	Ranger Classic	Ross 308	Ranger Classic
Sample codification	ARO	ARA	ORO	ORA
Age	48 days	56 days	83 days	85 days
Sex	M	M	F	F
Average alive weight	3.47 Kg	3.22 Kg	3.96 Kg	2.99 Kg
Tunnel exit T°	4.5-4.9°C	3.6-4,3°C	7.7-9,5°C	4.9-8.5°C

Ross 308 chicken strain is the main used strain in chicken meat production because of its fast-growth rate and high percentage of breast muscle in the final carcass. The second chicken strain, Ranger Classic, has recently been introduced into the production by Fileni company as it is an hybrid strain described to have slower-growth rate, more suitable for organic production.

The overall summary of the experiments performed in this thesis is reported in Figure 11. The first parts of this thesis (experiments 1 and 2) focused mainly on Ross 308 chicken meat while the proteomic investigations involved all the four groups of samples to include chicken strain as additional variable to the production method.

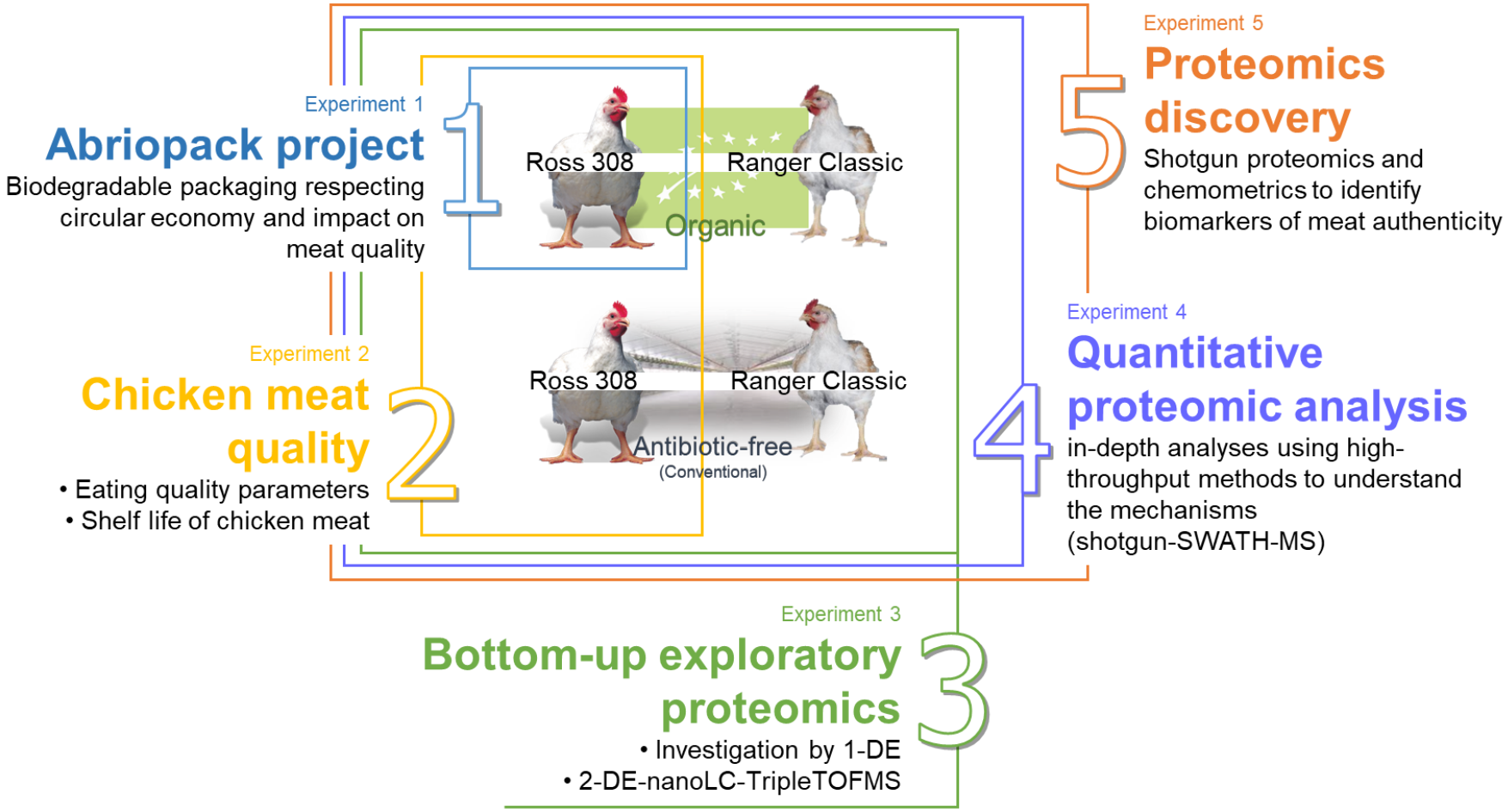

Figure 11 - Summary diagram of the experimental designs. The diagram illustrates the different parts of the thesis and the types of animals used. (1-DE: one dimensional electrophoresis, LC: liquid chromatography, TOFMS: Time of flight mass spectrometer, SWATH-MS: Sequential Window Acquisition of all Theoretical Mass Spectra - Mass Spectrometry)

Experiment 1. In the context of the Abriopack project, financed by Marche Region through PSR Marche 2014/2020 fund, the preservation capacity of a newly developed biopackaging, which is biodegradable and compostable was tested. The shelf-life of organic chicken meat preserved in the biopackaging was compared to the one of the same type of meat in a widespread plastic packaging. To obtain a complete view of the meat preservation status during the storage, chemical (biogenic amines and VOCs), microbiological and sensorial indicators (parameters) were analysed combining the expertise of two research groups at University of Camerino.

Experiment 2. This trial consisted of an preliminary on meat quality traits of organic and conventional antibiotic-free meats was performed to obtain an overview on the qualitative grade of Ross 308 samples. The investigated parameters were color, WHC, meat pH, tenderness, lipid fraction quantification, and lipid peroxidation. Moreover, a shelf-life comparative study was carried out to understand the differences in preservation of chicken meat from the two production systems. Chemical, microbiological, and sensorial parameters were investigated.

Experiment 3. The results of experiment 2 highlighted, among other differences, a marked dissimilarity in the production of biogenic amines between the two groups of samples analysed. Being these compounds are the products of the amino acids degradation in meat, this led me to suggest that the production system could influence the protein component of the meat. For this reason, I started to investigate the proteome of meat samples from different chicken strains and from different farming systems. Firstly, 1-DE electrophoresis of protein separations provided a first exploratory trial, which was subsequently combined with 2-DE-LC-MS/MS for in depth bottom-up proteomics analysis.

Experiment 4. Starting from previous experiments findings, it was decided to go more in depth in proteomic studies. The application of shotgun-SWATH-MS method allowed the quantification of the proteins that constitute the entire proteome of chicken breasts. This technique followed by statistical approaches allowed to compare the four groups to investigate the impact of both production system and chicken strain on the early post-mortem muscle proteome.

Experiment 5. The last part of my research was designed to couple data-independent acquisition proteomics and chemometric tools (such as but not limited to Partial Least Square Discriminant Analysis (PLS-DA)), for the discrimination of meat from the two chicken strains and the two farming systems. The combination of proteomics findings with chemometrics and multivariate data analyses allowed to propose for the first time putative protein biomarkers for chicken meat authenticity.

The workflow of the proteomics data analyses using both statistical methods and bioinformatics tools are summarized in Figure 12. Depending on the scientific questions fixed for each experiment, the methods were accordingly used and adapted to respond to my objectives described above.

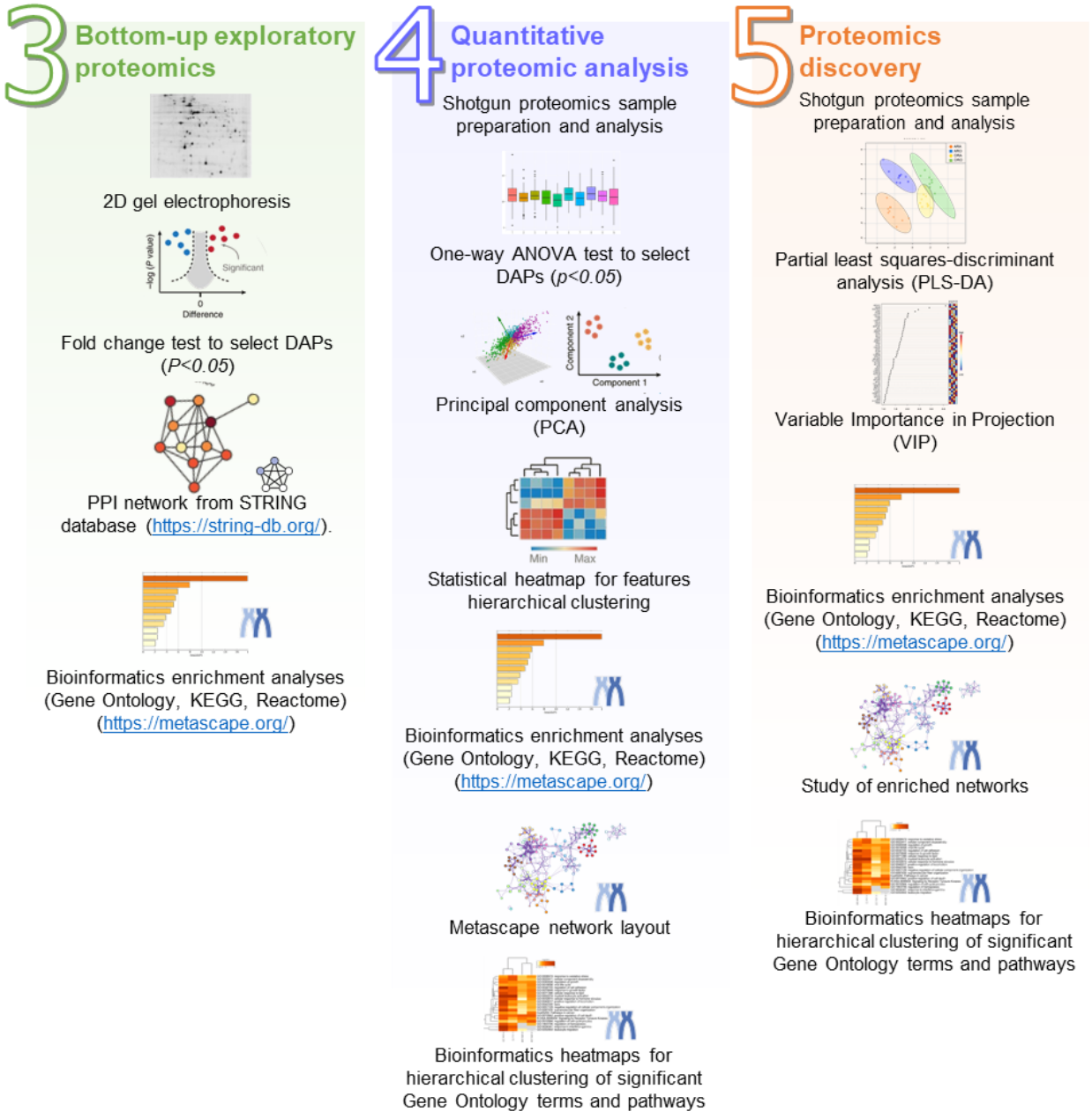

Figure 12 - Strategies of statistical and bioinformatics analysis

SECTION C
Results of the thesis

A shelf-life study for the evaluation of a new biopackaging to preserve the quality of organic chicken meat

Publication 1

A shelf-life study for the evaluation of a new biopackaging to preserve the quality of organic chicken meat

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Published in *Food Chemistry*, 371, 131134.
https://doi.org/10.1016/j.foodchem.2021.131134

Abstract

Widespread use of traditional packaging constitutes a serious ecological problem leading to a shift to biodegradable and compostable materials. The aim of this work is to study the ability of a new biopackaging (BP), based on biodegradable and compostable material, to preserve the quality of organic chicken meat for 14 days in comparison with a polyethylene terephthalate (PET) material. Results showed that the indices of Biogenic Amines (BAs) and the 18 monitored Volatile Organic Compounds (VOCs) have a similar trend in both packaged meats. For example, the total BAs concentration in meat increased from 390 to 961 mg Kg^{-1} in BP and from 393 to 800 mg Kg^{-1} in PET, as well as the microbiological counts. The new biopackaging (BP) showed similar properties of non-biodegradable material (PET) to preserve the shelf life of organic chicken meat and it could be used instead of plastic materials to promote a circular economy.

Keywords: Biopackaging organic chicken biogenic amines volatile organic compounds shelf life

1. Introduction

The global awareness of the environmental issues associated with the use of synthetic and non-degradable packaging has led to an increased interest in biopackaging based on biodegradable and natural polymers (Ganesh Kumar, Anjana, Hinduja, Sujitha, & Dharani, 2020). Biopackaging is thus a growing sector in terms of innovation, to face the sustainability challenges of the food packaging industry (Bajer, Janczak & Bajer, 2020). Indeed, biodegradable polymers offer a possible alternative to traditional non-biodegradable plastics when recycling is impractical or not economical (Magni et al., 2020). Compostable food packaging can solve the disposal problems of difficult-to-recycle plastic packaging, avoiding their disposal in landfills or incineration. At the same time, compostable food packaging allows the enhancement of organic waste, which, if properly treated, can generate quality compost to restore soil nutrition (Markus & Ramani, 2021). Among the biopolymeric materials used for biopackaging applications, polysaccharides such as cellulose, alginate, gelatin have been proposed. More recently, chitosan has been the most explored polysaccharide material for the development of biodegradable packaging (Díaz-Montes & Castro-Munoz, ˜ 2021a). Beyond polysaccharide-based materials, various studies proposed the use of proteins-based biopolymers (gluten, whey proteins, or casein) and lipids biopolymers (waxes, oils, free fatty acids). Moreover, biopackaging can be formulated with biopolymers synthesized from bioderived monomers (polylactic acid, polyesters) or produced directly from microorganisms (polyhydroxyalkanoate, polyhydroxybutyrate) (Chen et al., 2019; DíazMontes & Castro-Munoz, ˜ 2021b). However, from all the proposed materials, starch is the most used biopolymer for biopackaging formulation due to its mechanical properties close to those of traditional plastics like polyethylene and polystyrene (Jiang, Duan, Zhu, Liu & Yu, 2020). Nowadays, studying biopackaging and monitoring their quality to reduce economic loss and food waste is more important than ever. Therefore, a

key challenge is to assess the performance of biopackaging like starch-based packaging in preserving food quality during storage, especially easily perishable products. The preservation of quality and appearance of perishable food, such as raw meat, is essential during its distribution and merchandising. According to FAO, world poultry meat production soared from 9 to 122 million tonnes between 1961 and 2017, and in 2017, poultry meat represented about 37 % of global meat production, with chickens contributing 89 % of world poultry meat production (FAO, 2020). Among different systems, organic production has notably increased in importance over recent years (Rabadan ´ et al., 2020). The percentage of surface area dedicated to organic production has doubled in the last ten years (FIBL-IFOAM, 2019). Several factors affect meat shelf-life, such as the presence of oxygen, storage temperature, endogenous enzymes, exposure to light, and microorganisms, leading to deterioration and reduction of meat shelf life (Stopforth, 2017). Microbial deterioration, lipid oxidation, and autolytic enzymatic spoilage are the three main mechanisms for fresh meat spoilage. The spoilage processes lead to pH changes, appearance changes, slime formation, structural component degradation, which can cause the production of biogenic amines and volatile organic compounds (Dave & Ghaly, 2011). Off-odors released from meat during storage might suitably act as food freshness/spoilage indicators. Moreover, a sensory evaluation can be used to assess the deterioration of meat organoleptic characteristics. For example, the meat aspect is generally considered as the most important factor that affects consumer purchase (Sharif, Butt, Sharif, & Nasir, 2017). Currently, few studies have been performed on the application of starch-based biopackaging to maintain the quality of fresh meat. Research has been focused on the degradation, ecotoxicity, environmental impact, and mechanical characteristics of biopackaging materials (Johnson, Tucker & Barnes, 2003; Scaffaro, Morreale, Lo Re & La Mantia, 2009; Magni et al. 2020). Therefore, this study aims to assess the ability of a new biopackaging to preserve the quality of organic chicken meat

in comparison to classic polyethylene terephthalate packaging. This objective was achieved through the monitoring of chemical (biogenic amines, volatile organic compounds) and microbiological (meat microbiota) markers as well as sensorial parameters of packaged organic chicken breast meat.

2. Materials and methods

2.1. Packaging materials

The studied biopackaging (BP) consists of a completely biodegradable and compostable tray and film. The material used for BP is provided by Novamont (Novara, Italy). This material is obtained by means of Novamont's proprietary technologies, using bio-polyesters obtained by polycondensations of diacids and diol. The result is a compostable multilayer structure constituted by Novamont's bio-polymers. The geometry of the products are: a) film: weight 1,5 g, thickness 39 µm, (l1 × l2) 23 × 14,5 cm, b) tray: weight 23,5 g, thickness 600–700 µm; (l1 × l2 × h) 23 × 14,5 × 4 cm (Figure 1S). The packaging used for comparison consists of a traditional polyethylene terephthalate (PET) packaging (PET-EL L1523-27 TR1160000 O2WH, Linpac Packaging Pravia, Spain) combined with a cryovack film (LID 830X, Sealed Air Food Care, Charlotte, USA).

2.2. Sample collection

Fileni® industry (Cingoli, Italy) provided fresh organic chicken breast meat packaged with new BP composed by biodegradable and compostable tray and film. The same fresh organic chicken breast meat was wrapped with PET packaging combined with a cryovack film. All samples were wrapped in a modified atmosphere (MAP) (70 % O_2, 20 % CO_2, and 10 % N_2). Analyses were performed on chicken breast meat at day 0, day 3, day 6, day 10, and day 14, every time opening a new pack. During the analysis period, samples were held at 4 ◦C to simulate the consumer storage conditions. All analyses were performed in triplicate.

2.3. Chemicals and reagents

Spermine tetrahydrochloride (SPM, $C_{10}H_{26}N_4 \cdot 4HCl$, >98%, CAS No. 306-67-2), spermidine trihydrochloride (SPD, $C_7H_{17}N_3 \cdot 3HCl$, >98%, CAS No. 334-50-9), cadaverine dihydrochloride (CAD, $C_5H_{14}N_2 \cdot 2HCl$, >98%, CAS No. 1476-39-7), putrescine dihydrochloride (PUT, $C_4H_{12}N_2 \cdot 2HCl$, >98%, CAS No. 333-93-7), histamine dihydrochloride (HIS, $C_5H_9N_3 \cdot 2HCl$, >99%, CAS No. 56-92-8), tyramine hydrochloride (TYR, $C_8H_{11}NO \cdot HCl$, >98%, CAS No. 60-19-5), 2-phenylethylamine hydrochloride (PHE, $C_8H_{11}N \cdot HCl$, >98%, CAS No. 156-28-5) and tryptamine hydrochloride (TRY, $C_{10}H_{12}N_2 \cdot HCl$, >99%, CAS No. 343-94-2) for standard solutions preparation were supplied by Sigma-Aldrich (Milano, Italy). 1,7- diaminoheptane (98%, CAS No. 646-19-5) as the internal standard was supplied by Sigma-Aldrich (Milano, Italy). Hydrochloric acid (HCl, 37%, CAS No. 7647-01-0) trichloroacetic acid (TCA, ≥99.0%, CAS No. 76-03-9), acetone (≥99,5% CAS No. 67-64-1), sodium hydroxide anhydrous (NaOH, ≥98%, CAS No.1310-73-2), sodium carbonate anhydrous (Na_2CO_3, ≥99,5%, CAS No. 497-19-8), acetonitrile (CH_3CN, HPLC gradient grade, ≥99.9%, CAS No. 75-05-8), methanol (CH_3OH, HPLC gradient grade , ≥99.9%, CAS No. 67-56-1) and dansyl chloride ($C_{12}H_{12}ClNO_2S$, 98% CAS No. 605-65-2) for extraction and derivatization were from Sigma-Aldrich (Milano, Italy).

Individual stock solutions of biogenic amines (BAs) were prepared by dissolving 10 mg of each compound in 10 mL of HCl 0,1 M (Merck Darmstadt, Germany) and were then stored in glass-stoppered bottles at 4°C. Standard working solutions, at various concentrations, were prepared daily by appropriate dilution of different aliquots of the stock solutions with deionized water (<8MΩ cm resistivity) obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). Derivatization solution was prepared with dansyl chloride in acetone (10%).

2.5. Analysis of volatile organic compounds

5 g of chicken breast meat was finely minced and homogenized for 30 sec in an analytical blender (Tube Mill Control, IKA-Werke GmbH & Co. KG, Germany). Then, 2 g of the sample was weighted in a 10 mL vial with a perforable septum and conditioned at 40 °C for 20 min. The SPME fiber assembly was from Supelco (Bellefonte, PA, USA) and had a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coating with 1 cm length stationary phase. Then, the fiber was exposed in the headspace of the vial containing the sample for 30 min. Volatile organic compounds (VOCs) were analysed by GC-MS using a 6890N Network GC System coupled to a 5973 Network Mass Selective Detector both from Agilent Technologies (Santa Clara, CA, USA). A capillary column coated with polyethylene glycol (60 m x 0.25 mm x 0.25 µm film thickness, DB-WAX, Agilent Technologies, Santa Clara, CA, USA) was used. The initial carrier gas (helium) flow rate was 1.2 mL min^{-1}. Injector temperature was 260 °C, splitless time was 4 min. The oven temperature was held at 35°C for 4 min, then raised to 120 °C at 2.5°C min^{-1} and then went up to 250°C at 15 °C min^{-1} and held at this final temperature for 3.3 min, for a total run time of 50 min. Mass analysis was performed in scan mode in the range of 25-400 Da. The transfer line was maintained at 260°C, ion source at 230°C, and quadrupole at 150°C. The SPME fiber was left exposed in the injector for 10 min to be cleaned after desorption and reactivated. Straight chain alkanes were used to calculate retention indices. Thus, the detected VOCs were identified by comparing their retention indices and mass spectra with those of standards from the US National Institute of Standards and Technology database (NIST-USA, http://webbook.nist.gov). An external standard (3-octanol in acetone, 0.5 mg mL^{-1}) was used to control the repeatability of the method. A proper aliquot of the standard solution (10 µL) was put in a 2 mL vial and analysed in duplicate each day of analysis under the same conditions reported above.

2.6 Microbiological analysis

Chicken breast meat microbiota was monitored by determining total aerobic mesophiles, mesophilic lactic acid bacteria (LAB), β-glucosidase-positive Escherichia coli, Enterobacteriaceae, presumptive Pseudomonas spp., coagulase-positive staphylococci,

anaerobic sulfite-reducing bacteria, Clostridium perfringens. The analysed parameters followed the criterion of CeIRSA (2017) and the applied procedures were in accordance with the respective ISO guidelines. 10 g of chicken breast meat from each pack (PET and BP) were aseptically weighted inside sample bag (Whirl-Pak®, Seward, UK) and 90 mL of sterile 0.9% sodium chloride solution (NaCl) (Sigma-Aldrich, Co., St. Louis, USA) were added into sample bags and homogenized for 2 min by Stomacher. Then, 10-fold dilutions were prepared using a saline solution, and 0.1 mL of the corresponding dilutions was spread and inoculated into selective agar media. The aerobic mesophilic bacteria count was performed onto Plate Count Agar (PCA, Oxoid, Basinstoke, UK) under aerobic conditions at 30 °C for 72 h (ISO 4833). For the enumeration of mesophilic LAB, de Man, Rogosa, Sharpe agar (MRS Agar at pH 5.7, VWR, Leuven, Belgium) was used (ISO 15214:1998), while Tryptone Bile X-glucuronide Agar (TBX, VWR) for the detection of β-glucosidase-positive E. coli was aerobically incubated for 18 h to 24 h at 44 °C (ISO 16649-2). The detection and enumeration of Enterobacteriaceae, were performed onto violet red bile glucose agar (VRBGA, VWR) inoculated with samples at 37 °C for 24 h (ISO 21528-2). The enumeration of presumptive Pseudomonas spp. was carried out by aerobically inoculating samples onto Pseudomonas Selective Agar (CFC, Liofilchem s.r.l., Roseto degli Abruzzi, Italy) at 25 °C for 44 h ± 4 h (ISO 13720:2010[E]). The presence of coagulase-positive staphylococci was checked through the aerobic inoculation onto the Baird-Parker agar medium (VWR) at 35-37 ºC after 24 to 48 h of incubation (ISO 6888-1:1999 [E]). Sulfide-reducing bacteria were enumerated by using iron sulfite agar plates (Liofilchem) incubated under anaerobic conditions at 37 ± 1 ºC for 48 h (ISO 15213:2003 [E]). Cl. perfringens count was on tryptose sulfite cycloserine (TSC) agar (VWR) under anaerobic conditions at 37 ºC for 20 h ± 2 h (ISO 7937:2004 [E]).

2.7. pH measurement

The pH of chicken breast meat has been measured in triplicate, using a digital pH meter (Mettler Toledo, Columbus, UK) equipped with a probe for food through direct penetration in meat.

2.8. Sensory analyses

Sensorial analyses were conducted on raw chicken breast meat aspect (slime), odor, color, texture (elasticity), overall acceptability using a three-point hedonic scale, ranging from extremely dislike/reject (Score: 1) to extremely like/satisfy (Score: 3) on the same time points of the analysis (Huang et al., 2020) (Table 1S). The sensory panel was composed of 10 panelists from the laboratory, they had been trained before performing tests on selected samples in order to familiarize chicken breast meat sensorial attributes and terminology. Each sample was assigned a code; the panelists gave scores based on the coded samples.

2.9. Statistical analysis

Significant differences between the storage in the two different packaging (BP and PET) and between the different times of analysis were evaluated by one-way analysis of variance (ANOVA). Differences with $p < 0.05$ were considered statistically significant. Data elaboration was carried out using the PAST software package (Hammer, Harper & Ryan, 2001). Each experiment was performed in triplicate.

3. Results and discussion

3.1. Analysis of biogenic amines

The analytical method was previously validated according to the criteria of European Regulations for quantitative methods of confirmation. The HPLC-DAD chromatogram of BAs mixture at 25 mg L^{-1} and 1,7- diaminoheptane, used as internal standard, is shown in Figure 2S. The calibration curve of each BA was calculated using the response factor, which

is the ratio between the BA peak area and the internal standard peak area. All calibration curves of BAs showed good linearity ($r^2 > 0.9956$). Under these HPLC conditions, each BA and internal standard are clearly resolved, indicating that this method can be used for the quantitative determination of BAs in food samples. Figure 1 reports the Total content of BAs (A), the Biogenic Amine Index (B), and the spermidine/spermine ratio (C) related to chicken breast meat wrapped in BP and PET packaging during the shelf-life study. Samples were prepared according to the procedure described in section 2.4; each sample was analysed in triplicate. Several studies reported biogenic amines as indicators of meat freshness. Concentrations of some biogenic amines such as tyramine, putrescine, and cadaverine, normally increase during storage of meat and meat products, whereas spermidine and spermine decrease or remain constant (Onal, Tekkeli, & Onal, 2013; Wojcik, Łukasiewicz, & Puppel, 2021; Yusoff, Jaffri, & Azhari, 2021). The Total content of BAs showed a similar trend of growth both for BP and PET packaged meat, with two significant differences at day 3 and day 10 in favour of BP and PET, respectively (Figure 1A). The Biogenic Amine Index (BAI) (Douny et al, 2019) increased rapidly from day 6 to day 10 in the meat wrapped both in PET and BP packs. PET packaged meat values shifted from 65 mg Kg^{-1} at day 6 to 159 mg Kg^{-1} at day 10, while meat wrapped in BP from 61 mg Kg^{-1} to 245 mg Kg^{-1} in the same range of time (Figure 1B), without statistically significant differences. Concerning the ratio between SPD and SPM that is considered one of the most important indexes for evaluating the quality of chicken meat because is independent of the type of flora (Sirocchi et., 2013), the values are quite constant for both packaged meat (BP and PET) during the shelf-life study (Figure 1C). No statistically significant differences have been recorded for SPD/SPM between the two different packaging. Similar results of SPD/SPM ratios were reported for chicken (Silva & Gloria, 2002), fish (Biji, Ravishankar, Venkateswarlu, Mohan, & Srinivasa Gopal, 2016), and pork (Ngapo & Vachon, 2017) food matrices.

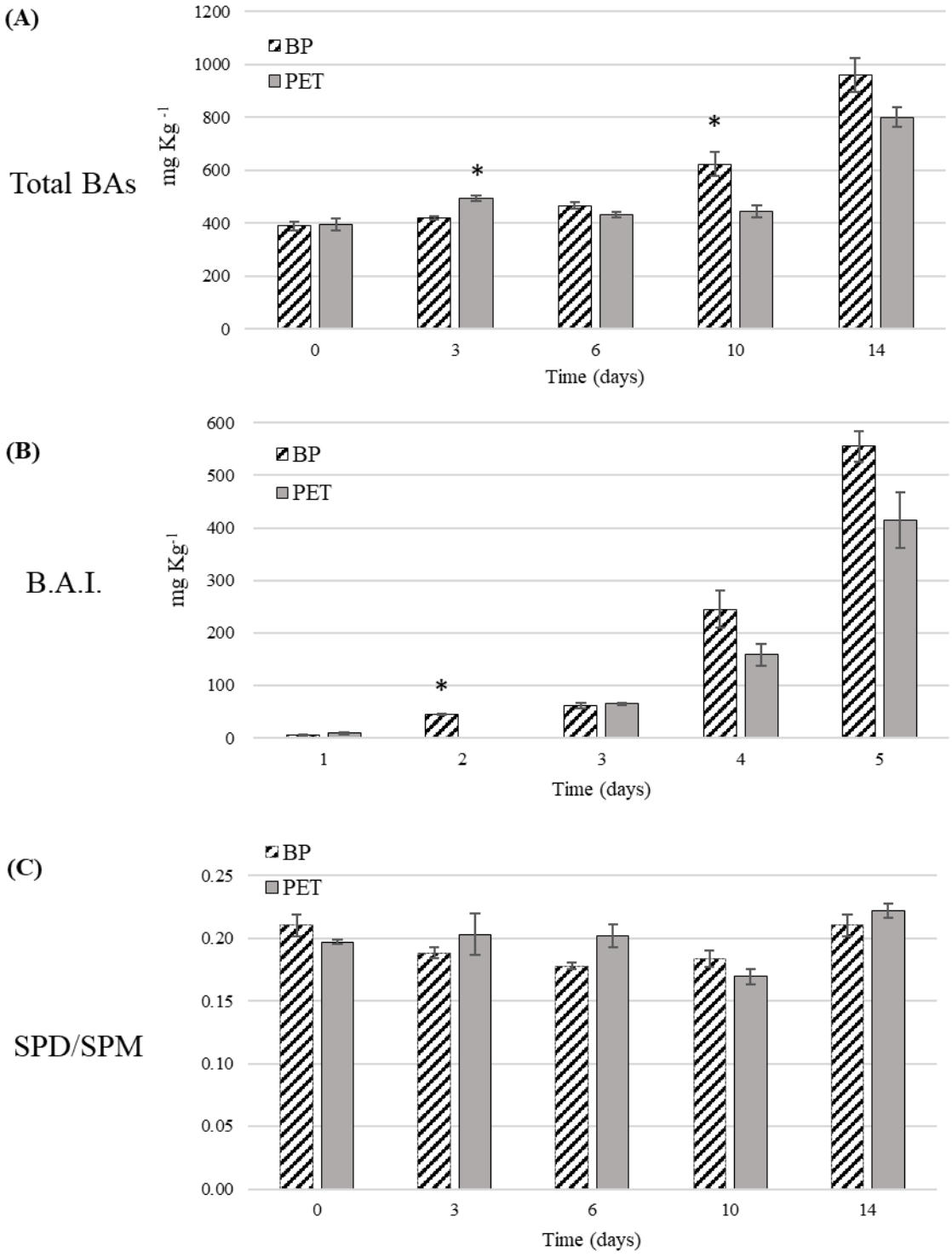

Figure 1. *Comparison (± standard deviation) of the sum of average concentrations of analysed BAs (A), Biogenic Amine Index (B) and Spermidine/spermine ratio (C) of chicken breast meat in the two types of packaging (BP and PET) during the storage period. Significant differences ($p < 0.05$) between the two packaging in each day are indicated by the asterisk.*

These results are in agreement with those published by Silva and Gloria (2002) that report the SPM prevalence over SPD level during the 14-days storage period, assessing that SPM decreases because microorganisms take up this polyamine as a nitrogen source. Finally, the comparison between the BAs level of chicken meat wrapped in BP and PET packaging shows a similar trend in the concentrations of Total BAs, BAI, and the ratio SPD/SPM

3.2. Analysis of volatile organic compounds (VOCs)

The formation and release of VOCs by chicken breast meat in two different packagings (BP and PET) over a 14-days storage period was assessed by HS-SPME-GC–MS. VOCs that are in part spoilage byproducts were monitored on different days: at time zero and after 3, 6, 9, and 14 days. A number of 18 VOCs were identified in the chicken breast meat packed in PET and BP and they are listed in Table 1. These compounds were alcohols, phenols, ketones, acids, and sulfur-containing compounds. Most of them are reported as common VOCs detected during fresh meat storage (Casaburi, Piombino, Nychas, Villani & Ercolini, 2015). Some of these compounds (e.g. 3-methyl butanol, 1-pentanol, 3- hydroxy-butanone, and acetic acid) increased during the storage period in both PET and BP packaging, and some VOCs (e.g. 1-octanol, 1-octen3-ol) could be found only at the end of the storage period (after 9 or 14 days). A selection of identified compounds is plotted in Figure 2. Alcohols can be produced by specific microorganisms during the storage of fresh meat, such as Pseudomonas and Carnobacteria (Casaburi et al. 2015). 1-Octen-3-ol and 3-methyl-1-butanol are the most frequently detected alcohols in raw meat (Casaburi et al., 2015). 1-Octen-3-ol mainly derives from the oxidation of linoleic and linolenic acids (Curioni & Bosset, 2002) and is known to contribute significantly to the aroma of meat (Casaburi et al., 2015). After day 3, it was detected in PET packaging and after day 6 in BP packaging, showing an increase in both packaging during the storage period. At day 14, 1-octen-3-ol is the only VOC that resulted to be significantly more abundant in BP than in PET packaging.

3-Methyl-1-butanol is known to derive from the proteolytic pathway of leucine, and it has been used as a chemical marker for chicken meat spoilage in previous studies (Casaburi et al., 2015; Alexandrakis, Brunton, Downey & Scannell, 2011). In this study, it was detected at day 9 in both packaging, indicating that it could be considered as a marker of the latter stages of the spoilage process, as 1- octanol, that was detected only after 14 days of storage in both packaging. 1-Pentanol was present in both the fresh and aged samples, with a higher presence in the latter for both packagings. Both 1-pentanol and 1- octanol are well-known lipid oxidation products (Shahidi, 1994).

Table 1. *Volatile compounds detected by HS-SPME-GC–MS during the storage of chicken breast meat in BP and PET packaging, their experimental linear retention indices (LRI) on a polyethyleneglycol coated column, their odour attribute, their abundances in terms of peak areas and % relative standard deviation (RSD, n = 2). Significant differences (p < 0.05) between the two packaging in each day are indicated by the asterisk. Significant differences (p < 0.05) between the different days for each of the two packaging are indicated by different letters in the same row.*

Compound	Odour attribute	LRI[1] (exptl)	LRI[2] (lit)		Day of storage 0		3		6		10		14		SI[3](%)
					Area	RSD%	Area	RSD%	Area	RSD%	Area	RSD%	Area	RSD%	
Alcohols and phenols															
Isopropyl alcohol	Musty	929	926	BP	5,04E+06^{a*}	8.0	4,73E+06^{a*}	4.3	2,14E+06^{b*}	7.8	2,38E+06^{b*}	0.3	7,22E+06^{c*}	3.0	80
				PET	1,93E+07^{a}	4.5	2,07E+07^{a}	1.6	6,47E+06^{b}	8.9	1,48E+07^{c}	14.8	1,18E+07^{c}	4.3	
1-Propanol	Musty	1038	1036	BP	2,86E+07^{a*}	8.5	5,13E+07^{b*}	0.7	1,49E+07^{c*}	10.1	1,20E+07^{c*}	11.0	4,25E+07^{d*}	2.3	86
				PET	1,24E+08^{a}	1.1	1,27E+08^{a}	0.8	5,96E+07^{b,d}	11.9	9,04E+07^{c,d}	10.5	8,08E+07^{d}	2.1	
3-Methylbutanol	Roasted	1213	1208	BP	nd	nd	nd	nd	nd	nd	4,70E+05^{a}	27.6	2,08E+06^{b}	17.9	80
				PET	nd	nd	nd	nd	nd	nd	8,46E+05	29.3	1,63E+06	6.4	
1-Pentanol	Fusel	1254	1252	BP	1,08E+05^{a}	10.5	1,57E+05^{a*}	1.7	6,64E+05^{b}	9.7	8,68E+05^{c}	1.6	9,56E+05^{c}	5.2	88
				PET	1,58E+05^{a}	12.1	6,97E+05^{b}	18.3	1,02E+06^{b}	11.4	7,72E+05^{b}	14.2	1,03E+06^{b}	6.6	
1-Hexanol	Fruity	1356	1354	BP	nd	nd	nd	nd	2,72E+05^{a}	2.0	1,77E+05^{b}	2.0	2,92E+05^{a}	11.4	82
				PET	nd	nd	3,33E+05	11.1	2,61E+05	0.9	2,97E+05	22.3	3,66E+05	14.7	
1-Octen-3-ol	Earthy	1451	1449	BP	nd	nd	nd	nd	1,92E+05^{a}	20.1	3,63E+05^{b}	5.4	7,66E+05^{c*}	2.1	83
				PET	nd	nd	1,57E+05^{a}	26.0	3,25E+05^{a,b}	17.9	4,76E+05^{b}	15.9	5,27E+05^{b}	14.7	
1-Octanol	Waxy	1566	1565	BP	nd	nd	nd	nd	nd	nd	nd	nd	3,15E+05*	33.3	85
				PET	nd	nd	nd	nd	nd	nd	nd	nd	9,48E+05	7.8	
Phenol	Sweet	1998	1996	BP	1.6E+05	10.4	nd	nd	1,29E+05	2.1	1,08E+05	16.8	1,30E+05 *	22.3	83
				PET	1,23E+05^{a}	28.7	2,41E+05^{a}	21.8	nd	nd	2,82E+05^{a}	30.9	1,02E+06^{b}	2.5	
Ketones															
2-Butanone	Fruity	892	894	BP	3,76E+05^{a}	22.7	2,82E+05^{a}	13.8	8,70E+05^{b*}	3.7	7,89E+05^{b*}	0.5	2,99E+05^{a}	18.8	82
				PET	nd	nd	2.75E+05^{a}	5.1	3,64E+05^{a,b}	8.4	3,96E+05^{b}	11.9	1,31E+05^{c}	4.4	
2,3-Butanedione	Buttery	966	970	BP	nd	nd	7,87E+05^{a}	25.3	3,25E+05^{b}	1.5	6,64E+05^{a,b}	5.1	1,54E+06^{c}	4.3	81
				PET	3.75E+05^{a}	30.1	1,45E+06^{b}	21.6	2,70E+05^{a,c}	30.2	1,04E+06^{a,b}	23.4	1,71E+06^{b,d}	7.6	
3-Hydroxy-2-butanone	Creamy	1280	1278	BP	nd	nd	nd	nd	7,88E+05^{a}	1.2	1,80E+06^{b*}	6.7	4,96E+06^{c*}	3.2	84
				PET	nd	nd	nd	nd	nd	nd	1,11E+06^{a}	6.7	6,41E+06^{b}	3.8	
Acids															
Acetic acid	Vinegar	1446	1453	BP	1,84E+05^{a}	25.3	1,52E+05^{a}	4.7	4,02E+05^{a,b}	23.5	2,64E+05^{a}	14.6	7,36E+05^{b}	23.3	90

Propanoic acid	Pungent	1539	1538	PET	nd	nd	1,66E+05^{a}	7.0	2,82E+05^{a,b}	6.6	3,08E+05^{b}	17.1	1,12E+06^{c}	0.8	89
				BP	nd	nd	7,68E+05^{a*}	6.3	6,96E+04^{b}	23.8	nd	nd	8,75E+04^{b*}	35.2	
Isovaleric acid	Cheesy	1670	1670	PET	nd	nd	9,55E+05^{a}	2.2	6,33E+04^{b}	13.3	2,98E+05^{c}	5.6	2,22E+05^{d}	3.6	83
				BP	nd	nd	nd	nd	6,22E+05	28.0	6,43E+05*	12.6	2,41E+05*	16.8	
Hexanoic acid	Sour	1843	1839	PET	nd	nd	nd	nd	1,41E+05^{a}	4.7	4,00E+06^{b}	8.5	9,29E+05	4.0	80
				BP	1,62E+05^{a,b}	4.5	1,49E+05^{a,b}	2.3	2,84E+05^{a}	12.5	1,33E+05^{b}	12.8	1,79E+05^{a,b}	34.3	
Nonanoic acid	Rancid	2161	2168	PET	1,55E+05^{a}	16.7	nd	nd	2,12E+05^{a,b}	1.3	2,63E+05^{a,b}	22.3	3,32E+05^{b}	11.6	81
				BP	2,25E+05	28.3	1,86E+05	30.1	3,32E+05	21.9	2,19E+05	14.2	2.74E+05	21.9	
				PET	2,19E+05	31.1	2.25E+05	12.8	2,85E+05	9.3	2,06E+05	17.8	2,76E+05	18.2	
Sulfur containing compounds															
Carbon disulfide	Ether-like	714	710	BP	2.27E+05^{a}	31.8	2,05E+05^{a}	8.9	2,00E+06^{b}	33.0	8.57E+05^{a,b}	16.1	2,05E+05^{a}	28.9	94
				PET	1,15E+06^{a,b}	11.0	1,95E+05^{a}	1.7	2,14E+06^{b}	38.9	7,66E+05^{a,b}	22.6	nd	nd	
Dimethyl sulfone	Cabbage-like	1904	1911	BP	4,11E+05^{a*}	8.6	9,78E+04^{b}	14.2	1,16E+05^{b,c}	39.3	1,72E+05^{b,c}	11.7	2,40E+05^{c}	13.6	96
				PET	5,38E+04^{a}	7.1	1,14E+05^{a}	26.7	3,68E+05^{b}	27.4	8,17E+04^{a}	33.0	2,42E+05^{a,b}	0.6	

[1] Experimental linear retention index;
[2] Linear retention indices reported in literature (NIST 2008);
[3] Similarity index.
nd: not detected (peak area value below 5E+04).

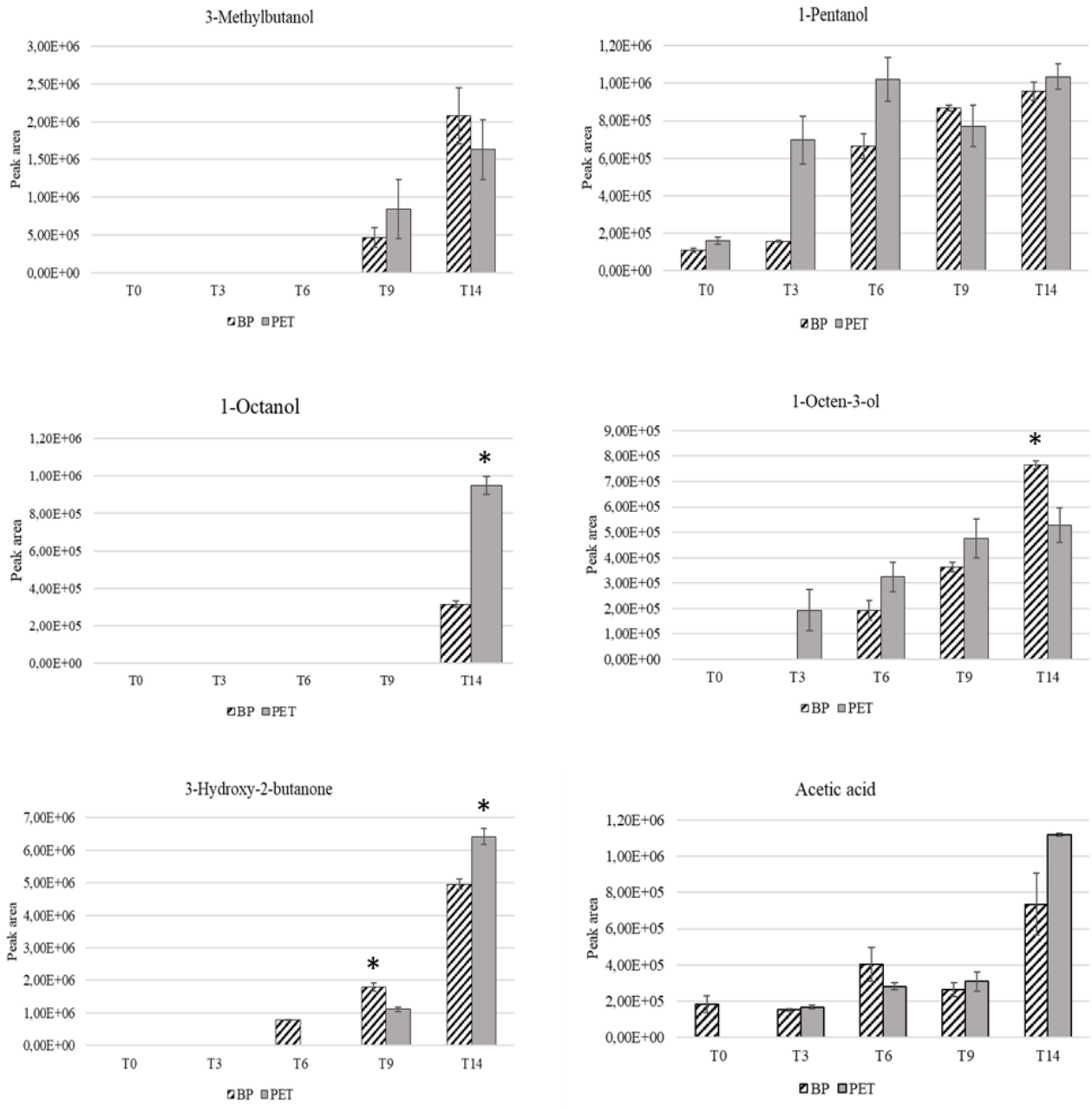

Figure 2. *Comparison of the average peak areas (±standard deviation) of selected VOCs in chicken breast meat in the two types of packaging (BP and PET) during the storage period. Significant differences ($p < 0.05$) between the two packaging in each day are indicated by the asterisk.*

Ketones are known to derive from fatty acids oxidation and have been used as meat-aging indicators also in previous studies (Est´evez, Morcuende, Ventanas & Cava, 2003; Zareian et al., 2018; Klein, Maurer, Herbert, Kreyenschmidt & Kaul, 2017). Ketones detected in this study include 2-butanone, 2,3-butanedione (diacetyl) and 3-hydroxy-2-butanone (acetoin). Acetoin a was detected at day 6 in BP and at day 9 in PET packaging, showing a significant increase in 14-day old samples. In particular, on day 14 acetoin quantity was significantly higher in PET as compared to BP packaging. Short and medium-chain free fatty acids detected in the samples are acetic, propionic, isovaleric, hexanoic, and nonanoic acids. Typically, LAB are the major responsible for the production of volatile fatty acids during meat storage (Casaburi et al., 2015). Acetic acid, the most abundant short-chain fatty acid detected, was found in both fresh and aged samples, showing a significant increase for both packaging after 14 days of storage, but with no significant differences between the two packagings. In general, the development of VOCs associated with meat spoilage was not significantly more pronounced in the BP packaging, thus contributing to demonstrate BP packaging suitability for meat chilled storage.

3.3. Microbiological analysis

Data of each analysed microbial group of both PET and BP samples are shown in Figure 3. The counts of β-glucosidase-positive E. coli, anaerobic sulfite-reducing bacteria, Cl. perfringens were under the detection limit throughout the study period. The bacterial counts of PET and BP samples increased during the storage period, with no significant difference found between the two types of packaging materials. Both samples showed the initial value of total aerobic mesophile around 4.2 log CFU g^{-1} which represented the analysed meat with good quality (Dawson et al., 1995; Latou et al., 2014). As the criterion of FCD (2009) suggested that the acceptable limit of total aerobic mesophiles is within 6 log CFU g^{-1} to 8 log CFU g^{-1}. As Figure 3A demonstrates, BP and PET samples reached the limit

approximately at 12 days and 13 days of chill storage, respectively. Lactic acid bacteria (LAB) are facultative anaerobic bacteria that can grow with the presence of oxygen or not, and some species of LAB constitute part of the natural microflora and some were found to be the main microorganisms related to meat spoilage (Jay et al., 2005; Casaburi et al., 2015). In both samples, the level of LAB increased during the study period which is corresponding to the decreased pH. The amount of Enterobacteriaceae is a good indicator of the general hygiene condition of the fresh poultry meat and temperature abuse during storage (Zeitoun et al., 1994). The modified air together with chill storage conditions slows down the proliferation of Enterobacteriaceae. The count was slightly above 3 log CFU g^{-1} at the starting point and it maintained for the first three days of storage, then increased about up to 6 log CFU g^{-1}. Pseudomonas spp. have been identified as the main spoilage microorganisms of chilled poultry meat (Wickramasinghe et al., 2019). Although the final count of Pseudomonas spp. was the same in both samples, PET samples showed a lower amount of this bacteria group than BP samples during day 3 to day 14. Some studies demonstrated that the Pseudomonas spp. were resistant to high levels of oxygen, while sensitive to high concentrations of carbon dioxide (Meredith et al., 2014). In the study performed by Chmiel et al. (2018), the count of Pseudomonas increased during the study period and with a significantly higher amount in air condition compared to MAP with 80% O_2 during storage. Coagulase-positive staphylococci (CPS) have been reported to the most common species related to foodborne illness (Do Carmo et al., 2004). Figure 3E shows that CPS of both samples showed a similar increasing trend with 4 log CFU g^{-1} increase during the 14 days storage at 4 °C. Finally, concerning the microbiological evaluation, no significant differences have been reported between BP and PET packaging materials in the preservation of chicken meat.

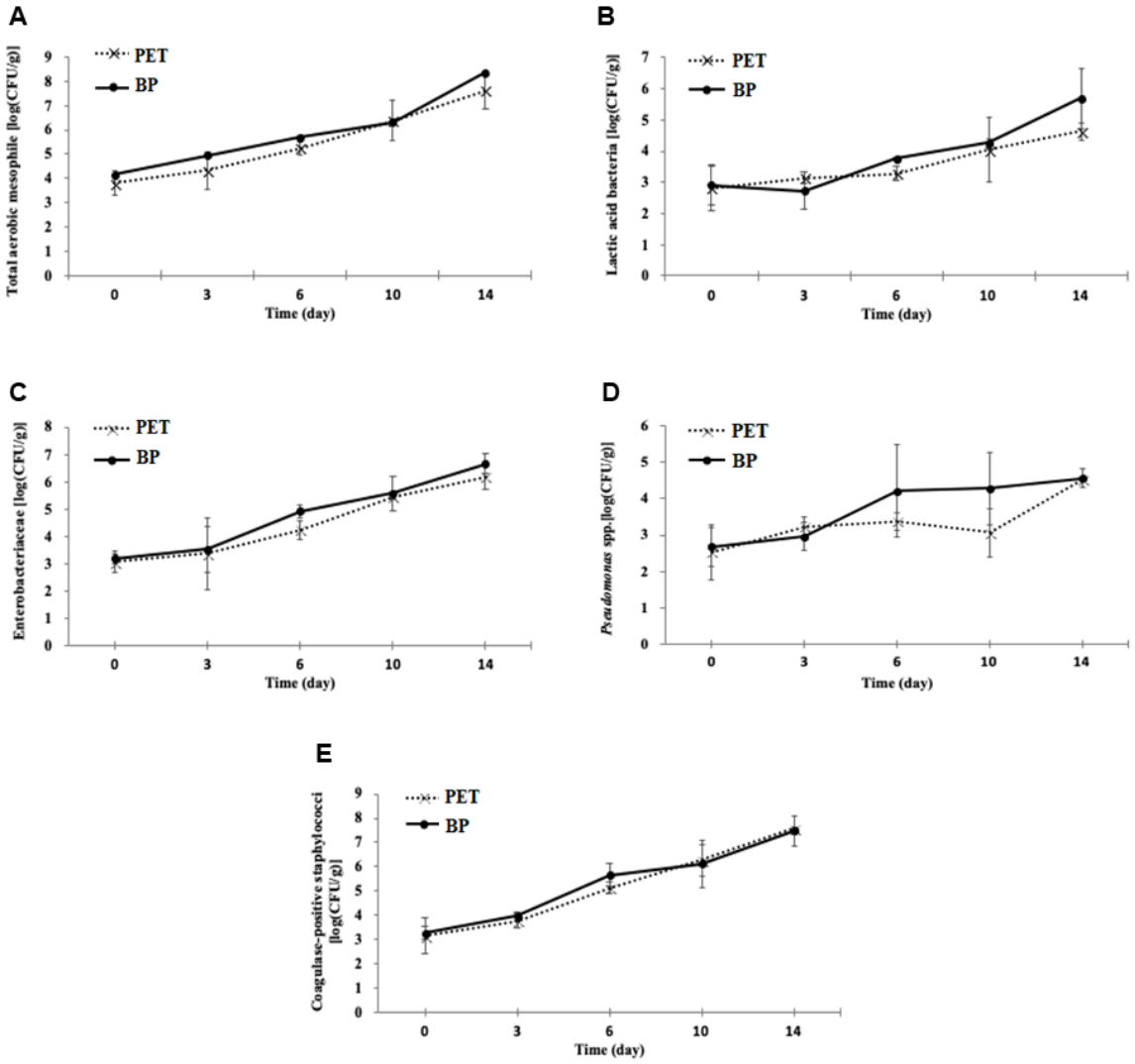

Figure 3. *Bacterial counts of total aerobic mesophile (A), mesophilic lactic acid bacteria (B), Enterobacteriaceae (C), presumptive Pseudomonas spp.(D), coagulasepositive staphylococci (E) during 0, 3, 6, 10, 14 days storage at 4 ◦C, detected in meat samples inside PET and BP packaging. Error bars represented standard deviations of the mean value.*

3.4. pH measurement

The pH values of both samples remained during the first three days of storage and slightly decreased during the following period (Figure 4). For meat stored in BP packaging system, it started with a value of 6.5, following a slight decrement at day 3 to 6.3 reaching 6.0 at the end. PET samples initial value was maintained during the first three days, and it also decreased to 6 at day 14. In general, there were small variations found between the two packaging systems throughout study period and this effect could be attributed to the strong buffering capacity of chicken fat.

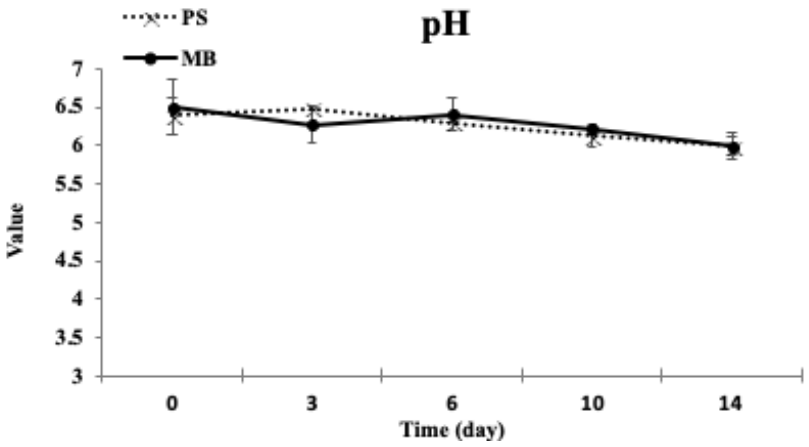

Figure 4. *pH values of raw chicken breast meat stored in PET and BP packaging.*

3.5. Sensory evaluation

The scores of meat sensorial attributes, aspect, color, odour, elasticity, and overall acceptability, were illustrated in Figure 5 and Table 1S. In general, attributes scores of both samples showed a decreasing trend and BP samples obtained higher scores than PET samples for meat aspect, color, overall acceptability on days 3, 6, and 10. However, on the last day of evaluation, panelists indicated PET samples were generally better than BP samples for all examined attributes. As for the other sensorial attributes, such as odour and elasticity (Figure 5B and 5D), both samples exhibited similar results for the first 10 days of storage, with higher scores obtained from PET samples at day 14. According to Chmiel et al. (2018),

chicken breast meat wrapped in PET was acceptable up to 9 days under MAP (75% O_2 and 25% CO_2) in the cooling room.

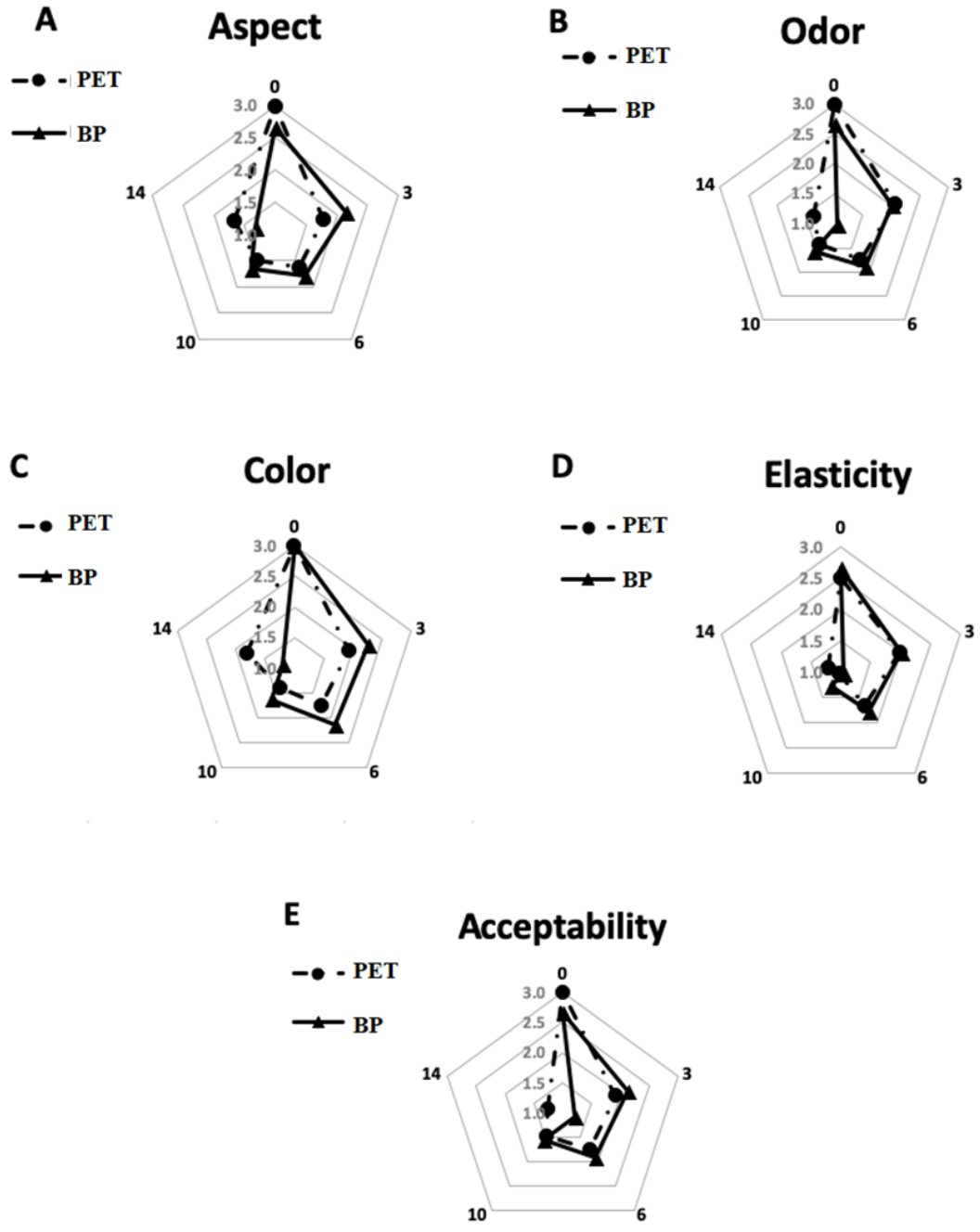

Figure 5. *Sensory evaluation of PET and BP samples at 0, 3, 6, 10, 14 days stored at 4 ◦C. Organoleptic descriptors of meat are reported in Table 1S (evaluation score from 1 to 3): A - aspect. B - odour. C - color. D - elasticity. E - acceptability.*

3.6. Discussion

In this research, the ability of a new biopackaging (BP) to preserve the quality of organic chicken meat in comparison to a classic polyethylene-terephthalate packaging (PET) has been evaluated. Chemical and microbiological markers of chicken meat (BAs, VOCs, selected microorganisms), pH, and sensorial parameters were monitored for 14 days in both packaged meat (BP and PET). The studied indices of BAs, i.e. Total BAs, BAI, and SPD/SPM ratio during the shelf life of meat showed a similar trend for both packs, and similar results have been obtained for the analysis of VOCs. The counts of total aerobic mesophile, mesophilic lactic acid bacteria, Enterobacteriaceae, presumptive Pseudomonas spp., and coagulase-positive staphylococci were not significantly different in the meat wrapped in BP and PET; moreover, the pH value was maintained during 14 days at chilled temperatures with slightly decrement. Concerning the sensorial evaluation, no significant differences were observed between meat BP and PET. Therefore, this new biopackaging showed similar performances in fresh chicken conservation compared to classic packaging. The assessment of the performances of biodegradable packaging in fresh meat preservation has been reported in few studies (Cheng et al., 2021). Compared to the literature, Moreno et al. (2018), reported that the storage of chicken breast fillets in oxidized starch biopackaging enhanced the microbiological shelf life of chicken but promoted lipid oxidation affecting the meat pH and color. The BP performances could be further improved through the incorporation of bioactive additives with antioxidant or antimicrobial activities (Cheng et al., 2019; Baek, Kim, & Song, 2019). Indeed, Hassan et al. (2019) developed a new starch-based biopackaging incorporated with rosehip extract, which limited the lipid oxidation in chicken breasts during storage. The proposed material is a result of a customized multi-layers structure that has been developed by exploiting the different properties of Novamont's bio-polymers, such as:

- HDT properties (Heat Deflection Temperature) that allowed to overcome the main problems related to storage temperature of trays before use (i.e. warehouse)

- Barriers properties that enabled the final structure to be used for different protective packaging with controlled atmosphere for all meats and fresh food. Thanks to the compostability of different layers all the packaging can be completely organic recycled (differently from the not recyclable traditional multi-layers structures).

4. Conclusions

This study showed that the new biopackaging (BP) is able to preserve chicken meat as a common plastic material (PET). Indeed, chemical, microbiological, and sensorial evaluations of chicken during storage revealed no statistically significant differences among the tested packaging, with the great advantage that the BP is completely biodegradable, compostable, and sustainable for the environment thanks to the complete organic recyclability in organic waste treatment plants (according to European standard EN13432). Further developments will consist to assess the preservation of other foods in the same packaging to promote a circular economy that can be only reached with the use of biodegradable and low environmental impact materials.

Declaration of Competing Interest

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

Acknowledgements

We are grateful to Marche Region through the “Programma di Sviluppo Rurale (PSR) 2014/2020 Misura 16.1” (ID 29057) for financially supporting the project.

Supplementary material

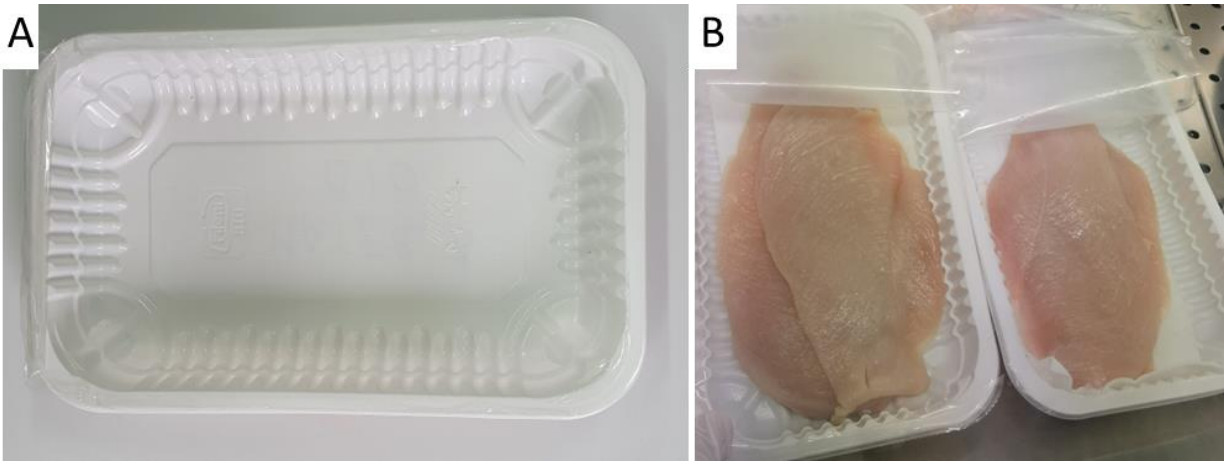

Figure 1S. *The studied Biopackaging (BP) without (A) and with (B) chicken meat*

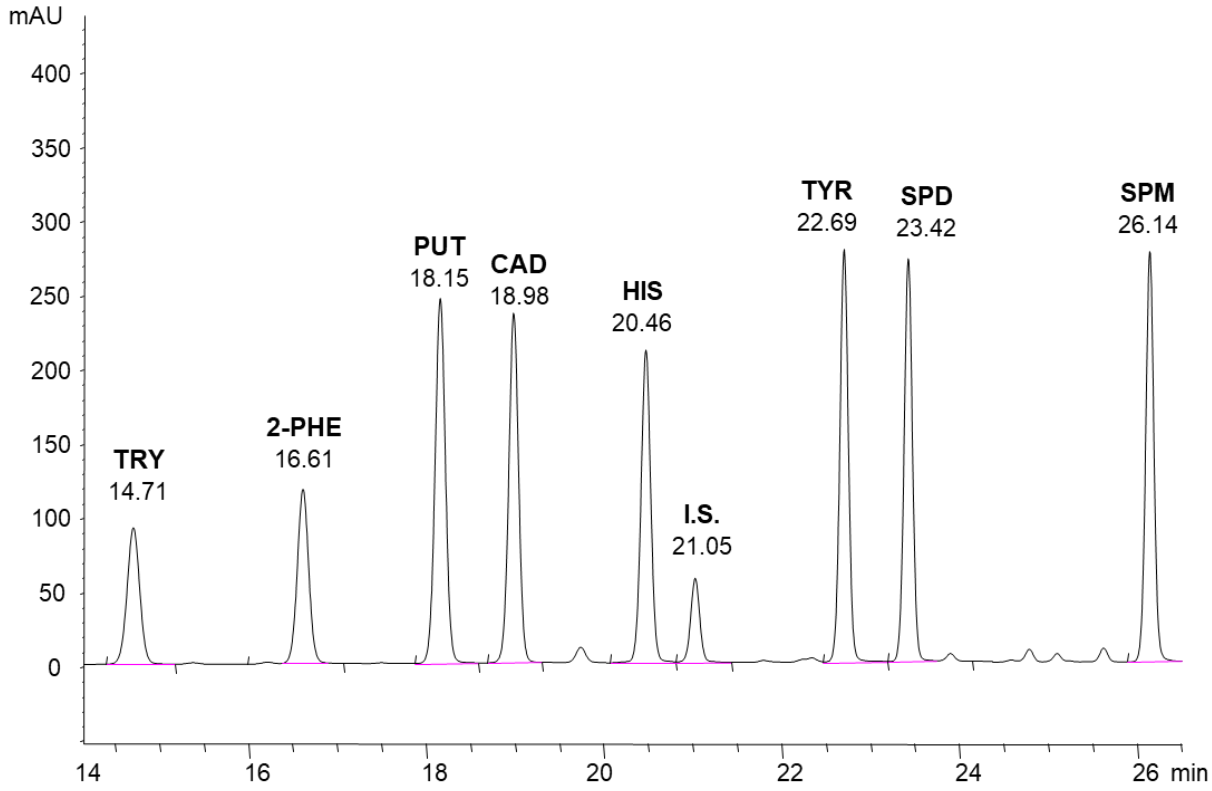

Figure 2S*. HPLC-DAD chromatogram of a 25 mg L-1 standard solution of all analysed BAs and Internal Standard. Legend: Tryptamine (TRY), 2-Phenylethylamine (2-PHE), Putrescine (PUT), Cadaverine (CAD), Histamine (HIS), 1,7- Diaminoheptane Internal Standard (I.S.), Tyramine (TYR), Spermidine (SPD), Spermine (SPM)*

Table 1S. *Chicken breast meat sensorial table.*

Attributes	Description	Score*
Aspect (Slime)	Without slime	3
	Slime present in some parts	2
	Slime on all surface	1
Odour	Characteristic	3
	Off-odour	2
	Foreign	1
Color	Pink to light red	3
	Pale pink to slight brown	2
	Earthly to brown	1
Elasticity	Fast return	3
	Slow return	2
	No return	1
Acceptability	Satisfied	3
	Acceptable	2
	Reject	1

*Intensity: 1-3.

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SECTION C
Results of the thesis

Organic and antibiotic-free chicken meat eating quality

Unpublished data

Organic and antibiotic-free chicken meat eating quality

1. Introduction

From the literature review carried out and summarized in section A, it emerged that many factors such as feeding, pre-slaughter stress, outdoor access, lifespan, etc. affect the final quality of chicken breast meat, although using the same chicken genotype. All these variables differ between divergent farming systems, such as antibiotic-free and organic farming. In the first one, in fact, the chickens are raised on an inside ground, fed with controlled feed, and without access to an open space. Organic breeding, on the other hand, provides an internal space but also an outdoor area where the animal can freely choose to go, they are fed with organic feeding and reared for longer times.

This preliminary trial consisted of assessment of meat quality traits of organic and antibiotic-free meats to obtain an overview on the qualitative grade of Ross 308 samples. The investigated parameters were color, WHC, meat pH, tenderness, lipid fraction quantification, and lipid peroxidation. Results were used as starting point for further research on the impact of farming system on chicken breast meat intrinsic characteristics.

2. Materials and Methods

2.1 Sample collection

The analysis carried out involved two groups of Ross 308 chicken samples provided by Fileni industry (Cingoli, Italy). Organic chickens were reared according to European Commission Regulation No 848/2018 for organic systems for poultry and livestock production. Antibiotic-free chicken meat according to the European Directive 2007/43/EC.

All the animals were reared and slaughtered in standardized conditions by the company and post-mortem Pectoralis major muscle (breast) biopsies were taken within 3 h after slaughter.

2.2 Meat colour

The instrument used was a Chroma Meter CR-400 (Konica Minolta ®, Tokyo, Japan). The measurement was performed using the CIELAB method. Lightness, redness, and yellowness values, L*, a*, and b*, respectively, are presented according to this method. Ten chicken breasts of each type were analysed performing 10 measurements at 10 different points on the surface of each entire breast.

2.3 Water Holding Capacity evaluation and total water content

Analyses were performed on Ross 308 chicken breast fresh meat using 10 breasts of each group, organic and antibiotic-free. Water Holding Capacity (WHC) was assessed applying the drip loss method described by (Bower & Zhuang 2015). A weighted sample from the central portion of the right part of each breast was removed, placed on a mesh screen in a covered plastic container, stored at 4°C, and reweighed after 24 hours. WHC was calculated using the following formula:

$$\text{WHC (\%)} = \frac{(\text{initial meat weight} - \text{final meat weight})}{\text{initial meat weight}} \text{ x } 100$$

To calculate the total content of water, chicken breast samples were trimmed to 5 × 2 ×1 cm size and the initial weight was determined. Samples were dried at 100 °C for 24 h in a stove (BINDER GmbH, Tuttlingen, Germany). Then the final weight was determined, and the percentage of water was calculated as followed:

$$\text{Total water content (\%)} = \frac{(\text{initial meat weight} - \text{final meat weight})}{\text{initial meat weight}} \text{ x } 100$$

2.4 pH monitoring

Analyses were performed on chicken breast meat using 10 breasts of each group. pH was measured using an Xs pH7 Vio pH meter (XS Instruments, Carpi (MO)) with XS 2 PORE T electrode for solid food matrices. The measurement was made by direct immersion of the electrode in the meat in ten different points of each breast.

2.5 Shear force

The Warner-Bratzler shear force (WBSF) method was used to determine instrumental tenderness of chicken raw meat expressed in Newtons. Three cubes of the size of 1 cm^3 from the right side of 10 breasts of each group of samples were cut parallel to the direction of muscle fibres. The shear strength of the sample meat is plotted with a computer graph that draws a curve as it is cut. The maximum shear strength is represented by the highest peak of the curve.

2.6 Lipid fraction quantification

A total of ten Ross 308 chicken breast meat of each group of samples was freeze-dried using an Lyovapor L-200 (BUCHI, Switzerland). The lipid fraction was extracted using a Soxhlet method. For each extraction, freeze-dried chicken meat was weighed and packed in a thimble and extracted with 250 mL of diethyl ether at 40°C for 6 hours. The extraction conditions were obtained from preliminary experiments performed in our laboratory. The extracts were dried by removing the residual solvent with a Rotavapor R-100 (BUCHI, Switzerland) and the residual lipids were weighed. The amount of total lipids of the meat was calculated according to the following formula:

$$\text{Fat content (\%)} = \frac{\text{extracted fraction weight}}{\text{initial freezedried meat weight}} \text{ x } 100$$

2.7 Lipid peroxidation (TBARS assay)

Lipid peroxidation was monitored in 10 Ross 308 chicken breasts from antibiotic-free and organic production systems by TBARS spectrophotometric assay. Briefly, the procedure was as follows: 3 g of each breast were homogenised using an Ultra-Turrax S 18N-10G (IKA-Werke Gmbh & Co., Germany) with 20 mL of MilliQ water. Then 5 mL of trichloroacetic acid (TCA, ≥99%, Sigma-Aldrich, Milano, Italy) 25% was added before vortex agitation so samples were left at 4 °C for 15 minutes and centrifuged at 4 °C for 20 minutes. From the paper filtered supernatant, 3.5 mL were transferred in a tube with 1.5 mL of 0.6 % 2-thiobarbituric acid (TBA) ($C_4H_4N_2O_2S$, ≥ 98 %, Sigma-Aldrich, Milano, Italy) solution and left in a water bath for 20 minutes at 70 °C. The reaction was stopped taking out the tubes and putting them in an ice bath for 5 minutes. Malonaldehyde reacts with TBA to produce a red color, its absorbance at 532 nm monitored using a spectrophotometer (Shimadzu, UV-160 A) and is proportional to the concentration of malonaldehyde. A stock solution of 1,1,3,3-Tetraethoxypropane ($(C_2H_5O)_2CHCH_2CH(OC_2H_5)_2$, ≥ 96 %, Sigma-Aldrich, Milano, Italy) was prepared in HCl 0.1 N and used for calibration curve. The TBARS are expressed as mg of malonaldehyde per kg meat sample.

2.8 Statistical analysis

Significant differences in each quality parameter between organic and antibiotic-free chicken breast samples were evaluated by one-way analysis of variance (ANOVA). Differences with $p < 0.05$ were considered statistically significant.

3. Results and discussion

The results of meat quality parameters analysed in Ross 308 chicken breast meat from organic and antibiotic-free are summarized in Table 1. Reported values are the means of ten analysed samples in each group and the calculated standard deviations. No statistically

significant difference was emerged between organic and antibiotic-free samples within each measured parameter of meat quality.

Table 7 - Summary of the results of chicken meat eating quality parameters in the comparison between organic and antibiotic-free (± standard deviation).

Eating quality parameters		Antibiotic-free	Organic
Meat color	Lightness (L*)	51.78 ± 4.41	56.02 ± 3.58
	Redness (a*)	2.23 ± 0.43	1.13 ± 0.56
	Yellowness (b*)	7.89 ± 2.15	10.60 ± 3.39
WHC	%	7.65 ± 0.38	8.10 ± 0.50
Total water content	%	76.21 ± 0.91	76.80 ± 1.50
Meat pH		5.92 ± 0.11	5.85 ± 0.12
Meat tenderness	N	9.43 ± 3.04	10.73 ± 4.00
Lipid content	%	2.75 ± 0.37	4.44 ± 0.34
Lipid peroxidation	mg MDA kg^{-1}	2.82 ± 0.97	1.34 ± 0.54

In meat color results, organic samples reported higher L* and yellowness value together with a lower redness although these differences were not statistically significant. Similarly, Lázaro *et al.* (2014) observed no difference in lightness values of organic and non-organic chicken meat during 9 day of refrigerated storage. In partial accordance with the present research results, Grashorn and Serini (2006) reported that breast meat colour was less red but more yellow in organic carcasses, without differences in brightness Moreover, Lee *et al.* (2008) proposed that other factors such as a lower WHC due to myofibril shrinkage are potentially responsible for the lower lightness values in non-organic samples. On the contrary, Husak *et al.* (2008); Viana *et al.* (2017) observed that organic broilers breast meat presented lower lightness and higher redness than non-organic ones. Previous studies have reported that breast meat from fast-growing broilers usually is less red when compared to meat from broilers with a slower growth-rate (Debut *et al.* 2003; Bihan-Duval *et al.* 2008; Viana *et al.* 2017).

The WHC values of organic and antibiotic-free compared chicken breast meat were very similar. Several studies reported that WHC can decrease over the frozen storage due to myofibrillar shrinkage caused by the formation of ice crystals, which damage muscle cells and cause protein denaturation (Xiong *et al.* 1999; Yoon 2002; Lee *et al.* 2008).

According to Viana *et al.* (2017), the production system seems not to affect pH values of chicken breast meat, as no statistically significant differences were found. Similar results were published by Souza *et al.* (2011) that documented that the pH values of breast meat of broilers produced under intensive and extensive rearing systems were not different. In contrast to our statistics, previous studies have documented that increased outdoor exercise during organic rearing systems can affect muscle traits, for example reducing meat pH and pre-slaughter stress (Castellini *et al.* 2002b; Castellini *et al.* 2006; Heyer *et al.* 2006).

Meat pH, WHC and color parameters are closely correlated in meat quality. The meat pH was reported to influence myofibrils structure as a lower pH value can cause the shrinkage of the contractile fibres reducing the WHC and therefore increases light scattering. Furthermore, a low pH reduces the myoglobin capacity in absorbing green light, resulting in less red and more yellow meat (Castellini *et al.* 2002b).

Antibiotic-free breast meat was slightly more tender than the organic one, without statistically significant differences. In general, it is well known that tenderness decreases when the chicken become older, in fact, organic chickens lifespan is longer resulting a less tender meat (Sirri *et al.* 2011). The same conclusion can be assessed for the lipid content results of the present study.

The TBARS values, as lipid peroxidation parameter, were slightly higher in antibiotic-free chicken breast meat but this difference was not statistically significant. Pre-slaughter stress and muscle-to-meat process conditions are the main factors that affect lipid peroxidation mechanism. In the comparison between conventional and organic chicken meat, lipid

peroxidation can be considered as an oxidative stress parameter related both to the breeding conditions and to the slaughter and technological processes (Castellini *et al.* 2002b; Dal Bosco *et al.* 2021). According to our findings, other studies reported that rearing system did not significantly affect lipid oxidation, but refrigerated storage can increase TBARS levels (de la Torre *et al.* 2012; Viana *et al.* 2017).

4. Conclusions

Rearing chickens by the organic system seems to be a possible alternative to the conventional antibiotic-free method because it increases the physical activity which favour the development of the muscle, and make animals less sensitive to stressors, improving their response to pre-slaughter treatments. Despite this, the data reported in the present study demonstrate that the farming system (antibiotic-free and organic) do not significantly affects the color, pH, WHC, tenderness and lipid oxidation proprieties of chicken *pectoralis major* muscle. Further studies at the molecular level should be conducted to elucidate the biochemical effects of organic production systems on meat quality and authenticity.

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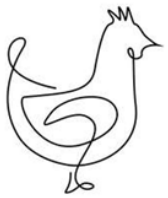

SECTION C
Results of the thesis

Assessing chicken meat shelf-life as meat quality parameter within divergent production systems (organic *versus* conventional) using a chemical, microbiological and sensorial markers

Publication 2

Assessing chicken meat shelf-life as meat quality parameter within divergent production systems (organic versus conventional) using a chemical, microbiological and sensorial markers

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Submitted to *Journal of Food Composition and Analysis*
http://dx.doi.org/10.2139/ssrn.4272315

Abstract

In recent years, the increasing interest in healthier and more sustainable food behaviours, led to a greater demand for organic products, especially in animal foods. This study aims to increase knowledge on the organic meat quality through a comparative shelf-life analysis of organic and conventional broilers (Ross 308) breast meat. A total of 11 biogenic amines and 21 volatile organic compounds (VOCs) were monitored combining the results with meat pH, microbiological and sensorial analysis during 10 days of storage (day 0, 3, 6, 10). The organic meat showed generally a lower value in spoilage markers than the conventional one. Meat spoilage related VOCs concentrations underlined important differences especially at day 10. The same trend emerged by the total biogenic amines concentration at day 10 with values of 853.24 mg Kg^{-1} in organic and 354.12 mg Kg^{-1} in conventional meat. Microbiological analysis reported a delayed bacterial proliferation in organic meat. Conventional meat maintains a better aspect, but odour and elasticity scores were higher in organic one. In conclusion, chicken breast meat from organic production systems showed overall higher shelf-life than chicken meat from conventional inside ground farms.

Keywords: Organic meat, meat shelf-life, chicken meat, meat quality.

1. Introduction

Nowadays, the demand for animal foods with fundamental nutritional qualities is ever increasing. The choice of poultry is influenced by its sensorial and nutritive values coupled with lower prices than that of red meat and other animal products (Yenilmez et al. 2014). The Food and Agriculture Organization (FAO) reported that the world poultry meat production has risen from 9 to 122 million tons in the last 50 years. Poultry meat accounts today for about 37% of globally produced meat, and chicken represent the 89% of it (FAO, 2020). The increasing demand for poultry meat has been a consequence of rising consumer concern about food in recent years. Most of them are willing to pay higher prices for animal products with a guaranteed high level of quality (Nguyen et al., 2019; Kaygisiz et al., 2019). One result is that organic production has experienced and is still experiencing a remarkably positive trend; in fact, worldwide, the area spent on organic production has doubled in last 10 years (Rabadán, 2020).

From 2020, the COVID-19 pandemic has intensified consumers attention on health values of food products. This led to an ever-growing attention on labels, origins, ingredients (Śmiglak-Krajewska and Wojciechowska-Solis, 2021). The increase in organic products purchases can be valued as a consequence of novel healthier and sustainable food behaviours (Ćirić et al. 2020; Castellini et al. 2021; Muresan et al. 2021).

Organic farming systems are based on exacting standards of animal welfare which can provide products with high levels of quality and safety (Fanatico et al., 2007; El-Deek and El-Sabrout, 2019). In Europe, organic farming and production are regulated by Council Regulation (EC) No 834/2007 and by two implementing regulations, No 889/2008 and No 1235/2008. These regulations are designed to provide a clear guideline for origin of animals, livestock housing and husbandry practices, feed, disease prevention and veterinary treatment

allowed, but also regulate collection, packaging, transport, storage, import and export trading of organic meat and derivatives.

Organic guidelines application is considered beneficial for animal welfare because it allows to live healthier lives, to have space for exercise and to perform natural behaviours. In broilers specific case, it has been reported that meat quality traits can be positively affected by organic production (Bogosavljević-Bošković et al., 2012; Vanhonacker et al., 2012). Although the current interest topic, in scientific literature, there is a paucity of studies on chicken meat with the focus on differences between organic and non-organic production systems in terms of quality of the meat product.

A recent study by Çapan, et al. 2021 reported that organic chicken breast meat has higher content of fat (with increased percentages of linoleic and docosahexaenoic acids), proteins, minerals together with better cooking loss and water holding capacity values than the conventional one. In meat science, safety, shelf-life, appearance, and sensorial characteristics are key aspects of distribution and selling meat and meat products. The trend of meat quality parameters, during meat shelf-life, is function not only of preservation method but also of meat intrinsic characteristics (Stopforth, 2017; Alessandroni et al. 2021). For this reason, the aim of this study is to increase our knowledge about organic meat quality through a comparative shelf-life analysis between breast meat from chicken reared according to organic regulations and from conventional inside ground farming.

2. Materials and Methods

2.1 Meat sample collection

A total of 24 Ross 308 chicken *pectoralis major* muscles were provided the day of slaughter by Fileni® industry (Cingoli, Italy). Half of them were males reared in an antibiotic-free inside ground farming (CC), slaughtered after 48 days at average live weight of 3473 g. The other half were females reared following European organic regulations (OC), slaughtered

after 83 days at average live weight of 3962 g. Shelf-life was assessed monitoring chemical, microbiological and sensorial parameters of the meat. Three samples from each group were straight analysed the day of slaughter (T0), while the others were stored at 4 °C. To avoid any impact of chicken meat conventional packing methods, the breasts were individually wrapped in an insulated plastic film with the sole purpose of avoiding environmental contamination during the storage period. Shelf-life parameters of three chicken breasts from each group were investigated after 3, 6, and 10 days of storage (T3, T6, T10).

2.2 Chemicals and Reagents

Spermine tetrahydrochloride (SPM, > 98 %), spermidine trihydrochloride (SPD, > 98 %), cadaverine dihydrochloride (CAD, > 98 %), putrescine dihydrochloride (PUT, > 98 %,), histamine dihydrochloride (HIS, > 99 %), tyramine hydrochloride (TYR, > 98 %), 2-phenylethylamine hydrochloride (PHE, > 98 %), tryptamine hydrochloride (TRY, > 99 %), (-)-epinephrine (E, > 99 %), dopamine hydrochloride (DA, 98 %) and serotonin (5-HT, > 98 %) for standard solutions preparation and 1,7- diaminoheptane (98 %) as the internal standard were supplied by Sigma-Aldrich (Milano, Italy). Hydrochloric acid (37 %) trichloroacetic acid (TCA, ≥ 99 %), acetone (≥ 99.5 %), sodium hydroxide anhydrous (≥ 98 %), sodium carbonate anhydrous (≥ 99.5 %), ammonia solution (28-30 %) acetonitrile (HPLC gradient grade, ≥ 99.9 %), methanol (HPLC gradient grade, ≥ 99.9 %) and dansyl chloride (98 %) were provided by Sigma-Aldrich (Milano, Italy).

The stock solutions (1000 mg L^{-1}) of the 11 biogenic amines (BAs) were prepared by adding 10 mL of 0.1 M HCl to 10 mg of each standard and were then stored in the dark at 4 °C. Working solutions at various concentrations were diluted with 0.1 M HCl. The internal standard solution was made at 10 mg mL^{-1} in 0.1 M HCl. The derivatizing solution was prepared each time by dissolving dansyl chloride in acetone (10 %).

For VOCs identification, ethanol (gradient grade for liquid chromatography LiChrosolv®), 3-methylbutanol (analytical standard), 1-pentanol (analytical standard), 1-hexanol (analytical standard), butanone (ACS reagent, ≥ 99.0 %), 2-pentanone (analytical standard), hexanal (analytical standard), 2-heptanone (analytical standard), 3-hydroxy-2-butanone (acetoin, analytical standard), propionic acid (analytical standard), hexanoic acid (analytical standard) and dimethyl sulfide (analytical standard) were provided by Supelco (Bellefonte, PA, USA) and 1-octen-3-ol (98 %), 1-octanol (≥ 99 %), 2-octen-1-ol (97 %), phenol (ACS reagent, 99.0-100.5 %), 2-nonanone (≥ 99 %), acetic acid (ACS reagent, ≥ 99.8 %), 2-methylbutyric acid (98 %), carbon disulfide (ACS reagent, ≥ 99.9 %) and dimethyl sulfone (98 %) were provided by Sigma-Aldrich (Milano, Italy).

2.3 Biogenic amines quantification

The BAs quantification procedure is based on a method previously published by Sirocchi et al. (2013) which has been slightly modified. Analyses were performed on three samples of CC and OC chicken breast meat the day of slaughter and after 3, 6, and 10 days. 5 g of each sample were weighted in a centrifuge tube and homogenized with 25 mL of TCA solution 5 % using an Ultra-Turrax S 18N-10G homogenizer (IKA-Werke Gmbh & Co., Germany). After centrifugation, 0.2 mL of a 10 mg L^{-1} internal standard solution, 0.3 mL of Na_2CO_3 saturated solution, and 100 μL of NaOH 2 N were added to 1 mL of supernatant. Derivatization was performed at 45 °C for 45 min in agitation adding 2 mL of 10 % dansyl chloride solution. Dansyl chloride excess was removed by adding 100 μL of NH_4OH 28 % and acetone was removed by N_2 drying. SPE STRATA X 33 μm Cartridges, 200 mg/6 mL (Phenomenex, Bologna, Italy) were used for purification step and samples were eluted with 4 mL of CH_3CN and filtered using a 0.45 μm PTFE filter (Supelco Bellefonte, PA, USA) before High-Performance Liquid Chromatography (HPLC) injection. All samples were analysed in triplicate. BAs were separated using a Kinetex C18 analytical column (100 × 4.6

mm I.D., particle size 4 μm) provided by Phenomenex (Torrance, CA, USA). The column temperature was 40 °C. Mobile phase for HPLC analysis flowed at 0.8 mL min^{-1} and consisted in MilliQ water (A) and a solution of MeOH/ACN 70:30 (B). The gradient program started from 60% B and goes to 70% B in the first 10 min, to 80% B in 20 min, to reach 100% on the 26^{th} minute B, isocratic flow until 29^{th} minute, and 60% B from 32 to 36 min. 10 μL of sample were injected. HPLC system (1260 Infinity II, Agilent Technologies, Santa Clara, CA, USA) was coupled with Fluorescence Detector (FLD) (Agilent Technologies, Santa Clara, CA, USA). To establish the best excitation and emission wavelengths, a multiexcitation and a multiemission analyses were performed in the range of 200 - 400 nm and 400 – 700 nm respectively. Excitation and emission wavelengths chosen were 256 nm and 520 nm, respectively. BAs working solutions at different concentrations were derivatized, as described above, and injected to obtain the 11 calibration curves shown in Figure 1S (Supplementary material). Response factors (Rf = BA peak area / internal standard peak area) were used to calculate the calibration curve of each BA. All calibration curves showed a good resolution and linearity (R^2> 0.9971).

2.4 Analysis of volatile organic compounds

Each day of analysis, three CC and three OC meat samples (5 g from each breast) were minced and homogenized in an analytical blender (Tube Mill Control, IKA-Werke GmbH & Co. KG, Germany) for 30 seconds. A proper aliquot (2 g) of the homogeneous sample was weighted in a 10 mL vial with a pierceable septum and conditioned at 40 °C for 20 min in a water bath. The solid phase microextraction (SPME) fibre assembly was purchased from Supelco (Bellefonte, PA, USA) and had a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coating with 1 cm length stationary phase. Then, the time of exposure of the fibre in the vial headspace (HS) was 30 min. Volatile compounds were analysed by GC-MS using a 6890N Network GC

System coupled to a 5973 Network Mass Selective Detector from Agilent Technologies (Santa Clara, CA, USA). The separation of the analytes was performed using a capillary column coated with polyethylene glycol (60 m x 0.25 mm x 0.25 μm film thickness, DB-WAX, Agilent Technologies, Santa Clara, CA, USA). The initial carrier gas (helium) flow rate was 1.2 mL min^{-1}. Injector temperature was 260 °C and splitless time was 4 min. Oven temperature was set at 35 °C for 4 min, then increased to 120 °C at 2.50 °C min^{-1} and then raised to 250 °C at 15 °C min^{-1} and maintained for 3.33 min, for a total run time of 50 min. Mass analysis was performed in scan mode in the range of 25-400 Da. Transfer line was held at 260 °C, ion source at 230 °C and quadrupole at 150 °C. The SPME fiber was left exposed in the injector for 10 min to be well cleaned and reactivated after the desorption. A mix of straight chain alkanes was used for the calculation of retention indices. VOCs were identified by obtaining structural information from the mass spectrum, and by comparison of their retention indices and their mass spectra with those of analytical standards individually analysed and those from NIST-USA US National Institute of Standards and Technology (http://webbook.nist.gov). The repeatability of the method was controlled using an external standard (3-octanol in acetone, 0.5 mg mL^{-1}); each day of the analysis 10 μL of the standard solution was put in a 10 mL vial and analysed in duplicate at the same condition of the sample.

2.5 Microbiological analysis

The microbial quality of three CC and three OC chicken breast was studied each day of analysis according to the guidelines from CeIRSA (2017) that transpose ISO guidelines. Briefly, 10 g of meat from each breast was homogenized with 90 mL sterile sodium chloride solution (NaCl, 0.9%, Sigma-Aldrich, Co., St. Louis, USA). Then, 10-fold serial dilutions were made, and 0.1 mL of each dilution was further plated on selective agar for enumerating the following microbial groups, including total aerobic mesophiles (PCA), mesophilic lactic

acid bacteria (MRS), *β*-glucosidase-positive *E. coli* (TBX), Enterobacteriaceae (VRBG), *Pseudomonas* spp. (Pseudomonas agar), coagulase-positive staphylococci (BPA), sulfide-reducing bacteria (ISA), *Cl. perfringens* (TSC). The description of selective media in details and the incubation condition for each bacterial group were described by Alessandroni et al., (2021). After incubation, the bacterial count of each group was calculated and expressed in colony forming unit (Log CFU g^{-1} of meat).

2.6 pH monitoring

pH was measured using an Xs pH7 Vio pH meter (XS Instruments, Carpi (MO)) with XS 2 PORE T electrode for solid food matrices. The measurement was made by direct immersion of the electrode in the meat. Each day of analysis (0, 3, 6, 10), the pH of three breasts from each group of samples was determined by calculating the average values of ten different measurement in ten scattered points of each breast.

2.7 Sensorial parameters

Chicken breast samples were prepared and selected to exclude factors that cause inhomogeneity and could potentially affect panelists evaluation, such as blood vessels, bruise, tenders. Meat samples were further divided into pieces with defined size - 3 cm x 3 cm x 2 cm. The sensory evaluation was conducted on raw meat samples by the same ten panelists each day of analysis (0, 3, 6, 10) according to the international standards (ISO, 2007) and the method was adapted from Yimenu (2019). The panel was composed by pretrained food faculty members of University of Camerino to familiarize sensorial vocabulary, including overall meat aspect, odor, color, elasticity, and overall acceptability (Baston and Barna, 2010). For each evaluation, three samples of conventional (CC) and organic (OC) meat were labelled with 3-number codes and presented to the panel. A hedonic scale of 0-3 that ranging from 0 (unacceptable) to 3 (optimum) was used for each sensorial attribute as describe by Alessandroni et al. (2021). Moreover, the sensorial index of each

sample at different time points was calculated using the following formula (Kreyenschmidt, 2003):

$$SI = 2A + 2O + 2C + 2OA/9$$

with: SI, sensorial index; A, aspect; O, odour; C, colour; OA, overall acceptability.

2.8 Statistical analysis

Significant differences of BAs indices, volatile organic compounds, and pH values between the two types of chicken meat in each day of analysis were evaluated by one-way analysis of variance (ANOVA). Significant differences between the microbial groups of samples were evaluated by two-way ANOVA using Šídák's multiple comparisons test. The test was performed using GraphPad Prism version 9 (Mac Os X, GraphPad Software, San Diego, California USA, www.graphpad.com). The same statistics were applied to study differences of each kind of meat among sampling days for each analysed parameter. Differences with $p < 0.05$ were considered statistically significant. Data elaboration was carried out using PAST software package (Hammer et al., 2001). Each experiment was performed in triplicate.

3. Results and discussion

3.1 Biogenic amines quantification

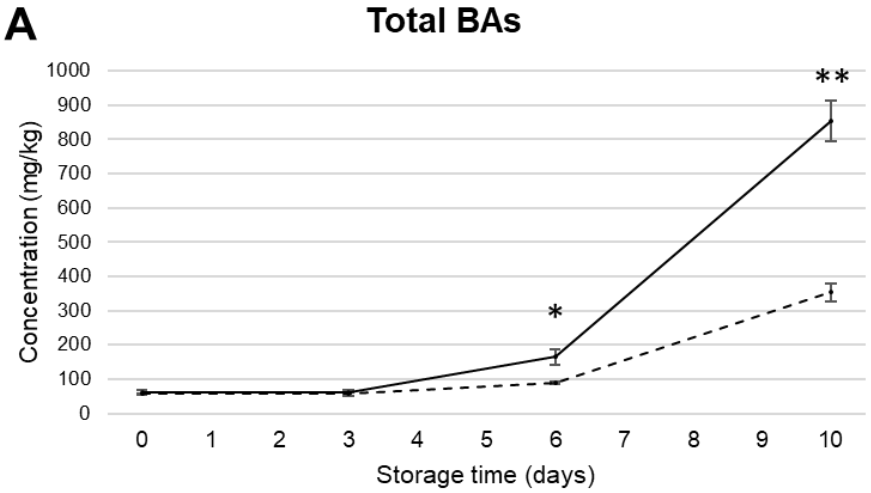
BAs extraction, derivatization, purification, and quantification using HPLC-FLD was performed each day of analysis on three chicken breasts of each group. Results are reported in Figure 1 using two indices: Total BAs (Figure 1A), which is the sum of the concentrations of the 11 monitored BAs and B.A.I (Biogenic Amines Index) (Figure 1B) which is the sum of the concentrations of PUT, CAD, HIS and TYR, both expressed in mg Kg^{-1} (Douny et al. 2019). BAs concentrations are expected to report an increasing trend during the shelf-life of meat. As shown in Figure 1A, during the first 3 days of shelf-life, both OC and CC groups of muscles showed low BAs levels. Then CC presented an important increase, reported on

day 6 with a statistically significant difference compared to OC and to its own value at day 3. On day 10, this accelerated BAs production in conventional meat samples led to value more than 2 times higher in CC than in OC, 853.24 mg Kg^{-1} and 354.12 mg Kg^{-1} respectively. B.A.I. values followed the same trend of the Total BAs. They were low until sampling day number 3 for both analysed meat groups. CC samples reported a faster increasing from day 6 respect to OC with a statistically significant difference maintained also in day 10 (Figure 1B). Moreover, focusing on the conservation time factor for each type of meat, both monitored BAs indices underlined statistically significant differences at day 6 and 10 in CC samples but only at day 10 in OC samples.

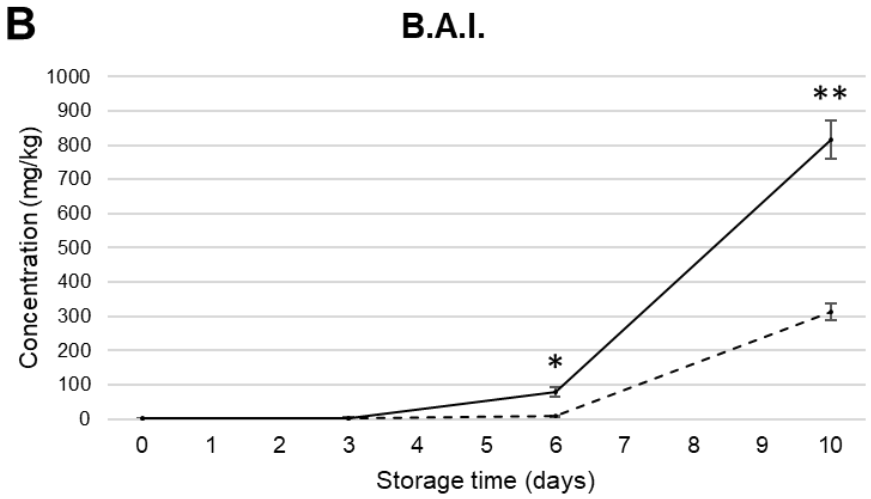
From a more in-depth analysis it emerges that the higher Total BAs and B.A.I. values at day 6 and 10 was due to a rapid increase of two specific BAs in both samples groups. Indeed, CAD and PUT production increased rapidly from day 6 of storage and their concentration grew particularly fast in CC samples during the latest stage of spoilage process. The trend of these BAs in CC and OC chicken meat spoilage is reported in Figure 2S (Supplementary material). PUT concentration grew from 1.48 mg Kg^{-1} (day 6) to 15.00 mg Kg^{-1} (day 10) in OC samples and from 8.65 mg Kg^{-1} to 253.11 mg Kg^{-1} in CC in the same shelf-life period. These values underline that specific BAs production starts before

in CC respect to OC. CAD values further mark this concept with statistically significant differences between CC and OC and from day 6 and day 10 among the same meat group (from 6.21 mg Kg^{-1} to 262.76 mg Kg^{-1} and from 36.50 mg Kg^{-1} to 388.01 mg Kg^{-1} for OC and CC respectively). BAs in meat are principally produced by bacterial decarboxylation of corresponding amino acids through substrate-specific decarboxylase enzymes (Alessandroni et al. 2021). The underlined differences between CC and OC in terms of BAs formation suggest not only a difference in meat microbiota (see section 3.3), but also a probable dissimilarity in amino acids and protein expression. Tyramine, putrescine and cadaverine are

reported to normally increase their concentration during the shelf-life of meat and meat products, while other BAs such as spermidine and spermine tend to decrease or remain constant (Alessandroni et al. 2021; Yusoff et al., 2021; Wójcik et al., 2021). Vinci and Antonelli (2002) observed that chicken meat conservation was critical, because non physiological BA increased earlier and more rapidly than in red meat. Accordingly, Ruiz-Capillas and Jiménez-Colmenero (2005) assessed those shorter muscular fibres in chicken and consequently to shorter chain proteins, facilitating proteolytic enzymes action and promoting BAs biosynthesis from amino acids.



	T0	T3	T6	T10
CC	61.1±7.1[a]	59.9±9.3[a]	164.4±21.2[b]	853.2±58.6[c]
OC	56.9±2.2[a]	56.1±4.6[a]	90.5±3.2[a]	354.1±26.7[b]



	T0	T3	T6	T10
CC	0.951±0.14[a]	3.29±0.25[a]	80.4±13.9[b]	814.6±55.8[c]
OC	2.73±0.44[a]	3.46±0.47[a]	9.08±1.47[a]	312.3±23.9[b]

Figure 1. *Biogenic amines quantification during 10-days shelf-life of chicken breast meat from different production systems (CC and OC). A) sum of average concentrations of analysed BAs (Total BAs); B) Biogenic Amines Index (B.A.I.) of OC and CC during the storage period (±standard deviation). Significant differences ($p<0.05$) between the two meats in each day are indicated by the asterisks. Significant differences ($p<0.05$) between the days for each kind of meat are indicated in the tables below by different letters.*

3.2 Analysis of volatile organic compounds

The formation of VOCs from the two types of chicken breast meat (OC and CC) over 10-days storage period was evaluated by HS-SPME-GC-MS. VOCs were monitored at different days: at time zero and after 3, 6 and 10 days. A total of 21 VOCs were identified in both samples, and they are listed in Table 1. These compounds were organic acids, ketones, aldehydes, alcohols, phenols, and sulfur containing compounds. Most VOCs identified in this study were previously detected in the headspace of both unspoiled and spoiled chicken meat (Mikš-Krajnik et al, 2015; Casaburi et al., 2015; Ioannidis et al., 2018). As reported in Table 1, some compounds were detected in both types of meat, but after different times of storage (e.g., 1-octen-3-ol, 2-methylbutyric acid, 2-pentanone and hexanal), while other analytes, such as acetic acid, dimethyl sulfide, 2-heptanone and propionic acid could be found only at the latter days of storage of the samples, usually after 10 days. Alcohols can be produced by some microorganisms (e.g. *Pseudomonas* and *Carnobacteria*) during meat storage, deriving from different metabolic pathways, such as proteolytic activity and amino acid metabolism, methyl ketones reduction and reduction of aldehydes coming from lipid oxidation (Casaburi et al., 2015). For instance, 3-methylbutanol is one of the most detected alcohols in raw meat; deriving from the proteolytic pathway of leucine, it has been considered as a chemical marker for poultry meat spoilage in previous studies (Alexandrakis et al., 2011; Casaburi et al., 2015). In this work, it was detected at day 6 in CC and at day 10 in both samples (higher concentration in CC) indicating that it could be considered a marker of the latter stages of the spoilage process. Ethanol developed during storage time in CC and at day 10 in OC samples. As for other alcohols, it was considered a meat spoilage indicator, as reported by Mikš-Krajnik et al. (2015) and Alexandrakis et al. (2011). Ketones could derive from fatty acid oxidation or from different metabolic reaction, such as the glucose catabolism and from the microbial degradation of aspartate as for the acetoin (3-hydroxy-

2-butanone). Acetoin is the ketone most produced in meat by several microbial species, and it is the molecule involved in the development of the cheesy odour in spoiling meat (Zareian et al., 2018). Table 1 shows that acetoin was detected in CC samples after 6 and 10 days of storage but not in OC samples. Free organic acids detected in the analysed meat were short chain fatty acids (SCFAs), namely acetic, propionic, 2-methylbutyric and hexanoic acids. They could derive from the degradation of amino acids, oxidation of aldehydes or from metabolic activities of some bacteria, mainly LAB and *B. thermosphacta* (Zareian et al., 2018). All the detected SCFAs were present after 10 days only in CC samples, except for 2-methylbutyric acid, which developed during time only in OC samples. Acetic acid was the most prevalent fatty acid CC samples. In several studies, it has been considered as a good indicator of meat spoilage with its characteristic vinegar and pungent odour (Mikš-Krajnik et al., 2015; Zareian et al., 2018). Volatile sulphur compounds are responsible for the sulphury and putrid odour that is predominant in the meat at advanced storage. They mostly originate from degradation of sulphur-containing amino acids. Dimethyl sulphide is known to be the most common volatile sulphur compound in meat (Casaburi et al., 2015). In the present study, it was detected after 10 days in both samples, in greater quantity in OC. Dimethyl sulfone was found instead only in the CC meat. Considering the VOCs composition of the two types of chicken meat after 10 days of storage, it was found that CC samples produced the highest number of spoilage related VOCs, thus showing a slower deterioration respect to antibiotic-free conventional chicken meat. Mancinelli et al. (2020) indicated that the poultry genotype is responsible for the major differences detected in the VOC profiles of the meat. Each detected compound may become also more or less dominant depending on animal species/age, feeding, processing and storage (Casaburi et al, 2015). There is not much information in literature about the differences in VOCs composition between organic and conventional chicken meat, but some studies indicated some nutritional

and sensorial differences. For instance, Gashorn and Serini (2006) reported higher contents of dry matter, crude protein, ash, fat, and *n*-3 polyunsaturated fatty acids (PUFAs) in organic chicken meat. Then sensory panellists indicated organic broiler meat as tougher and tastier than conventional one, demonstrating a slight superior quality of organic chicken meat. Also, Mancinelli et al. (2020) indicated a higher percentage of *n*-3 PUFAs in slow-growing chickens compared to fast-growing ones. This content could increase in free-range and organic systems because of the high pasture intake. Then, intake of grass improves antioxidant responses and prevents PUFAs from oxidation. The differences found in the lipid composition of organic and conventional chicken meat are highly important, because VOCs development is linked to the contents of PUFAs and antioxidants (Mancinelli et al., 2020). Also, microorganisms content is strictly related to VOCs production, and it is minor in organic chicken

Table 1. *Volatile compounds detected by HS-SPME-GC-MS during the storage of chicken breast meat from the two farming method, their experimental linear retention indices (LRI) on a polyethyleneglycol coated column (LRI reported in literature (NIST, 2017)), their odour attribute, abundances in terms of peak areas and % relative standard deviation (RSD, n=3) and their similarity index (SI). Significant differences ($p < 0.05$) between the two types of meat (OC and CC) in each day are indicated by the asterisk. Significant differences ($p < 0.05$) between the different days for each of the two meats are indicated by different letters in the same row.*

Compound	Odour attribute	LRI[1] (exp)	LRI[2] (lit)		Sampling days 0 Area	0 RSD%	3 Area	3 RSD%	6 Area	6 RSD%	10 Area	10 RSD%	SI[3] (%)
Alcohols and phenols													
Ethanol	Vinous	929	930	OC	nd	nd	nd	nd	nd	nd	3.36E+06*	13.91	80
				CC	6.29E+05^{a}	3.37	nd	nd	1.30E+06^{a,b}	23.78	3.30E+06^{b}	25.86	
3-Methylbutanol	Roasted	1213	1208	OC	nd	nd	nd	nd	nd	nd	5.96E+06*	4.78	86
				CC	nd	nd	nd	nd	1.00E+06	30.33	2.27E+07	37.93	
1-Pentanol	Fusel	1254	1252	OC	5.25E+05^{a}	2.16	6.72E+05^{a}	30.22	7.42E+05*^{a}	8.85	1.39E+06^{b}	4.76	82
				CC	nd	nd	nd	nd	1.70E+06	20.2	nd	nd	
1-Hexanol	Fruity	1356	1354	OC	nd	nd	3.55E+05^{a}	36.38	4.76E+05^{a}	1.31	2.01E+06*^{b}	2.72	78
				CC	nd	nd	nd	nd	8.75E+05	74.29	7.73E+05	14.99	
1-Octen-3-ol	Earthy	1451	1449	OC	7.70E+05^{a}	4.59	1.27E+06^{b}	9.05	1.45E+06*^{b}	7.73	5.78E+06*^{c}	2.57	86
				CC	nd	nd	nd	nd	2.45E+06	7.5	2.53E+06	8.53	
1-Octanol	Waxy	1566	1565	OC	1.48E+06^{a}	9.53	3.49E+05*^{b}	16.98	8.68E+05^{c}	0.09	6.99E+05^{c}	6.22	80
				CC	1.23E+06	11.49	7.86E+05	13.67	1.97E+06	25.44	1.87E+06	27.88	
2-Octen-1-ol	Green	1615	1620	OC	nd	nd	nd	nd	3.14E+05	42.95	6.03E+05	4.67	80
				CC	nd	nd	nd	nd	nd	nd	nd	nd	
Phenol	Sweet	1985	1987	OC	nd	nd	nd	nd	nd	nd	nd	nd	86
				CC	nd	nd	nd	nd	1.23E+06	22.04	1.31E+06	18	
Ketones and aldehydes													
2-Butanone	Fruity	892	894	OC	9.21E+05*	3.07	9.21E+05	35.03	9.84E+05	18.45	1.35E+06*	4.87	79
				CC	1.05E+06^{a}	1.61	4.50E+05^{b}	18.16	4.76E+05^{b}	18.16	6.03E+05^{b}	5.25	
2-Pentanone	Fruity	961	970	OC	nd	nd	nd	nd	5.34E+05^{a}	32.25	4.44E+06*^{b}	1.78	72
				CC	3.25E+05^{a}	4.35	nd	nd	6.08E+05^{a}	23.17	1.80E+06^{b}	0.13	
Hexanal	Green	1064	1065	OC	2.37E+06*^{a}	8.96	nd	nd	5.53E+05*^{b}	13.6	3.53E+05*^{b}	24.17	90
				CC	1.59E+06^{a}	8.88	nd	nd	1.33E+06^{a}	13.56	2.65E+06^{c}	10.2	
2-Heptanone	Cheesy	1166	1173	OC	nd	nd	nd	nd	nd	nd	4.17E+06	4.5	80

3-Hydroxy-2-butanone	Creamy	1280	1278	CC	nd	nd	nd	nd	nd	nd	9.84E+05	8.9	90
				OC	nd	nd	nd	nd	nd	nd	nd	nd	
2-Nonanone	Fruity	1377	1380	CC	nd	nd	nd	nd	2.70E+06[a]	24.62	1.25E+07[b]	5.71	84
				OC	nd	nd	nd	nd	nd	nd	1.59E+06*	9.92	
Acids				CC	nd	nd	nd	nd	nd	nd	5.23E+05	8.9	
Acetic acid	Vinegar	1446	1453	OC	nd	nd	nd	nd	nd	nd	nd	nd	91
				CC	nd	nd	nd	nd	nd	nd	4.53E+06	35.59	
Propionic acid	Pungent	1539	1538	OC	nd	nd	nd	nd	nd	nd	nd	nd	82
				CC	nd	nd	nd	nd	nd	nd	6.89E+05	36.39	
2-Methylbutyric acid	Cheesy	1665	1668	OC	nd	nd	4.21E+05[a]	11.26	4.52E+05[a]	7.48	8.02E+05[b]	2.14	76
				CC	nd	nd	nd	nd	nd	nd	nd	nd	
Hexanoic acid	Sour	1843	1839	OC	nd	nd	nd	nd	nd	nd	nd	nd	83
				CC	nd	nd	nd	nd	nd	nd	1.15E+06	38.3	
Sulfur containing compounds													
Carbon disulfide	Sulfurous	707	710	OC	4.43E+05	1.6	nd	nd	1.60E+06	33.24	1.05E+06	3.16	83
				CC	1.12E+06	12.59	4.55E+05	50.01	3.73E+05	23.4	nd	nd	
Dimethyl sulfide	Sulfurous	724	720	OC	nd	nd	nd	nd	nd	nd	5.16E+05	29.03	94
				CC	nd	nd	nd	nd	nd	nd	4.09E+05	2.85	
Dimethyl sulfone	Cabbage-like	1904	1911	OC	nd	nd	nd	nd	nd	nd	nd	nd	87
				CC	nd	nd	nd	nd	4.20E+05[a]	0.53	9.15E+05[b]	11.55	

nd: not detected (peak area value below 3E+05)

meat (Kim et al., 2020). Considering the total content of volatile compounds detected at the end of the evaluated storage period, it was found that the CC samples released the highest number of VOCs. Also, Mancinelli et al. (2020) reported a lower VOCs content in slow-grown animals with respect to fast-grown ones (244.1 μg Kg^{-1} vs 1771.3 μg Kg^{-1}, respectively).

3.3 Microbiological analysis

The amount of five different microbial groups detected from CC and OC samples during 10 days under refrigerated storage are reported in Figure 2 (A-E). Anaerobic sulphite-reducing bacteria and of *Cl. perfringens* cell counts were under detection limit (not detectable) throughout the study period due to the aerobic storage conditions. In general, both samples showed excellent quality, with low initial microbial load, especially OC sample, in which a delayed microbial growth was observed. The growth of total mesophiles in OC samples was observed after 6 days storage and after 10 days for the other bacterial groups. Lactic acid bacteria count was under detection limit during all studied period in OC and it was counted from day 6 in CC samples. Despite the delayed microbial growth, the level of total aerobic mesophiles and *Pseudomonas* spp. – meat spoilage indicators and *β*-glucuronidase *E. coli* was higher in OC than CC at day 10. Although the level of Enterobacteriaceae and *β*-glucosidase-positive *E. coli* exceeded the defined threshold (CeIRSA, 2017) at the end, the amount of other bacterial groups, including total mesophiles, *Pseudomonas* spp. and lactic acid bacteria were below the acceptance limit throughout storage (red line). In contrast to our results, one study found that the organic chicken breast contained similar coliform bacteria and more total aerobic mesophiles than conventional meat (Berna and Bagdatli, 2021). Several studies demonstrated the initial microbial counts that were generally higher than our results, suggesting the good hygiene processing conditions in the current study (Çiftçi and Güran, 2019).

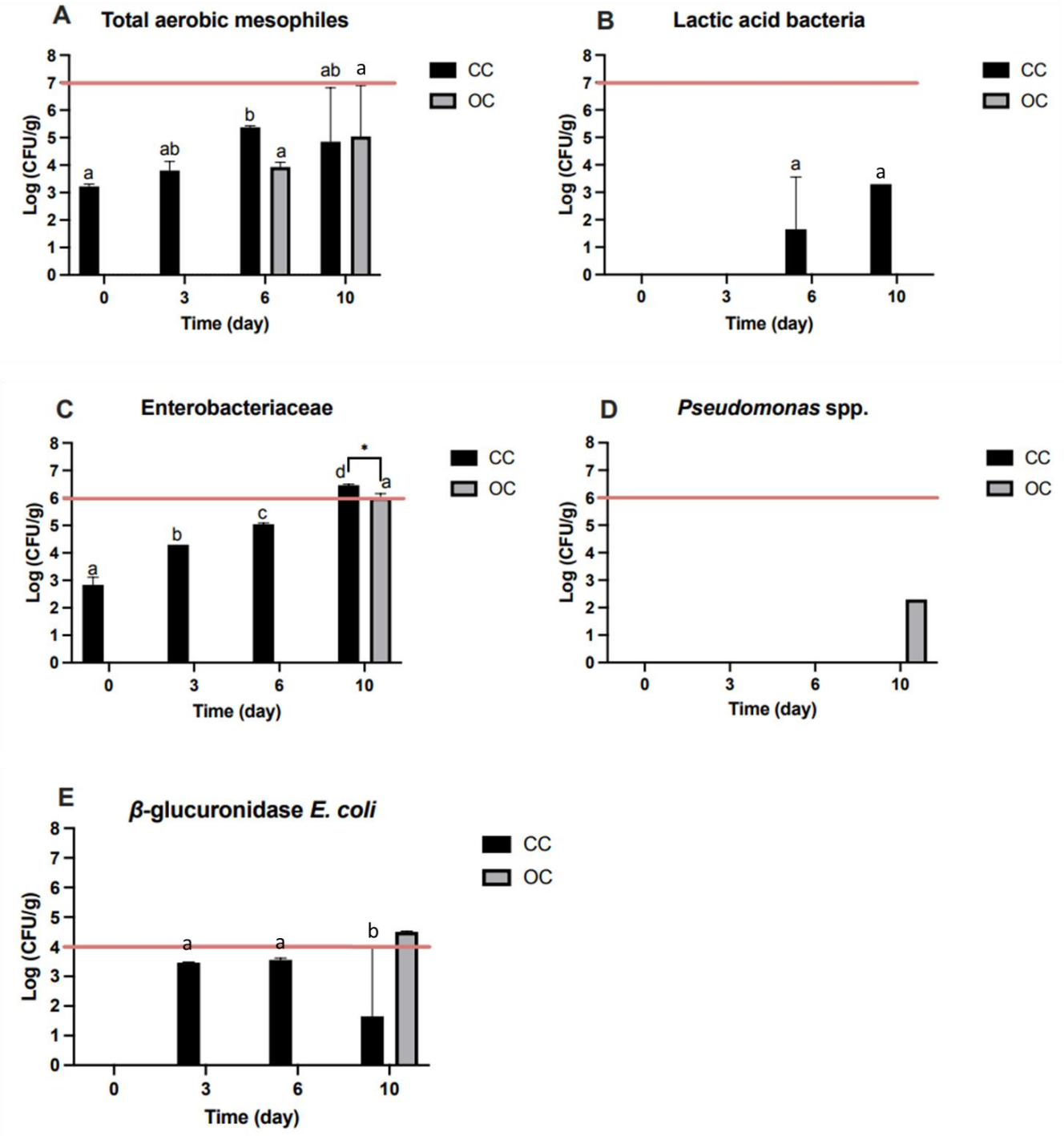
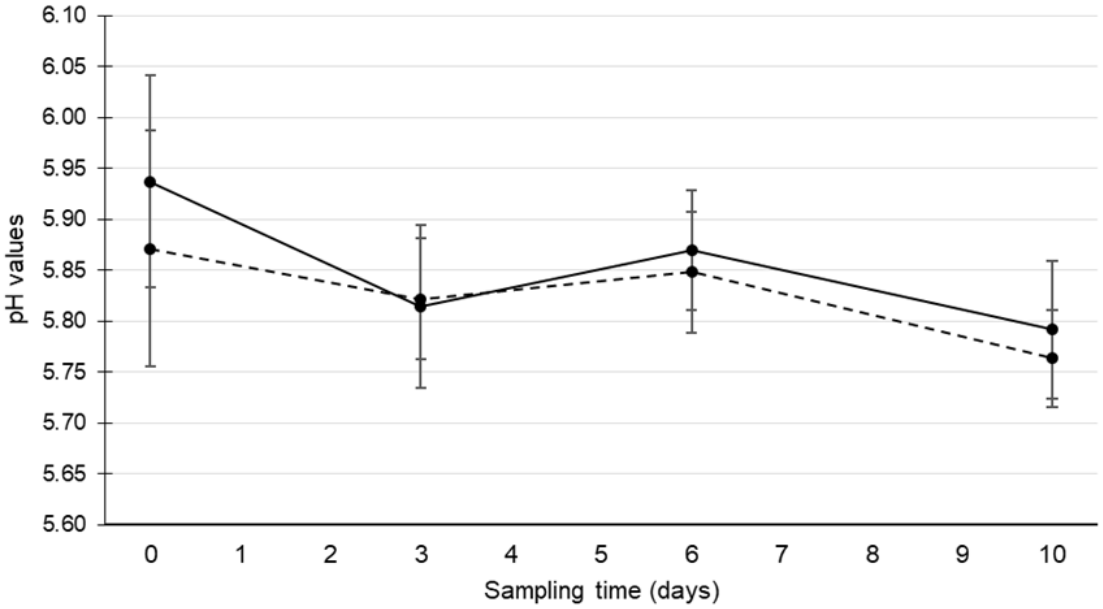


Figure 2. *Dominant bacterial groups detected in organic meat (OC) and conventional meat (CC) at 0, 3, 6, 10 days storage. A) total aerobic mesophiles, B) lactic acid bacteria, C) Enterobacteriaceae, D) Pseudomonas spp., E) β-glucuronidase Escherichia coli. Acceptance limit is represented by a red line in each graph. Statistically significant differences ($p < 0.05$) between the two kinds of meat in each day are indicated by the asterisks. Significant differences ($p<0.05$) between the days for each kind of meat are indicated by different letters.*

3.4 pH measurement

The pH of chicken entire *pectoralis major* muscle was monitored on day 0, day 3, day 6 and day 10 *post-mortem* by direct immersion of electrode in the meat. As shown in Figure 3, pH values of both meat groups during the whole shelf-life period were in the range of 5.76 - 5.94. Besides being an index of spoilage, pH is also an index of quality. Meat pH values of all analysed samples were between the limits of dark, firm, dry (DFD) (pH > 6) and pale, soft, exudative (PSE) (pH < 5.3) described by Adzitey and Nurul (2011). Despite that the two groups of meats presented very similar trend and no statistically significant differences between them, OC samples shelf-life pH line is below the one of CC samples for the entire period, except for day 3 when pH values were very similar. Le Bihan-Duval et al. (2008) reported meat pH as a relevant selection criterion that can be strongly correlated to other meat quality traits such as meat colour, water-holding capacity, and texture. Obtained pH results underlined the high meat quality and a good animal welfare of all analysed chicken muscles from both farming methods. Castellini et al. (2002) reported that lower pH in organic broilers could be consequence of a better welfare condition that reduce pre-slaughter stress. Lower stress led to a lower production of endogenous adrenaline and noradrenaline. These two neurotransmitters can promote glycogenolysis and anaerobic glycolysis, so their reduction led to an inhibition of these biological pathways, resulting in glycogen accumulation that is immediately transformed in lactic acid after slaughter which contributes to *post-mortem* muscle acidification (Mach et al. 2008; Braghieri and Napolitano (2009).

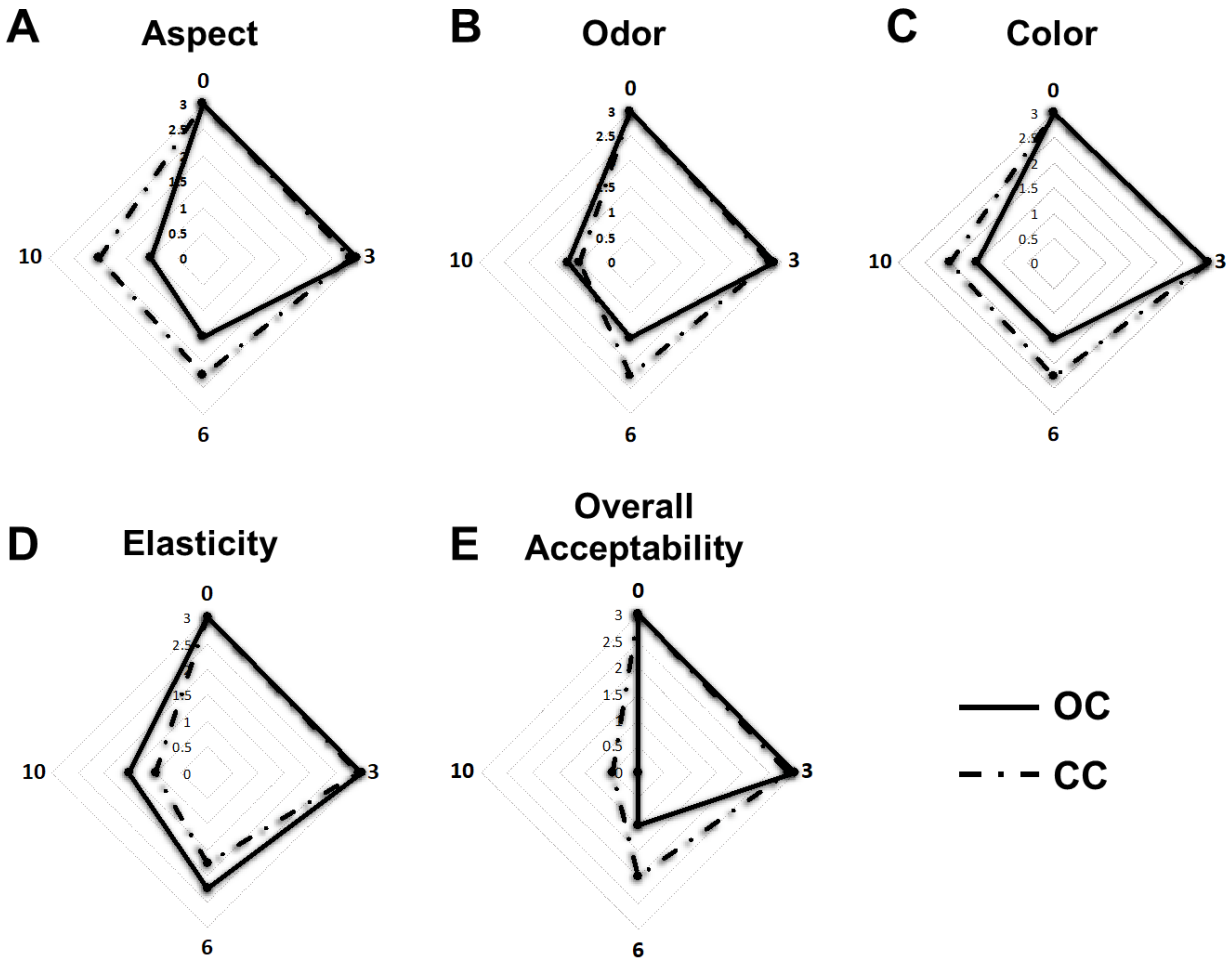


	T0	T3	T6	T10
CC	5.94±0.10^{a}	5.81±0.09^{b}	5.87±0.12^{a,b}	5.79±0.08^{b,c}
OC	5.87±0.11^{a}	5.82±0.06^{a,b}	5.85±0.10^{a}	5.76±0.05^{b}

Figure 3. *pH values of the organic (OC) and conventional (CC) chicken breast meat during 10-days of storage period. Significant differences ($p<0.05$) between the days for each type of meat are indicated in the table by different letters.*

3.5 Sensorial evaluation

As shown in Figure 4, the sensorial attribute of each meat sample (CC and OC) reduced during the storage period. In general, CC sample demonstrated higher scores of aspects, colour, and overall acceptability than OC sample during study. The panelists reported that organic chicken breast exhibited yellowness that mainly affected the aspect and overall acceptability scores. However, OC meat was more elastic than CC meat as well as the odor at the end of the study. The table below, in figure 4, revealed the sensory indexes of CC and OC samples at each time point. The sensory index (SI) of each sample shows a value of OC meat higher than CC meat at the early storage, suggesting the freshness and pleasant of the organic meat, but the scores were reversed by the yellow color of



F

Sampling time (days)	0	3	6	10
CC	18.67[a]	17.89[b]	13.94[c]	7.50[d]
OC	18.67[a]	18.37[a]	9.22[b]	7.00[c]

Figure 4. *Variation of meat sensorial attributes of organic (OC) and conventional chicken meat (CC) during the 10 days storage. A) Aspect; B) Odor; C) Color; D) Elasticity; E) Overall acceptability; F) Sensorial indices values table of OC and CC at different sampling time, significant differences ($p<0.05$) between the days for each type of meat are indicated by different letters.*

the OC meat at later storage (Brown et al. 2008). Similar effect (yellowness) was also observed by Grashorn and Serini (2006) through comparison between organic and conventional chicken meat. On the contrary, some authors stated that conventional meat was yellower than organic meat, which was probably ascribed to limited use of corn and soybean in organic feed (Husak et al. 2008; Castromán et al. 2013). The controversial organic meat color is presumably caused by the broiler species, the geographic regions where the study has been conducted as well as the feed composition.

4. Conclusions

In conclusion, the objective of this work was to investigate the preservation characteristics of organic chicken meat and to underline the differences compared to conventional chicken meat. Organic production system based on high standards of animal welfare can provide chicken meat with better preservation capacity compared to conventional farming. From a meat quality standpoint, shelf-life dissimilitude could be a consequence of physiological and metabolic functions of the animals due to the rearing system. The number of studies reported in literature and the knowledge on the meat quality related to organic production systems are still limited. Moreover, consumers have inaccurate or inadequate information about the organic production of poultry meat, so it would be remarkable to provide a scientific groundwork to inform the consumers in experimental terms.

Supplementary material

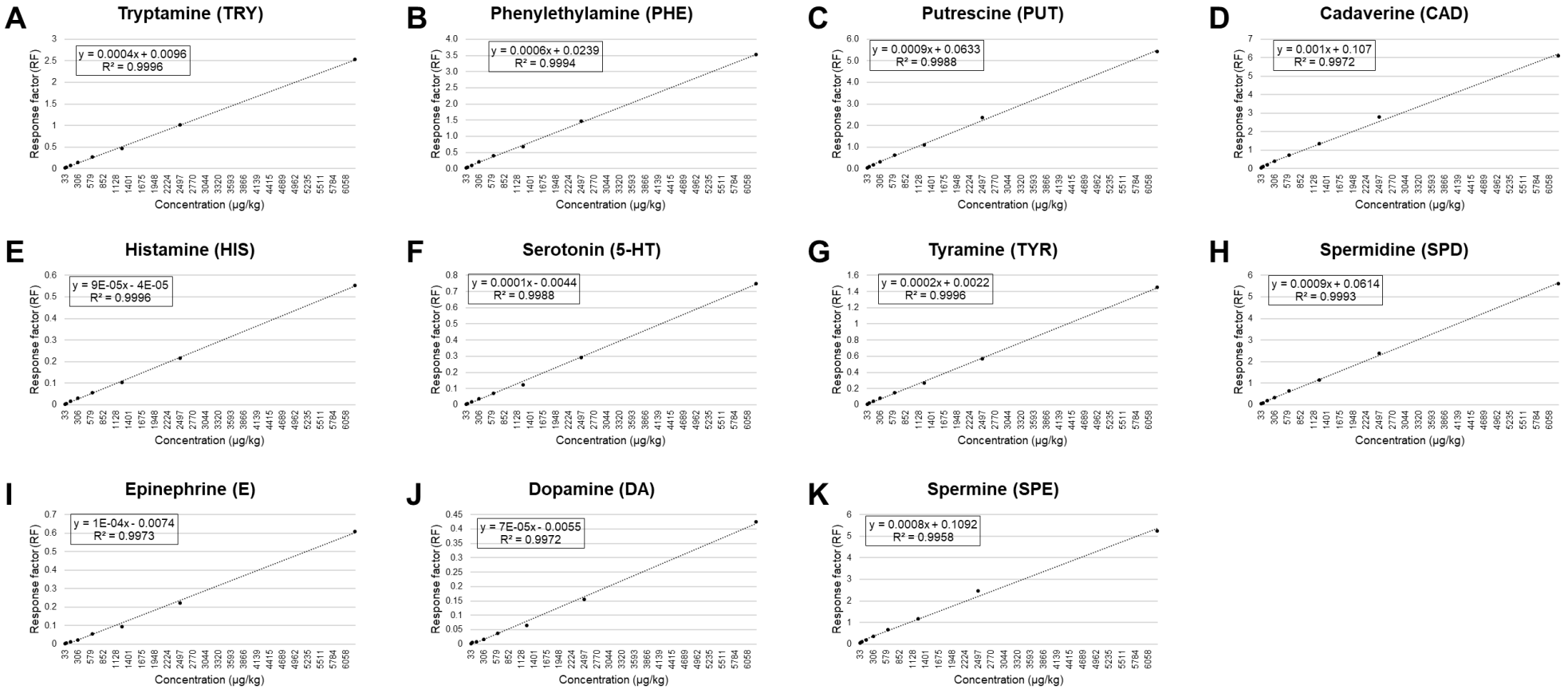
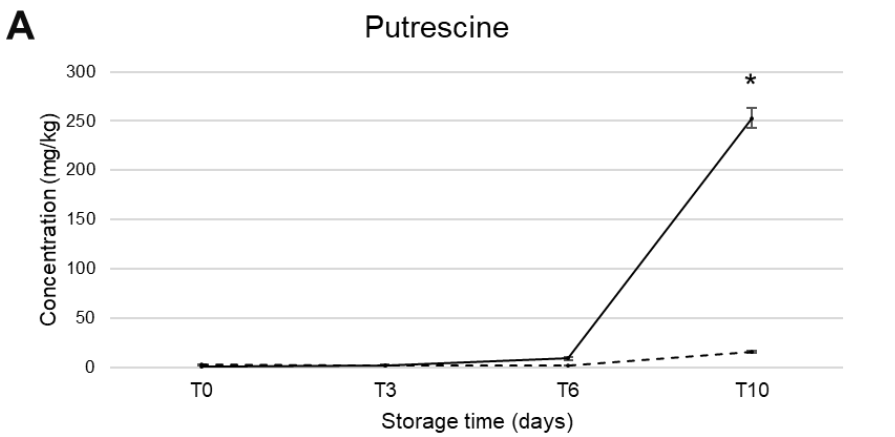
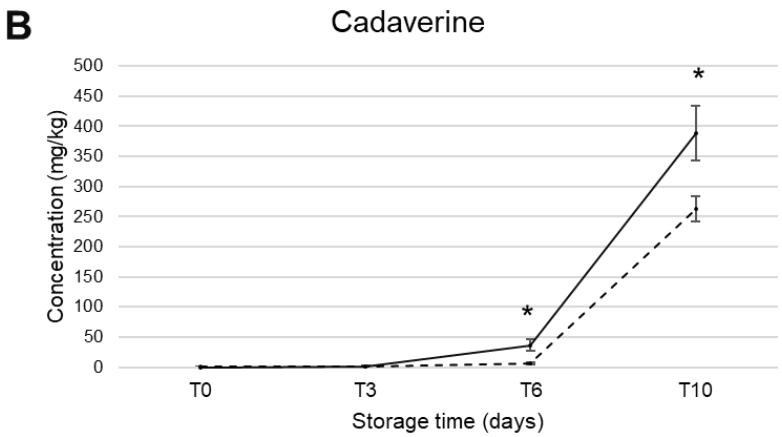


Figure 1S. *Calibration curves of the 11 quantified BAs. (Tryptamine (A), 2-phenylethylamine (B), putrescine (C), cadaverine (D), histamine (E), serotonin (F), tyramine (G), spermidine (H), epinephrine (I), dopamine (J) and spermine (K)). Calibration curves were obtained by injection of 8 different concentration of each BA. Response factor was calculated using 1,7-diaminoheptane (internal standard) peak area.*



	T0	T3	T6	T10
—— CC	0.951±0.140^{a}	1.76±0.30^{a}	8.65±1.66^{a}	253.1±10.1^{b}
– – – OC	2.33±0.33^{a}	1.92±0.23^{a}	1.48±0.38^{a}	15.00±1.1^{b}



	T0	T3	T6	T10
—— CC	n.d.	1.54±0.08^{a}	36.5±9.1^{b}	388.1±45.4^{c}
– – – OC	0.401±0.119^{a}	1.54±0.24^{a}	6.21±1.30^{a}	262.76±20.81^{b}

Figure 2S. *PUT (A) and CAD (B) quantification results as the two most influential BAs in the analysed samples. Tables show the average values of the triplicate (±standard deviation) . Significant differences ($p < 0.05$) between the two meats in each day are indicated by the asterisks. Significant differences ($p < 0.05$) between the different days for each type of meat are indicated in the tables by different letters.*

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SECTION C
Results of the thesis

A proteomics and bioinformatics approach to characterize protein changes in chicken breast meat from divergent farming systems: Organic versus antibiotic-free

Publication 3

A proteomics and bioinformatics approach to characterize protein changes in chicken breast meat from divergent farming systems: organic *versus* antibiotic-free

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Article in preparation for Poultry Science

Abstract

In recent years and among the foodomics approaches, proteomics has been extensively used in meat research for its key analytical potential to investigate the complexity of muscle proteomes and in deciphering the sophisticated biochemical changes occurring during the conversion of muscle into meat. This study aimed to characterize for the first time the differences and similarities in the early post-mortem muscle proteome of chicken raised under two farming systems, these being antibiotic-free and organic production systems. Thus, the total protein extracts of post-mortem *Pectoralis major* muscle sampled from two chicken strains (Ross 308 and Ranger Classic) reared under the two farming systems were compared using two-dimensional electrophoresis (2-DE) and liquid chromatography tandem mass spectrometry techniques (LC-MS/MS). Within antibiotic-free and organic farming systems, 12 spots (14 proteins) and 15 spots (16 proteins) were differentially abundant between Ross 308 and Ranger Classic, respectively. Within Ross 308 and Ranger Classic chicken strains, 16 spots (12 proteins) and 7 spots (18 proteins) were differentially abundant between organic and antibiotic-free, respectively. The changing proteins were found to belong to several molecular pathways, likely muscle structure (n = 12) and energy metabolism, mainly glycolysis (n = 7), followed by signal transduction (n = 4), protein transport (n = 3), proteolysis (n = 2), immune response (n = 2) and others (amino acids biosynthesis, chaperones and protein folding, metal binding, oxidative stress and tRNA processing). Effective differences reflecting the impact of chicken strain and production system were in depth investigated through bioinformatics analyses. Muscle contraction revealed to be the main enriched pathway in the antibiotic-free for the Ross 308 strain, while for organic and Ranger Classic they were mainly cellular processes. Signal transduction and proteolysis were overexpressed in organic meat, especially in Ranger Classic. This trial evidenced that antibiotic-free and organic production system can significantly impact the

muscle proteome of chicken breast meat. Furthermore, this study allowed proposing putative protein biomarkers for meat authenticity.

Keywords: Chicken meat, Proteomics, Meat authenticity, Meat proteome, Putative biomarkers

1. Introduction

Recent data suggest that the popularity of poultry meat continues to increase among consumers due to affordability, availability, and nutritional qualities. Chicken is the mainly consumed poultry meat in Europe and its production has experienced a cumulative rise of around 30 % over the past 10 years (Augère-Granier 2019). Commercial strains of chickens have been selected predominately for individual performance traits such as breast percentage in carcasses and feed efficiency, with little regard to behavioral traits and adaptability of the animal to the farming system (Dal Bosco et al. 2021).

Public demand regarding animal welfare and more humanely raised food has grown exponentially over the years, so producers are transitioning away from traditional farming systems towards alternative ones more focused on animal health and well-being (Cooreman-Algoed et al. 2022). Among the extensive production systems, the inside ground farming with an antibiotic-free approach is a widespread technique (Prache et al. 2021). In addition, organic animal production has experienced a rapid spread and development since the 1990s driven, among other factors, by the willingness of consumers to pay for this kind of products. Outdoor access, for at least a third part of the lifespan, is one of the European guidelines for organic animal production requirements, together with organic feeding, the prohibition of antibiotics and synthetic compounds dispensation and other measures aimed at maintaining high standards of animal welfare such as lighting, noise and ventilation (Commission 834/2007, 889/2008). Studies have shown that birds with outdoor access have improved plumage condition, and less prevalence in bone deformities development and dermatitis (Bari et al. 2020; Wurtz et al. 2022).

The recent progress in food science and technology brought about new analytical methods and a novel and modern approach concerning food quality and safety. Foodomics is a powerful tool that can be used to study at molecular level food nutrients and constituents

(Balkir et al. 2021). Proteomics has been one of the most appreciated and used foodomics approach by meat scientists as it offers the analytical opportunity to apply effective quantitative methods for the characterization of the global or partial proteome of the muscle tissues (Munekata et al. 2021). In meat science, proteomics allows a better understanding of the biochemical mechanisms underpinning meat quality and authenticity traits (Gagaoua & Zhu 2022). The combination of electrophoresis separation coupled with mass spectrometry (MS) methods is the most common approach in proteomics as it allows the detection, characterization, and quantification of many protein components. (Bonzon-Kulichenko et al. 2011; Meyer & Schilling 2017; Picard 2020; Zhu et al. 2021). The importance of proteomics in the field of meat science is illustrated by the large and ever increasing number of publications published during the last 20 years reviewed by (Gagaoua & Picard 2022). In poultry science, proteomics has been applied to investigate protein biomarkers related to chicken meat quality and safety parameters.

Meat quality parameters such as tenderness, growth performances, water holding capacity, meat pH, color, cooking loss, texture, fat and mineral contents were investigated to understand their linkage with proteomic profiles of chicken meat (Phongpa-Ngan et al. 2011; Kuttappan et al. 2017; Cai et al. 2018; Zhang et al. 2020a; Zhang et al. 2020b). Moreover, several studies focused on chicken meat abnormalities like woody breast myopathy, white striping or pale, soft, and exudative (PSE) through different proteomic approaches (Desai et al. 2016; Soglia et al. 2016; Zambonelli et al. 2016; Zhang et al. 2020b). Although the popularity of organic and antibiotic-free management practices is on the rise, the impact of such methods on chicken meat proteome are still not studied extensively.

The aim of the present study is the application of 2-DE-LC-MS/MS proteomic technique to assess differences in post-mortem proteome between chicken breast meat from antibiotic-

free and organic production systems and the impact of genotype investigating two different chicken strains.

2. Materials and Methods

2.1 Samples

A total of forty chicken breast divided in four groups, according to the chicken strain and production system were collected at Fileni® industry (Cingoli, Italy) and analyzed in the present work. Table 1 shows a summary of the characteristics of the four groups of samples. Organic meat was reared according to European Commission Regulation No 848/2018 for organic systems for poultry and livestock production, using organic feed, controlled housing, having access to an outdoor area with the presence of pasture for at least one third of their life. Antibiotic-free chicken meat featured a standard broiler inside-ground farming system, using concentrated feed and controlled housing (artificial light and climate control, automatic water, and feed supply) according to the European Directive 2007/43/EC. Pectoralis major muscles biopsies were performed immediately after slaughter by cutting a 2 cm3 section from the top right part of each breast. Samples were quickly frozen in liquid nitrogen and stored at -80°C until extraction day.

Table 1. *Summary of the four groups of analysed samples and main animal characteristics.*

	Antibiotic-free		**Organic**	
Chicken strain	Ross 308	Ranger Classic	Ross 308	Ranger Classic
Sample name	ARO	ARA	ORO	ORA
Age	48 days	56 days	83 days	85 days
Sex	Male	Male	Female	Female
Average alive weight	3.473 kg	3.218 kg	3.962 kg	2.998 kg
Tunnel exit T°	4.5-4.9 °C	3.6-4.3 °C	7.7-9.5 °C	4.9-8.5 °C

2.2 Protein extraction and quantification

Proteins of about 200 mg of muscle tissue were extracted using 3 mL of buffer containing 8.3 M urea, 2 M thiourea, 1% Dithiothreitol, 2% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) and 2% Pharmalyte® (Immobilized pH gradient (IPG) buffer pH 3–10) and homogenising with T 25 digital Ultra-Turrax® (Picard 2020; Picard & Gagaoua 2020). Protein homogenates were incubated for 30 min in wet-ice and centrifuged for 30 min at 10000 rpm at 4 °C. To have four representative samples, 100 µL of each supernatant were pooled in an Eppendorf for each group (ARO, ARA, ORO and ORA). Protein concentrations of obtained pooled extracts were determined using the dye-binding protocol of Bradford (Bradford 1976). A spectrophotometer (UV-1700, Pharmaspec, SHIMADZU) and bovine serum albumin as a standard (Bio-Rad Protein Assay kit, Bio-Rad, France) were used.

2.3 Two-dimensional electrophoresis (2-DE)

35 µg of each pooled sample was purify by MeOH/CHCl3 precipitation and resuspended in 150 mL of DeStreak solution (Biorad Laboratories, Hercules) adding 1% of IPG buffer solution (Biorad Laboratories, Hercules). The sample was loaded onto IPG strip (7-cm long, pH 3–10 linear gradient, ReadyStrip IPG strips, BioRad Laboratories, Hercules) and first-dimension isoelectric focusing of proteins in strip gel was performed using a PROTEAN IEF cell system (Bio-Rad Laboratories) for 20000 total kVh at 20 °C adding 12 hours of active hydration prior to the beginning of the voltage application. After equilibration, the second dimension was run on 10 % SDS polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-PROTEAN ® PrecastGels (Bio-Rad, USA). Total protein was staining using SYPRO Ruby protein gel fluorescent stain (Lonza, Rockland) following the manufacturer's indications.

2.4 2-DE image analysis

The 2-DE images from gels stained with SYPRO Ruby fluorescent dyes were captured with the ChemiDoc MP Imaging System (Bio-Rad Laboratories). Analysis of digitalized gel images was performed with SameSpots 5.1.012 (TotalLab Laboratories). Protein volumes of detected and matched spots over biological replicates were automatic measure by SameSpots software and manually validated. Only protein spots reproducibly detected in at the least two of tree biological replicates were selected for image analyses. Differential expression of a protein present in the gels was considered significant when the fold change was at least 1.2 and the p-value was below 0.05.

2.5 Protein identification by LC-MS/MS

2.5.1 Protein digestion

To identify the proteins in the spots of interest, spots were manually cut and submitted to a tryptic digestion following the protocol: reduction with 10 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO) in 50 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO) and alkylation with 55 mM iodoacetamide (Sigma- Aldrich, St. Louis, MO) in 50 mM ammonium bicarbonate. Then, modified porcine trypsin (Promega, Madison, WI, USA) at a final concentration of 20 ng/μL in 20mM ammonium bicarbonate was added and digestion was carried out incubating samples at 37 °C for 16 h. Peptides were purified using a C18 columns. The resulting peptide extracts were pooled, concentrated in a SpeedVac and stored at −20 °C.

2.5.2 Mass spectrometric analysis

Digested peptides of each spot were separated using Reverse Phase Chromatography. Gradient was developed using a micro liquid chromatography system (Eksigent Technologies nanoLC 400, SCIEX) coupled to high-speed Triple TOF 6600 mass spectrometer (SCIEX, Foster City, CA) with a micro flow source. The analytical column

used was a silica-based reversed phase column YMC-TRIART C18 150 × 0.30 mm, 3 µm particle size and 120 Å pore size (YMC Technologies, Teknokroma). The trap column was a YMC-TRIART C18 (YMC Technologies, Teknokroma) with a 3 µm particle size and 120 Å pore size, switched on-line with the analytical column. The loading pump delivered a solution of 0.1% formic acid in water at 10 µL/min. The micro-pump provided a flow-rate of 5 µl/min and was operated under gradient elution conditions, using 0.1% formic acid in water as mobile phase A, and 0.1% formic acid in acetonitrile as mobile phase B. Peptides were separated using a 90 minutes gradient ranging from 2% to 90% mobile phase B (Anfray et al. 2021; Peñas-Martínez et al. 2021; Chantada-Vázquez et al. 2022). Data acquisition was carried out using a Data dependent workflow. Source and interface conditions were as follows: ion spray voltage floating (ISVF) 5500 V, curtain gas 25, collision energy 10 and ion source gas 1 25. Instrument was operated with Analyst TF 1.7.1 software (SCIEX, USA). Switching criteria was set to ions greater than mass to charge ratio (m/z) 350 and smaller than m/z 1800 with charge state of 2–5, mass tolerance 250 ppm and an abundance threshold of more than 200 counts. Former target ions were excluded for 15 s. Instrument was automatically calibrated every 4 hours using as external calibrant tryptic peptides from pepcalMix.

2.6 Data analysis

After MS/MS analysis, data files were processed using ProteinPilotTM 5.0.1 software from Sciex which uses the algorithm ParagonTM for database search and ProgroupTM for data grouping. Data were searched using a chicken (gallus gallus) specific Uniprot database using trypsin as enzyme used in the digestion, iodoacetamide to perform the Cys carboxyamidomethylation and as a special feature a lys biotinylation. False discovery rate was performed using a non-lineal fitting method displaying only those results that reported a 1 % global false discovery rate or better (Shilov et al. 2007; Tang et al. 2008).

3. Results

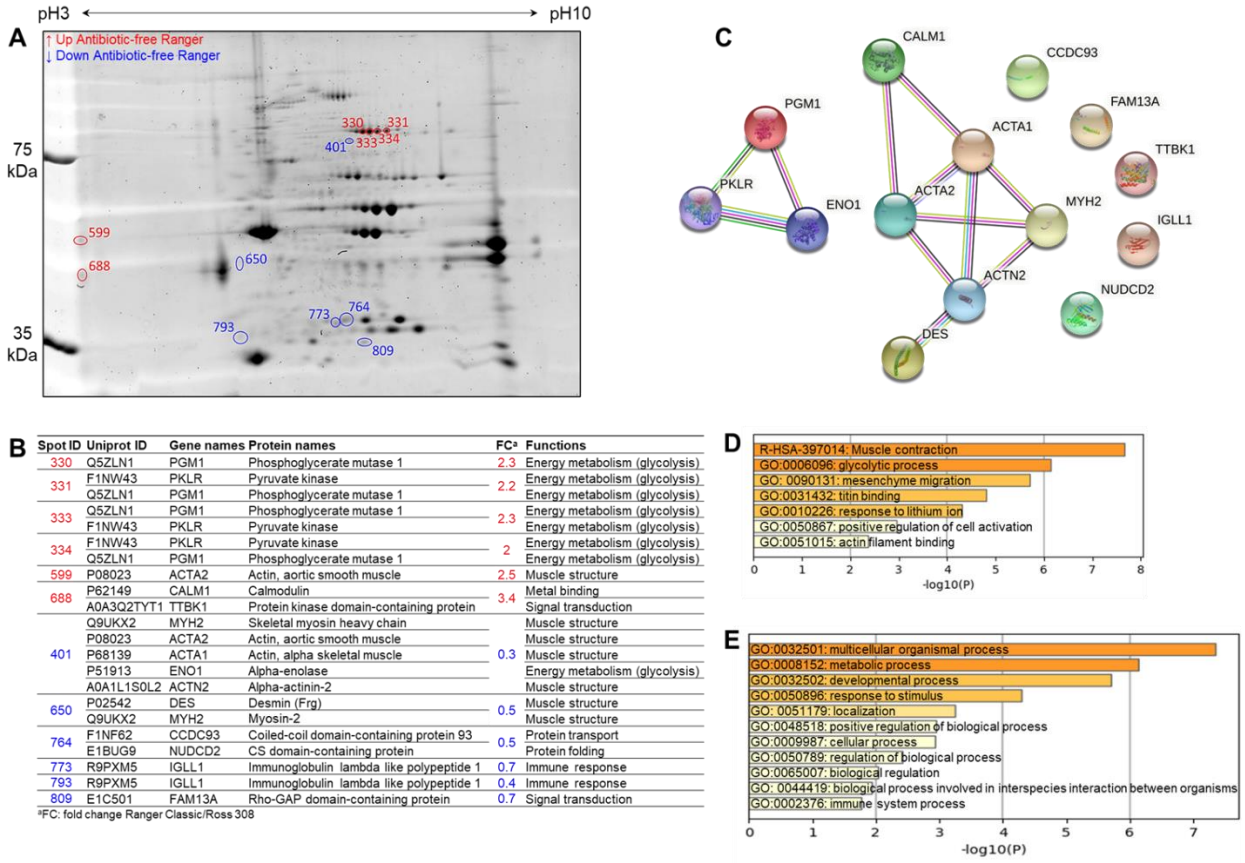
The 2-DE image analysis reveals protein spots with statistically significant differences identified using trough the fold change value. MS/MS allowed the characterization of the proteins related to differentially abundant proteins (DAPs). Results were divided according to chicken strain and production systems comparisons in the following sections.

3.1 Antibiotic-free chicken meat: DAPs among chicken strains

The overlap of 2-DE gels of antibiotic-free Ross 308 (ARO) and Ranger Classic (ARA) samples and the 12 differentially abundant spots are shown in Figure 1A. Gene names, Uniprot ID, protein names and functions are reported in Figure 1B together with their fold change values calculated in 2-DE image analysis. Calmodulin (CALM1), actin (ACTA2), phosphoglycerate mutase 1 (PGM1) and pyruvate kinase (PKLR) were more abundant in ARA samples while immunoglobulin (IGLL1), rho-GAP domain-containing protein (FAM13A), desmin (DES) and myosin-2 (MYH2) were the main overabundant proteins in ARO samples. These proteins can be a investigated as biomarkers to discriminate Ranger Classic from Ross 308 chicken breast meat from antibiotic-free inside ground farming. STRING bioinformatic analysis highlighted two main networks (Figure 1C). The larger related to muscle structure pathways is formed by various actin isoforms (ACTA1, ACTA2, ACTN2), MYH2, CALM1 and DES. The second is related to energy metabolism and is composed by PGM1, alpha-enolase (ENO1) and PKLR. Bioinformatics enrichment analyses on the 14 DAPs through Gene Ontology, KEGG and Reactome databases are given in Figure 1D-E. It resulted that 7 cluster terms were significantly enriched mainly dominated by “muscle contraction (R-HSA-397014)” and “glycolytic process (GO:0006096)”. 11 biological processes were enriched with “multicellular organismal process (GO:0032501)”, “metabolic process (GO:0008152)” and “developmental process (GO:0032502)” as the top three terms.

3.2 Organic chicken meat: DAPs among chicken strains

The overlap of 2-DE gels of organic Ross 308 (ORO) and Ranger Classic (ORA) samples and the 15 differentially abundant spots are shown in Figure 2A. Gene names, Uniprot ID, protein names and functions are reported in Figure 2B together with their fold change values calculated in 2-DE image analysis. Among the most abundant proteins in ORA, alpha-2-macroglobulin (A2M) was the mainly DAP with a fold change score of 5.9 followed by IGLL1 and FAM13A. NudC domain-containing protein 2 (NUDCD2) and beta-actin (ACTB) were two of the most abundant proteins in ORO samples. These proteins can be a investigated as biomarkers to discriminate Ranger Classic from Ross 308 chicken breast meat from organic farming. STRING bioinformatic analysis highlighted one main network related to muscle structure pathways (Figure 2C). The protein with highest number of interactions was ACTB with 7 interactions. Bioinformatics enrichment analyses on the 16 DAPs through Gene Ontology, KEGG and Reactome databases are given in Figure 2D-E. It resulted that 8 cluster terms were significantly enriched mainly dominated by “hypertrophic cardiomyopathy (hsa:05410)”, “UCH proteinases (R-HSA-5689603)” and “protease binding (GO:0002020)”. 10 biological processes were enriched with “multicellular organismal process (GO:0032501)”, “cellular process (GO:0009987)” and “developmental process (GO:0032502)” as the top three terms.



B

Spot ID	Uniprot ID	Gene names	Protein names	FC[a]	Functions
330	Q5ZLN1	PGM1	Phosphoglycerate mutase 1	2.3	Energy metabolism (glycolysis)
331	F1NW43	PKLR	Pyruvate kinase	2.2	Energy metabolism (glycolysis)
	Q5ZLN1	PGM1	Phosphoglycerate mutase 1		Energy metabolism (glycolysis)
333	Q5ZLN1	PGM1	Phosphoglycerate mutase 1	2.3	Energy metabolism (glycolysis)
	F1NW43	PKLR	Pyruvate kinase		Energy metabolism (glycolysis)
334	F1NW43	PKLR	Pyruvate kinase	2	Energy metabolism (glycolysis)
	Q5ZLN1	PGM1	Phosphoglycerate mutase 1		Energy metabolism (glycolysis)
599	P08023	ACTA2	Actin, aortic smooth muscle	2.5	Muscle structure
688	P62149	CALM1	Calmodulin	3.4	Metal binding
	A0A3Q2TYT1	TTBK1	Protein kinase domain-containing protein		Signal transduction
401	Q9UKX2	MYH2	Skeletal myosin heavy chain	0.3	Muscle structure
	P08023	ACTA2	Actin, aortic smooth muscle		Muscle structure
	P68139	ACTA1	Actin, alpha skeletal muscle		Muscle structure
	P51913	ENO1	Alpha-enolase		Energy metabolism (glycolysis)
	A0A1L1S0L2	ACTN2	Alpha-actinin-2		Muscle structure
650	P02542	DES	Desmin (Frg)	0.5	Muscle structure
	Q9UKX2	MYH2	Myosin-2		Muscle structure
764	F1NF62	CCDC93	Coiled-coil domain-containing protein 93	0.5	Protein transport
	E1BUG9	NUDCD2	CS domain-containing protein		Protein folding
773	R9PXM5	IGLL1	Immunoglobulin lambda like polypeptide 1	0.7	Immune response
793	R9PXM5	IGLL1	Immunoglobulin lambda like polypeptide 1	0.4	Immune response
809	E1C501	FAM13A	Rho-GAP domain-containing protein	0.7	Signal transduction

[a]FC: fold change Ranger Classic/Ross 308

Figure 1. *Comparison between the chicken breast muscle proteomes of Ross 308 and Ranger Classic within the antibiotic-free production system.* ***A)*** *Representative two-dimensional gel electrophoresis highlighting the differentially abundant protein spots between the two groups. Spots in blue colour correspond to the down-regulated proteins, those in red are up-regulated proteins in Ranger Classic chicken meat.* ***B)*** *Identify of differentially abundant proteins (DAPs) including their functions and fold change.* ***C)*** *Protein-Protein interaction (PPI) network using the list of the 14 DAPs in antibiotic-free chicken breast muscle. The PPI network was built using the STRING database (https://string-db.org/).* ***D-E)*** *Bioinformatics enrichment analyses (Gene Ontology, KEGG, Reactome) using the 14 DAPs.* ***D)*** *Top enriched cluster terms.* ***E)*** *Biological processes.*

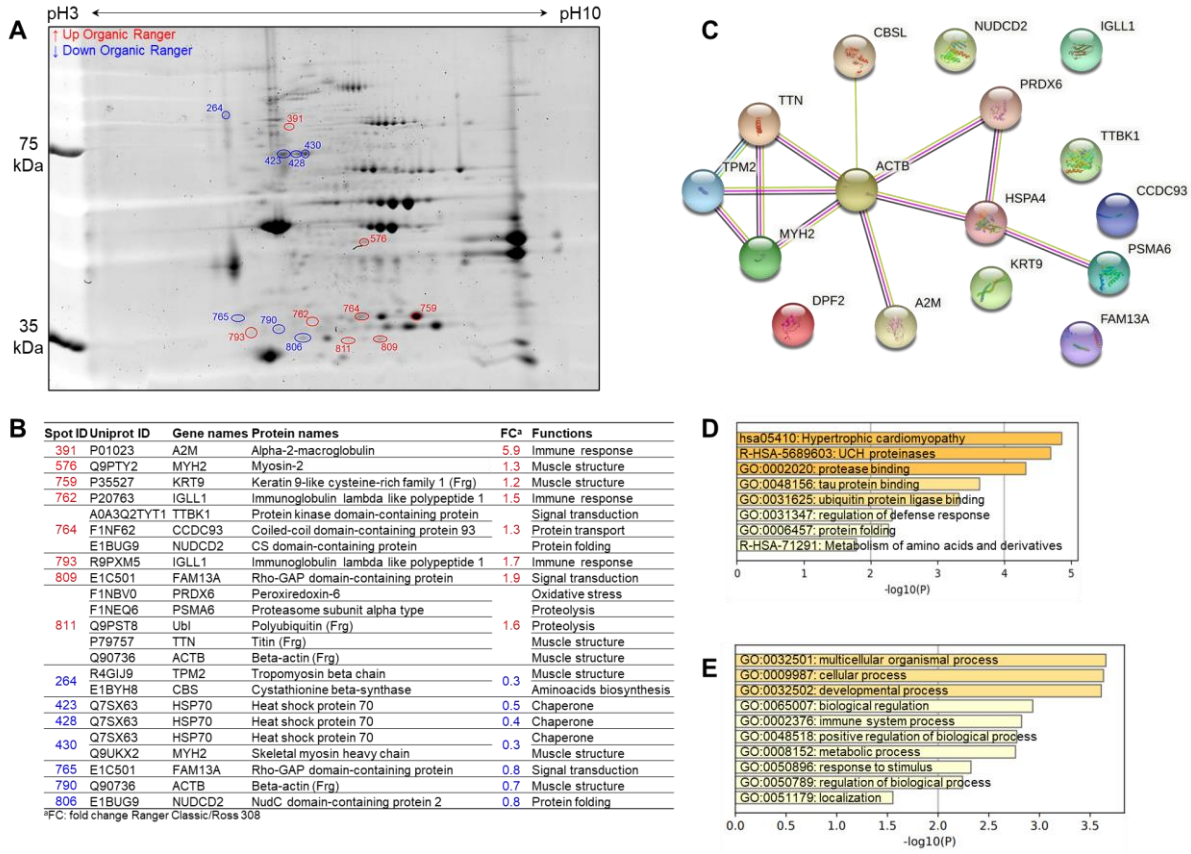


Figure 2. *Comparison between the chicken breast muscle proteomes of Ross 308 and Ranger Classic within the organic production system.* ***A)*** *Representative two-dimensional gel electrophoresis highlighting the differentially abundant protein spots between the two groups. Spots in blue colour correspond to the down-regulated proteins, those in red are up-regulated proteins in Ranger Classic chicken meat.* ***B)*** *Identify of differentially abundant proteins (DAPs) including their functions and fold change.* ***C)*** *Protein-Protein interaction (PPI) network using the list of the 16 DAPs in organic chicken breast muscle. The PPI network was built using the STRING database (https://string-db.org/).* ***D-E)*** *Bioinformatics enrichment analyses (Gene Ontology, KEGG, Reactome) using the 16 DAPs.* ***D)*** *Top enriched cluster terms.* ***E)*** *Biological processes.*

3.3 Ross 308 chicken meat: DAPs among meat production systems

The overlap of 2-DE gels of Ross 308 chicken meat from organic (ORO) and antibiotic-free (ARO) farming systems and the 12 differentially abundant spots are shown in Figure 3A. Gene names, Uniprot ID, protein names and functions are reported in Figure 3B together with their fold change values calculated in 2-DE image analysis. Glycerol-3-phosphate dehydrogenase (GPD1), troponin I (TNNI2) and Tau-tubulin kinase 1 (TTBK1) were more abundant in organic samples while ACTA2, phosphoglycerate kinase (PGK2) and MYH2 were the main overabundant proteins in antibiotic-free samples. These proteins can be a investigated as biomarkers to discriminate organic and antibiotic-free chicken meat production system from Ross 308 strain. STRING bioinformatic analysis highlighted one main 9-proteins network related to muscle structure pathways (Figure 3C). The proteins with highest number of interactions were ACTA1 with 5 interactions and ACTN2 and MYH2 with 4 each. Bioinformatics enrichment analyses on the 16 DAPs through Gene Ontology, KEGG and Reactome databases are given in Figure 3D-E. It resulted that 6 cluster terms were significantly enriched mainly dominated by “muscle contraction (GO:0006936)” and “mesenchyme migration (GO:0090131)”. 12 biological processes were enriched with “multicellular organismal process (GO:0032501)”, “developmental process (GO:0032502)” and “metabolic process (GO:0008152)” as the top three terms.

3.4 Ranger Classic chicken meat: DAPs among meat production systems

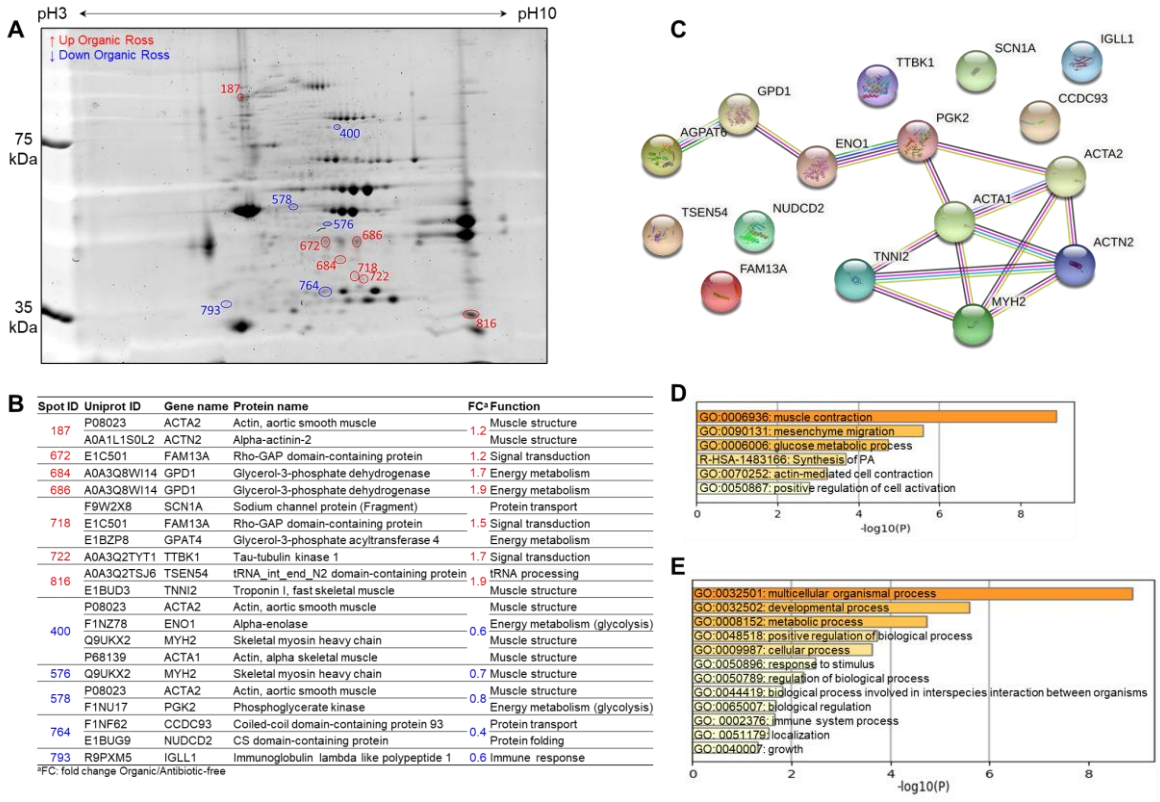
The overlap of 2-DE gels of Ranger Classic chicken meat from organic (ORA) and antibiotic-free (ARA) farming systems and the 7 differentially abundant spots are shown in Figure 4A. Gene names, Uniprot ID, protein names and functions are reported in Figure 4B together with their fold change values calculated on 2-DE image analysis. IGLL1, FAM13A and fructose-bisphosphate aldolase (ALDOC) were more abundant in organic samples while heat shock protein 70 (HSP70), MYH2 and CALM1 were the most abundant DAPs in

antibiotic-free samples. These proteins can be investigated as biomarkers to discriminate organic and antibiotic-free chicken meat production system from Ranger Classic chicken strain. STRING bioinformatic analysis highlighted one main 9-proteins network related to muscle structure pathways (Figure 4C). The proteins with highest number of interactions were ACTB with 6 interactions and CALM1 and HSPA4 with 4 each. Bioinformatics enrichment analyses on the 18 DAPs through Gene Ontology, KEGG and Reactome databases are given in Figure 4D-E. It resulted that 7 cluster terms were significantly enriched mainly dominated by "substantia nigra development (GO:0021762)", "downstream signaling events of B cell receptor (BCR) (R-HSA-1168372)" and "dilated cardiomyopathy (hsa:05414)". 10 biological processes were enriched with "developmental process (GO:0032502)", "biological regulation (GO:0065007)" and "cellular process (GO:0009987)" as the top three terms.

3.5 Overview

An overview on DAPs among antibiotic-free and organic chicken breasts is reported in Figure 5. A total of six proteins were in common and three of them reported the same trend in strain comparison. NUDCD2 and FAM13A were overabundant and TTBK1 was statistically less abundant in Ranger Classic samples. DAPs among Ross 308 and Ranger Classic chicken breasts are reported in Figure 6. A total of five proteins were in common and four of them reported the same trend in farming system comparison. MYH2 was down-regulated and FAM13A, GPAT4 (Glycerol-3-phosphate acyltransferase 4) and TTBK1 were statistically overabundant in organic samples. Figure 7 reports a summary of the resulting DAPs and their abundance among the four analyzed groups. MYH2, IGLL1 and TTBK1 were identified as DAPs whatever the condition (farming system and chicken strain). MYH2 was overabundant in antibiotic-free chicken meat, especially in Ross 308. TTBK1 was overabundant in organic and in Ranger Classic samples. IGLL1 expression results to be

influenced by both production system and chicken strain. It resulted overabundant in Ross 308 when comparing antibiotic-free samples and in Ranger Classic when comparing organics.

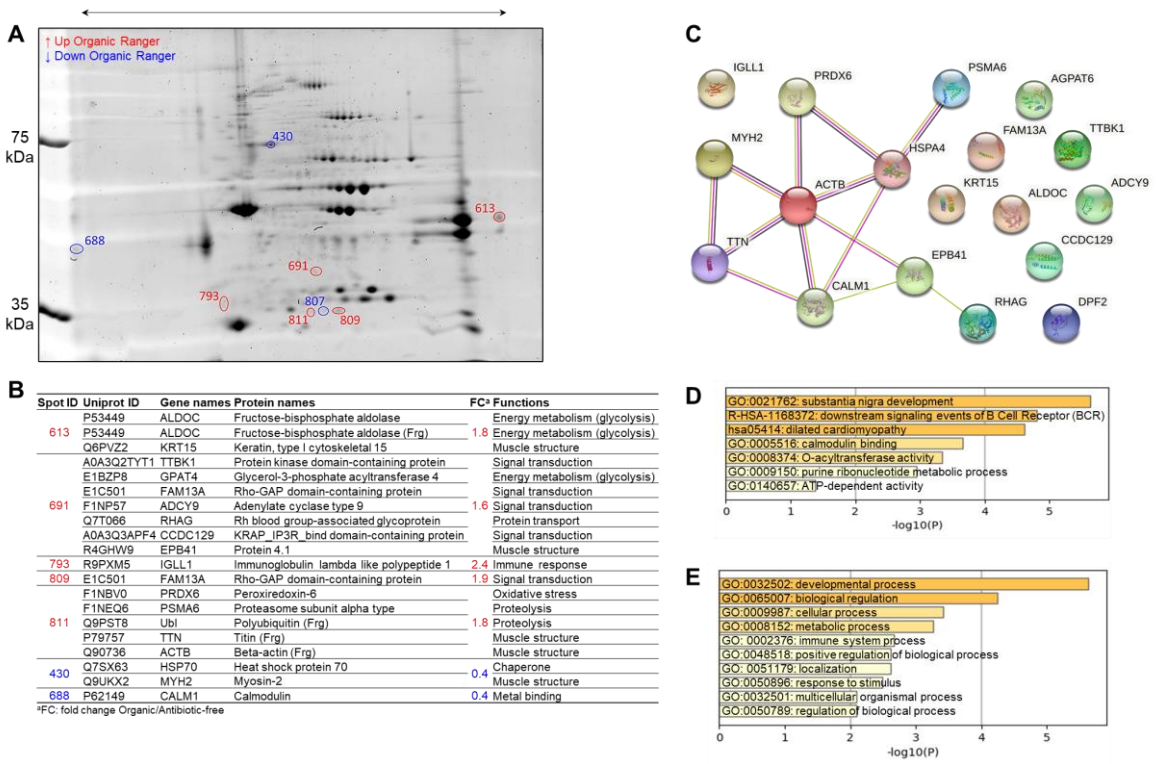


B

Spot ID	Uniprot ID	Gene name	Protein name	FC[a]	Function
187	P08023	ACTA2	Actin, aortic smooth muscle	1.2	Muscle structure
	A0A1L1S0L2	ACTN2	Alpha-actinin-2		Muscle structure
672	E1C501	FAM13A	Rho-GAP domain-containing protein	1.2	Signal transduction
684	A0A3Q8WI14	GPD1	Glycerol-3-phosphate dehydrogenase	1.7	Energy metabolism
686	A0A3Q8WI14	GPD1	Glycerol-3-phosphate dehydrogenase	1.9	Energy metabolism
718	F9W2X8	SCN1A	Sodium channel protein (Fragment)	1.5	Protein transport
	E1C501	FAM13A	Rho-GAP domain-containing protein		Signal transduction
	E1BZP8	GPAT4	Glycerol-3-phosphate acyltransferase 4		Energy metabolism
722	A0A3Q2TYT1	TTBK1	Tau-tubulin kinase 1	1.7	Signal transduction
816	A0A3Q2TSJ6	TSEN54	tRNA_int_end_N2 domain-containing protein	1.9	tRNA processing
	E1BUD3	TNNI2	Troponin I, fast skeletal muscle		Muscle structure
400	P08023	ACTA2	Actin, aortic smooth muscle	0.6	Muscle structure
	F1NZ78	ENO1	Alpha-enolase		Energy metabolism (glycolysis)
	Q9UKX2	MYH2	Skeletal myosin heavy chain		Muscle structure
	P68139	ACTA1	Actin, alpha skeletal muscle		Muscle structure
576	Q9UKX2	MYH2	Skeletal myosin heavy chain	0.7	Muscle structure
578	P08023	ACTA2	Actin, aortic smooth muscle	0.8	Muscle structure
	F1NU17	PGK2	Phosphoglycerate kinase		Energy metabolism (glycolysis)
764	F1NF62	CCDC93	Coiled-coil domain-containing protein 93	0.4	Protein transport
	E1BUG9	NUDCD2	CS domain-containing protein		Protein folding
793	R9PXM5	IGLL1	Immunoglobulin lambda like polypeptide 1	0.6	Immune response

[a]FC: fold change Organic/Antibiotic-free

Figure 3. *Comparison between antibiotic-free and organic chicken breast muscle proteomes within the Ross 308 strain. A) Representative two-dimensional gel electrophoresis highlighting the differentially abundant protein spots between the two groups. Spots in blue colour correspond to the down-regulated proteins, those in red are up-regulated proteins in organic chicken meat. B) Identify of differentially abundant proteins (DAPs) including their functions and fold change. C) Protein-Protein interaction (PPI) network using the list of the 16 DAPs in Ross 308 chicken breast muscle. The PPI network was built using the STRING database (https://string-db.org/). D-E) Bioinformatics enrichment analyses (Gene Ontology, KEGG, Reactome) using the 16 DAPs. D) Top enriched cluster terms. E) Biological processes.*

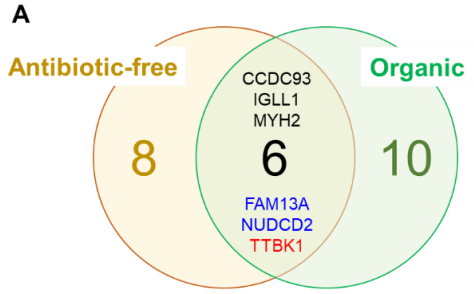


Spot ID	Uniprot ID	Gene names	Protein names	FC[a]	Functions
	P53449	ALDOC	Fructose-bisphosphate aldolase		Energy metabolism (glycolysis)
613	P53449	ALDOC	Fructose-bisphosphate aldolase (Frg)	1.8	Energy metabolism (glycolysis)
	Q6PVZ2	KRT15	Keratin, type I cytoskeletal 15		Muscle structure
	A0A3Q2TYT1	TTBK1	Protein kinase domain-containing protein		Signal transduction
	E1BZP8	GPAT4	Glycerol-3-phosphate acyltransferase 4		Energy metabolism (glycolysis)
	E1C501	FAM13A	Rho-GAP domain-containing protein		Signal transduction
691	F1NP57	ADCY9	Adenylate cyclase type 9	1.6	Signal transduction
	Q7T066	RHAG	Rh blood group-associated glycoprotein		Protein transport
	A0A3Q3APF4	CCDC129	KRAP_IP3R_bind domain-containing protein		Signal transduction
	R4GHW9	EPB41	Protein 4.1		Muscle structure
793	R9PXM5	IGLL1	Immunoglobulin lambda like polypeptide 1	2.4	Immune response
809	E1C501	FAM13A	Rho-GAP domain-containing protein	1.9	Signal transduction
	F1NBV0	PRDX6	Peroxiredoxin-6		Oxidative stress
	F1NEQ6	PSMA6	Proteasome subunit alpha type		Proteolysis
811	Q9PST8	Ubl	Polyubiquitin (Frg)	1.8	Proteolysis
	P79757	TTN	Titin (Frg)		Muscle structure
	Q90736	ACTB	Beta-actin (Frg)		Muscle structure
430	Q7SX63	HSP70	Heat shock protein 70	0.4	Chaperone
	Q9UKX2	MYH2	Myosin-2		Muscle structure
688	P62149	CALM1	Calmodulin	0.4	Metal binding

[a]FC: fold change Organic/Antibiotic-free

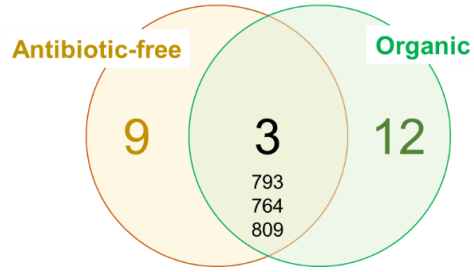
Figure 4. *Comparison between antibiotic-free and organic chicken breast muscle proteomes within the Ranger Classic strain.* ***A)*** *Representative two-dimensional gel electrophoresis highlighting the differentially abundant protein spots between the two groups. Spots in blue colour correspond to the down-regulated proteins, those in red are up-regulated proteins in organic chicken meat.* ***B)*** *Identify of differentially abundant proteins (DAPs) including their functions and fold change.* ***C)*** *Protein-Protein interaction (PPI) network using the list of the 18 DAPs in Ranger Classic chicken breast muscle. The PPI network was built using the STRING database (https://string-db.org/).* ***D-E)*** *Bioinformatics enrichment analyses (Gene Ontology, KEGG, Reactome) using the 16 DAPs.* ***D)*** *Top enriched cluster terms.* ***E)*** *Biological processes.*

A



Groups	Total	Proteins
AF ORG	6	MYH2 NUDCD2 IGLL1 TTBK1 CCDC93 FAM13A
AF	8	ACTN2 PKLR ENO1 ACTA2 PGM1 CALM1 DES ACTA1
ORG	10	PRDX6 ACTB TTN HSP70 CBS PSMA6 UBI A2M KRT9 TPM2

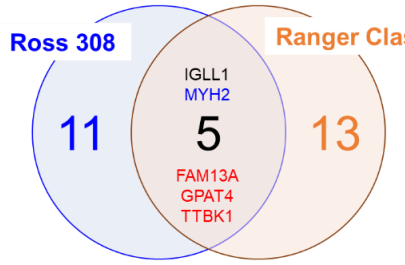
B



Groups	Total	Protein spots
AF ORG	3	793 764 809
AF	9	330 773 331 401 599 650 333 334 688
ORG	12	391 428 762 759 806 264 576 790 811 430 765 423

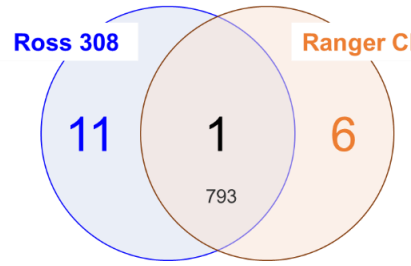
Figure 5. *Venn diagrams related to antibiotic-free (AF) and organic (ORG) chicken breast meat samples.* ***A)*** *Venn diagram highlighting the number of common proteins. In blue down-regulated and in red up-regulated proteins in Ranger Classic chickens compared to Ross 308 strain.* ***B)*** *Venn diagram related to the identified protein spots.*

A



Groups	Total	Proteins
RANGER ROSS	5	MYH2 IGLL1 TTBK1 GPAT4 FAM13A
ROSS	11	NUDCD2 ACTN2 TNNI2 ENO1 PGK2 TSEN54 ACTA2 GPD1 CCDC93 SCN1A ACTA1
RANGER	13	PRDX6 EPB41 UBI CCDC129 RHAG ACTB TTN HSP70 PSMA6 ADCY9 ALDOC CALM1 KRT15

B



Groups	Total	Protein spots
RANGER ROSS	1	793
ROSS	11	578 718 764 672 576 816 400 684 722 686 187
RANGER	6	613 811 809 430 688 691

Figure 6. *Venn diagrams comparing Ross 308 and Ranger Classic chicken breast meat samples.* ***A)*** *Venn diagram highlighting the number of common proteins. In blue down-regulated and in red up-regulated proteins in organic samples compared to antibiotic-free.* ***B)*** *Venn diagram related to the identified protein spots.*

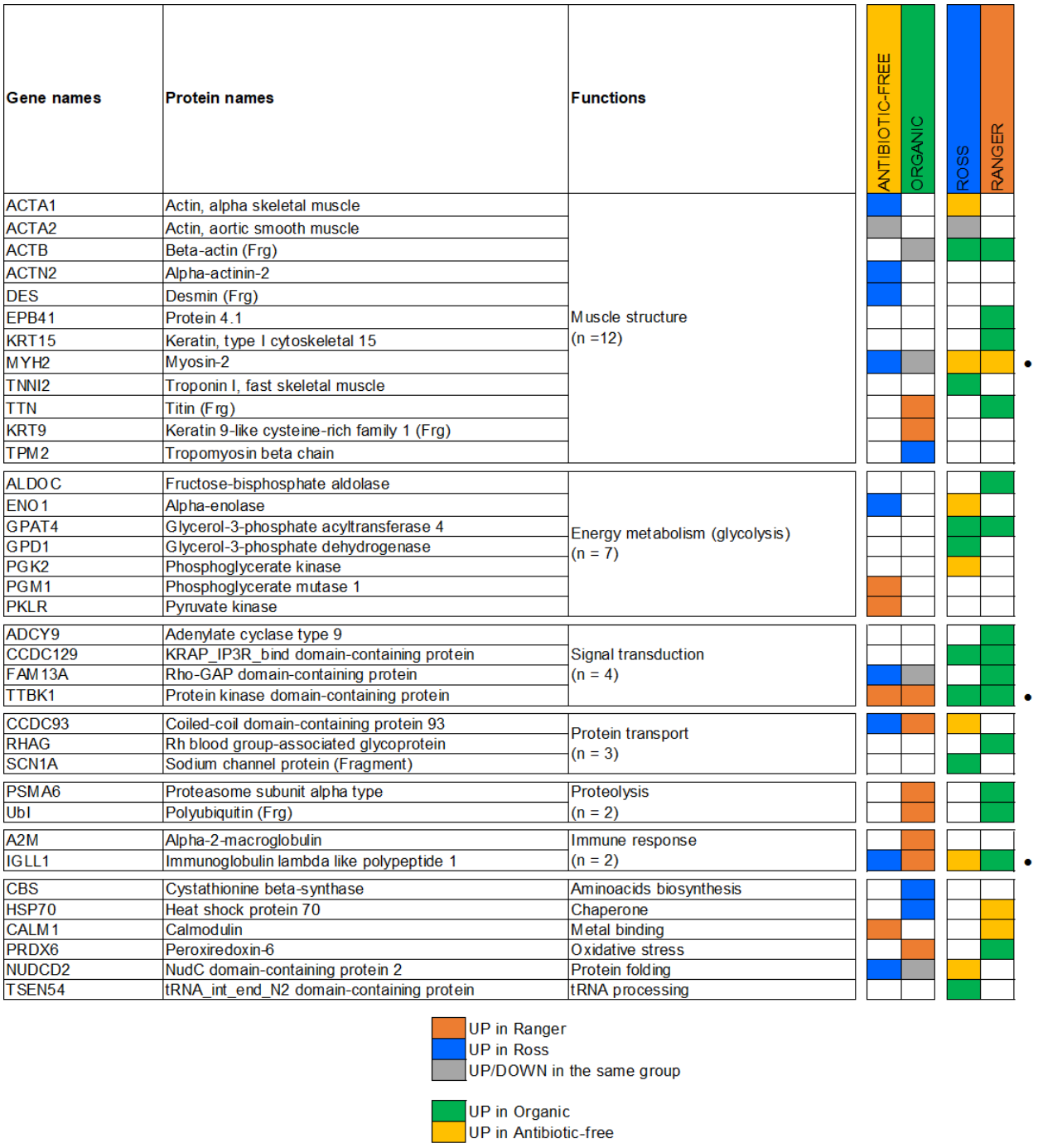


Figure 7. *Summary of the differentially abundant proteins (DAPs) organized by molecular function identified in this study to differ among the factors. The proteins identified whatever the condition are shown in the right of the panel with a symbol.*

4. Discussion

This study aimed to increase the knowledge on chicken meat proteome related to chicken strain and production system. The totality of results shows the complexity of proteome and its variation within different strains and different farming conditions. Specific proteomic signatures resulted statistically differentially expressed and they provide clues on biochemical processes responsible for meat quality traits and allow the investigation of biological markers that are indicators of meat authenticity.

Several research works studied the proteome of chicken breast meat using 2-DE followed by LC-MS/MS analysis to explore breast abnormalities and meat quality defects (Kuttappan et al. 2017; Cai et al. 2018; Zhang et al. 2020a; Zhang et al. 2020b; Zhang et al. 2021). However, there are no scientific evidence about the correlation of meat proteome with chicken farming system. (Kuttappan et al. 2017) compared the proteomic profiles of normal breasts with breasts that report severe myopathic lesions. Results showed that three biochemical pathways were up-regulated in breasts with severe lesions. These pathways were related to an increase in protein synthesis and cellular stress while others related to energy metabolism such as glycolysis and gluconeogenesis were up-regulated in normal breasts. According to these results, (Cai et al. 2018) underlined a decreased glycolytic activity and an increased activation of specific pathways in response to oxidative stress in woody breast meat.

In this article, most of DAPs related to energy metabolism pathways were overabundant in antibiotic-free Ranger Classic chicken breast meat when compared with Ross 308 from the same farming system. Chaperone activity and amino acids biosynthesis related proteins were overabundant in organic Ross 308 when compared to organic Ranger Classic breasts. Associating these results with those reported above from the literature, it can be concluded that Ranger Classic strain is less disposed to the development of myopathies and meat quality

defects than Ross 308 in both production systems. According to this, (Zhang et al. 2020a) compared the protein profiles between normal and woody breast of five chicken strains to identify biomarkers of myopathies. Results reported that one specific broiler strains had more woody breast specific overexpressed proteins respect to the others. These proteins were involved in apoptosis, protein synthesis, and oxidative stress.

Among the 12 statistically different proteins related to muscle structure, MYH2, a myosin isoform, showed a significative overabundance in Ross 308 breasts, especially from antibiotic-free farming. This protein, together with others related to muscle structure pathways, was reported to be more expressed in chicken breasts affected by PSE abnormality (Desai et al. 2016). Moreover, it was identified as putative biomarker of Korean native chicken meat when compared to commercial broiler one (De Liu et al. 2012). On the contrary, ACTB, an actin isoform, resulted overabundant in organic meat from both production systems together with GPAT4 and TTBK1.

The expression of proteins related to signal transduction pathways in Ranger Classic may be influenced by the production system, in fact, they were all overabundant in organic meat than in antibiotic-free.

The identified proteolysis related proteins (PSMA6 and UBI) were both significantly overabundant in organic Ranger Classic when compared to both antibiotic-free Ranger Classic and organic Ross 308. These proteins were reported as overexpressed in PSE breast by (Desai et al. 2016).

In conclusion, it was assessed that differences in strains and farming system can influence the chicken breast meat quality. Comparing the results with scientific literature it was found that specific differences in proteome due to differences in broiler genetic can lead more likely to have meat with qualitative defects. Moreover, this study focused on two farming systems,

organic and antibiotic-free, underlining differences in protein expression in chicken breast final product. This aspect should be more investigated by further research as a paucity of scientific literature was found about the impact of organic production system on chicken breast muscle proteome.

Acknowledgment

The authors are grateful to Fileni S.p.a. for financially support this project and Dr. Susana Bravo López from the Proteomic Unit, Health Research Institute (IDIS) of Santiago de Compostela, Spain for support in MS analyses.

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SECTION C
Results of the thesis

Organic versus antibiotic-free chicken meat: the contribution of shotgun proteomics to decipher the unknowns and discover biomarkers of authenticity

Publication 4

Organic *versus* antibiotic-free chicken meat: the contribution of shotgun proteomics to decipher the unknowns and discover biomarkers of authenticity

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Article in preparation for Journal of Proteomics

Abstract

The discovery of biomarkers for chicken meat quality and authenticity is gaining interest due to its potential implications for the poultry industry. Biomarkers are measurable indicators that can be used to assess the quality or authenticity of chicken meat. This study focuses on the identification of specific proteins or peptides that are associated with differences in chicken farming systems or strain differences using proteomics. The comparison of the muscle proteome of Ranger Classic and Ross 308, reared under an antibiotic-free production system, and organic production system, revealed potential biomarkers between the two strains. Bioinformatics enrichment analyses revealed significant differences in the muscle proteome between the two chicken strains, which may be related to their genetic background and rearing conditions. These results provide insights into the potential pathways that are associated with these differences, such as muscle contraction and energy metabolism, and may have important implications for the poultry industry. Moreover, the discovery of biomarkers for chicken meat quality and authenticity can also be used to detect food fraud, ensuring that consumers receive what they pay for. The findings revealed new pathways and insights that are worthy of validation under other sampling conditions and experimental design before setting up a targeted proteomics evaluation for the list of biomarkers. Overall, the study highlights the importance of genetic selection and management practices in improving the quality of chicken meat. Further studies are needed to confirm these findings and explore their practical implications in more detail.

Keywords: Poultry muscle; Omics; Proteome; Organic meat; Production systems; Biological pathways.

1. Introduction

Nowadays consumer concern about food quality and sustainability and most of them are willing to pay high prices for guaranteed and certified animal products (Kaygisiz et al. 2019; Nguyen et al. 2019). The exponential increase in the market for organic products in recent years is proof of this.

Organic meat production based on natural feed, produced without artificial fertilizers and chemical pesticides and high standards of animal welfare, aimed to provide more sustainable products with high levels of quality and safety than conventional meat production more dependent on chemical inputs to produce more in less time. (Kumm 2002; El-Deek & El-Sabrout 2019). In Europe, organic meat production is regulated by Regulation (EC) no. 834/2007 (2007) and two implementing regulations, no. 889/2008 and n. 1235/2008((EC) 2008). These guidelines regulate all the meat supply chain, from origin and breeding of animals, feed, veterinary treatments, slaughtering methods, packaging solutions, transport, storage, import and export of products themselves and their derivatives ((EC) 2008).

Among the variety of meat-based foods on the market, poultry meat is the primary protein source for people in the most areas of the world (Zaboli et al. 2019). Modern chicken breeds are the result of decades of artificial selection for commercial purposes. In fact, the most widely used strain in broiler production is the Ross 308 fast growing hybrid, raised to produce a lot of meat in a short period of time. (Karlsson 2016) Organic production involves a longer rearing period and this causes several welfare problems for fast-growing broilers, so the choice of more suitable strains for this production system is an open issue among poultry industries. (Sirri 2011; Rocchi et al. 2021).

Ranger Classic is a broiler strain with a naturally slower growth rate than the Ross 308 (Center 2018). Ranger Classic growth-rate meets more the rearing times required by European regulations for organic farming, without any feed restrictions (Karlsson 2016).

Moreover, it has been well documented that pre-slaughter stressors, such as farming conditions, temperature and handling procedures, affect physiological and metabolic functions of animals, with consequent repercussions on meat quality (Schwartzkopf-Genswein et al. 2012; Xing et al. 2019; Xing et al. 2020). Organic production regulations impose high standards of animal welfare that should provide high quality product.

Over the past two decades, -OMIC technologies have begun to be applied to the study of meat quality, as part of a Foodomics approach to investigate how biological mechanisms can affect meat quality traits. Proteomics turned out to be a very effective tool to analyze the dynamic biochemical mechanisms of post-mortem muscle. Techniques based on the combination of proteomics with mass spectrometry (MS) can provide a superior ability to separate and identify a large number of muscle proteins with a greater resolving power. Therefore, the application of these techniques can allow a deeper knowledge of the muscle-to-meat conversion mechanisms and their influence on meat quality traits (Picard & Gagaoua 2020).

Despite that poultry meat is one of the most consumed meats in the world, to date there has been a paucity of published literature on the application of high-throughput omics methods, such as proteomics, to such food matrices. A further understanding of the dynamics of protein models related to different growth-rates, diet, strains and production systems could lead to characterize the potential impacts of these parameters on meat quality. Therefore, this study aims to understand the impact of an organic production system, compared to an

antibiotic-free production system, at the level of the proteome of post-mortem pectoralis major muscle in the Ross 308 and in the Ranger Classic chicken strains.

2. Materials and methods

2.1 Animals and muscle tissue sampling

A total of 20 Ross 308 and 20 Ranger Classic chickens were applied to proteomic analysis in this study, all the samples were provided by Fileni® industry (Cingoli, Italy). From each group, 10 chickens were reared antibiotic-free inside ground farming and 10 according to European standards of organic farming and livestock ((EC) 2008). Figure 1 shows the 4 groups of samples, divided according to strains and production system and the applied workflow. Pectoralis major muscles were collected immediately after slaughter. A 2 cm3 section was cut using a sterile scalpel from the top right part of the muscle, frozen in liquid nitrogen and stored at -80°C until extraction day.

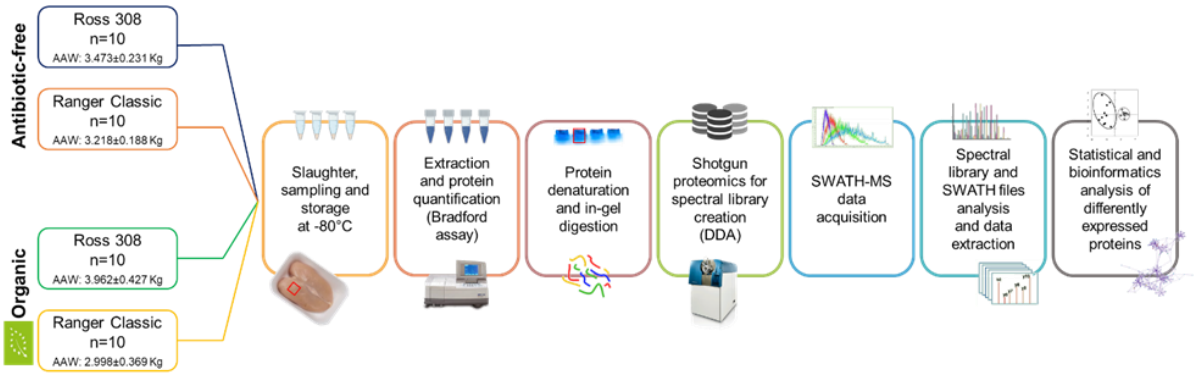

Figure 1. *Workflow of the bottom-up proteomic method applied to the 40 chicken pectoralis major samples in this study. (AAW: Average Alive Weight)*

2.2 Protein extraction and quantification

About 200 mg of muscle tissue were homogenized in 3 mL of extraction buffer containing 8.3 M urea, 2 M thiourea, 1% Dithiothreitol (DTT), 2% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) and 2% Pharmalyte® (Immobilized pH gradient (IPG) buffer pH 3–10) using a T 25 digital Ultra-Turrax® (Picard 2020; Picard & Gagaoua

2020). Protein homogenates were then incubated for 30 min on wet-ice and centrifuged for 30 min at 10000 rpm at 4°C. Supernatants were transferred into Eppendorf tubes and stored at -80 °C until protein quantification. Protein concentrations of obtained extracts were determined using the dye-binding protocol of Bradford (Bradford 1976). A spectrophotometer (UV-1700, Pharmaspec, SHIMADZU) and bovine serum albumin (BSA) as a standard (Bio-Rad Protein Assay kit, Bio-Rad, France) were used.

2.3 Protein bands preparation for LC-MS/MS

Muscle protein extracts were diluted and denatured by 1:1 Laemmli sample buffer (Bio-Rad Laboratories, Deeside, UK). Protein extracts quality was then assessed by SDS-PAGE gel electrophoresis on 12% acrylamide gels by the presence of distinct protein bands. The denatured protein extracts were concentrated on 1D stacking gel of SDS-PAGE. 20 µg of proteins were loaded in each gel lane and electrophoresis was run for about 15 min to concentrate the proteins. Subsequently, the gels were washed with MilliQ water, stained with EZ Blue Gel staining reagent (Sigma-Aldrich, St. Louis, USA) and kept in gentle agitation for 2 h and then washed with distilled. Stacking gels protein bands were excised with a sterile disposable scalpel and transferred into Eppendorf tubes with 150 µL of 50 mM ammonium bicarbonate 50% ethanol. Then, disulfide bonds were reduced with 200 µL of 10 mM dithiothreitol (Sigma-Aldrich, St. Louis, USA) in 50 mM ammonium bicarbonate buffer for 45 min at 56°C. Proteins alkylation was carried out with 200 µL of 55 mM iodoacetamide (Sigma-Aldrich, St. Louis, USA) in 50 mM ammonium bicarbonate buffer for 30 min in darkness. Subsequently bands were destained by 200 µL of 25 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, USA), 5% acetonitrile for 30 min and washed twice with 200 µL of 25 mM ammonium bicarbonate, 50% acetonitrile for 30 min each time. Finally, bands were dehydrated with 100% acetonitrile for 10 min then the liquid was discarded. Dried protein bands were stored at -80°C until SWATH-MS analysis.

2.4 Protein identification by LC-MS/MS

In order to make global protein identification and quantification (by SWATH analysis), an equal amount of protein (40 µg) from all samples were loaded on a 10% SDS-PAGE gel. The run was stopped as soon as the front had penetrated 3 mm into the resolving gel (Bonzon-Kulichenko et al. 2011; Perez-Hernandez et al. 2013). The protein band was detected by Sypro-Ruby fluorescent staining (Lonza, Switzerland), excised, and processed for in-gel, manual tryptic digestion as described elsewhere (Shevchenko et al. 1996). Peptides were extracted by carrying out three 20-min incubations in 40 µL of 60% acetonitrile dissolved in 0.5% HCOOH. The resulting peptide extracts were pooled, concentrated in a SpeedVac, and stored at −20 °C.

2.5 Protein quantification by SWATH (Sequential Window Acquisition of all Theoretical Mass Spectra)

2.5.1 Creation of the spectral library

To construct the MS/MS spectral libraries, the peptide solutions were analysed by a shotgun data-dependent acquisition (DDA) approach by micro-LC-MS/MS. To get a good representation of the peptides and proteins present in all samples, pooled vials of samples from each group (ORO, ORA, ARO and ARA) were prepared using equal mixtures of the original samples. 4 µL (4µg) of each pool was separated into a micro-LC system Ekspert nLC425 (Eksigen, Dublin, CA, USA) using a column Chrom XP C18 150 × 0.30 mm, 3 µm particle size and 120 Å pore size (Eksigent, SCIEX) at a flow rate of 5µL/min. Water and ACN, both containing 0.1% formic acid, were used as solvents A and B, respectively. The gradient run consisted of 5% to 95% B for 30 min, 5 min at 90% B and finally 5 min at 5% B for column equilibration, for a total run time of 40 min. When the peptides eluted, they were directly injected into a hybrid quadrupole-TOF mass spectrometer Triple TOF 6600plus (Sciex, Redwood City, CA, USA) operated with a data-dependent acquisition

system in positive ion mode. A Micro source (Sciex) was used for the interface between microLC and MS, with an application of 2600 V voltage. The acquisition mode consisted of a 250 ms survey MS scan from 400 to 1250 m/z followed by an MS/MS scan from 100 to 1500 m/z (25 ms acquisition time) of the top 65 precursor ions from the survey scan, for a total cycle time of 2.8 s. The fragmented precursors were then added to a dynamic exclusion list for 15 s; any singly charged ions were excluded from the MS/MS analysis.

The peptide and protein identifications were performed using Protein Pilot software (version 5.0.1, Sciex, Redwood City, CA, USA). Data were searched using a chicken specific Uniprot database, specifying iodoacetamide as Cys alkylation. This software uses the algorithm ParagonTM for database search and ProgroupTM for data grouping. This false discovery rate was performed using a non-linear fitting method displaying only those results that reported a 1% global false discovery rate or better for both peptides and proteins (Shilov et al. 2007). The MS/MS spectra of the identified peptides were then used to generate the spectral library for SWATH peak extraction using the add-in for PeakView Software (version 2.2, Sciex, Redwood City, CA, USA) MS/MSALL with SWATH Acquisition MicroApp (version 2.0, Sciex, Redwood City, CA, USA). Only peptides with a confidence score above 99% (as obtained from Protein Pilot database search) were included in the spectral library.

2.5.2 Relative quantification by SWATH acquisition

SWATH (Sequential Window Acquisition of all Theoretical Mass Spectra) – MS acquisition was performed on a TripleTOF® 6600plus LC-MS/MS system (AB Sciex, Redwood City, CA, USA). Samples were analysed using a data-independent acquisition (DIA) method (40 total samples). Each sample (4 μL from a mg/ml solution) was analysed using the LC-MS equipment and LC gradient described above for building the spectral library but instead using the SWATH-MS acquisition method. The method consisted of repeating a cycle that

consisted of the acquisition of 100 TOF MS/MS scans (400 to 1500 m/z, high sensitivity mode, 50 ms acquisition time) of overlapping sequential precursor isolation windows of variable width (1 m/z overlap) covering the 400 to 1250 m/z mass range with a previous TOF MS scan (400 to 1500 m/z, 50 ms acquisition time) for each cycle. Total cycle time was 6.3 s. For each sample set, the width of the 100 variable windows was optimized according to the ion density found in the DDA runs using a SWATH variable window calculator worksheet from Sciex.

2.5.3 Data analysis library free using Dia NN software

After the samples were acquired individually using the SWATH method, DIA-NN (1.8) was used to generate the protein values per samples using recommended settings. Mass ranges and charges were set appropriately: peptide length range 7 to 35, precursor charge range 2 to 5, precursor range 350 to 1400 m/z and fragment ion range 100 to 1500 m/z. For the search chicken database from Uniprot was added. Other important parameters were as neural network classifier were set as single pass mode, as quantify strategy were set Any LC (high accuracy) and Cross-run normalization was set as RT-Dependent. For the in silico predicted library search, the reduced memory option was additionally activated. Protein FDR was set to 1.0 %.

The integrated peak areas obtained from DIA NN were exported to the MarkerView software (Sciex, Redwood City, CA, USA) for relative quantitative analysis. The export generated information about individual ions, the summed intensity of different ions for a particular peptide and the summed intensity of different peptides for a particular protein. MarkerView has been used for analysis of SWATH-MS data reported in other proteomics studies (Luo et al. 2017; Meyer & Schilling 2017; Ortea et al. 2018; Tan & Chung 2018) because of its data-independent method of quantitation. MarkerView uses processing algorithms that accurately find chromatographic and spectral peaks direct from the raw SWATH data. Data alignment

by MarkerView compensated for minor variations in both mass and retention time values, ensuring that identical compounds in different samples were accurately compared to one another. To control for possible uneven sample loss across the different samples during the sample preparation process, we performed a MLR global normalization. Unsupervised multivariate statistical analysis using principal component analysis (PCA) was performed to compare the data across the samples. The average MS peak area of each protein was derived using the MarkerView from the replicates of the SWATH-MS of each sample followed by Student's t-test analysis software for comparison among the samples based on the averaged area sums of all the transitions derived for each protein. The t-test indicated how well each variable distinguishes the two groups, reported as a p-value. For each library, its set of differentially expressed proteins (p-value <0.05) with a 1.5 fold in- or decrease was selected.

2.6 Statistical analysis

Statistical analyses were performed using XLSTAT 2018.2 (AddinSoft, Paris, France). A one-way ANOVA was used to select differently abundant proteins of each comparison between groups. Protein abundances were considered differently expressed at $p < 0.05$.

Subsequently, all proteins characterized as differentially abundant in the four comparisons were screened using principal component analysis (PCA) with the corresponding strain or farming method variables. Furthermore, correlations among the differential proteins were used to build biological correlation networks following the procedure described earlier (Gagaoua et al. 2021).

2.7 Bioinformatics analysis

Differentially expressed proteins among the groups were compared for the enriched Gene Ontology (GO) terms using the web tool Metascape® (https://metascape.org/) as described earlier (Gagaoua et al. 2021).

3. Results

The SWATH-MS proteomics applied in this study on individual samples allowed the identification of 660 quantifiable proteins in chicken Pectoralis major muscle. The relative abundances of the muscle proteomes were compared based on the different conditions investigated in this trial these being within the strain under the same production system or within the production system under the same strain. The results of the different comparisons are given in the following subsections.

3.1 Comparison of the muscle proteome of Ranger Classic and Ross 308 reared under antibiotic-free production system

The comparison, using ANOVA test, between the two strains, Ranger Classic (ARA) versus Ross 308 (ARO) reared under antibiotic-free production system, revealed 38 differentially expressed proteins (DEPs) from which 18 were up- and 20 down-regulated in ARA (Table S1). The projection of the DEPs proteins by means of a principal component analysis (PCA) allowed a clear separation of ARA from ARO (Figure 2A). This is further evidenced by the hierarchical clustering analysis through the heatmap using the individual abundances of the DEPs (Figure 2B).

The bioinformatics enrichment analyses on the 38 DEPs through Gene Ontology (GO), KEGG and Reactome databases are given in Figure 2C. It resulted that 11 cluster terms were significantly enriched mainly dominated by “striated muscle contraction pathway (WP383)” followed by “ATP metabolic process (GO:0046034)” and “formation of tubulin folding intermediates (R-HSA-389960)” as the top three GO terms. These enriched cluster terms allowed to construct a process network of the pathways (Figure 2D). It confirmed the dominance of a sub-network muscle structure, contraction and associated pathways and another one related to ATP and energy metabolic processes.

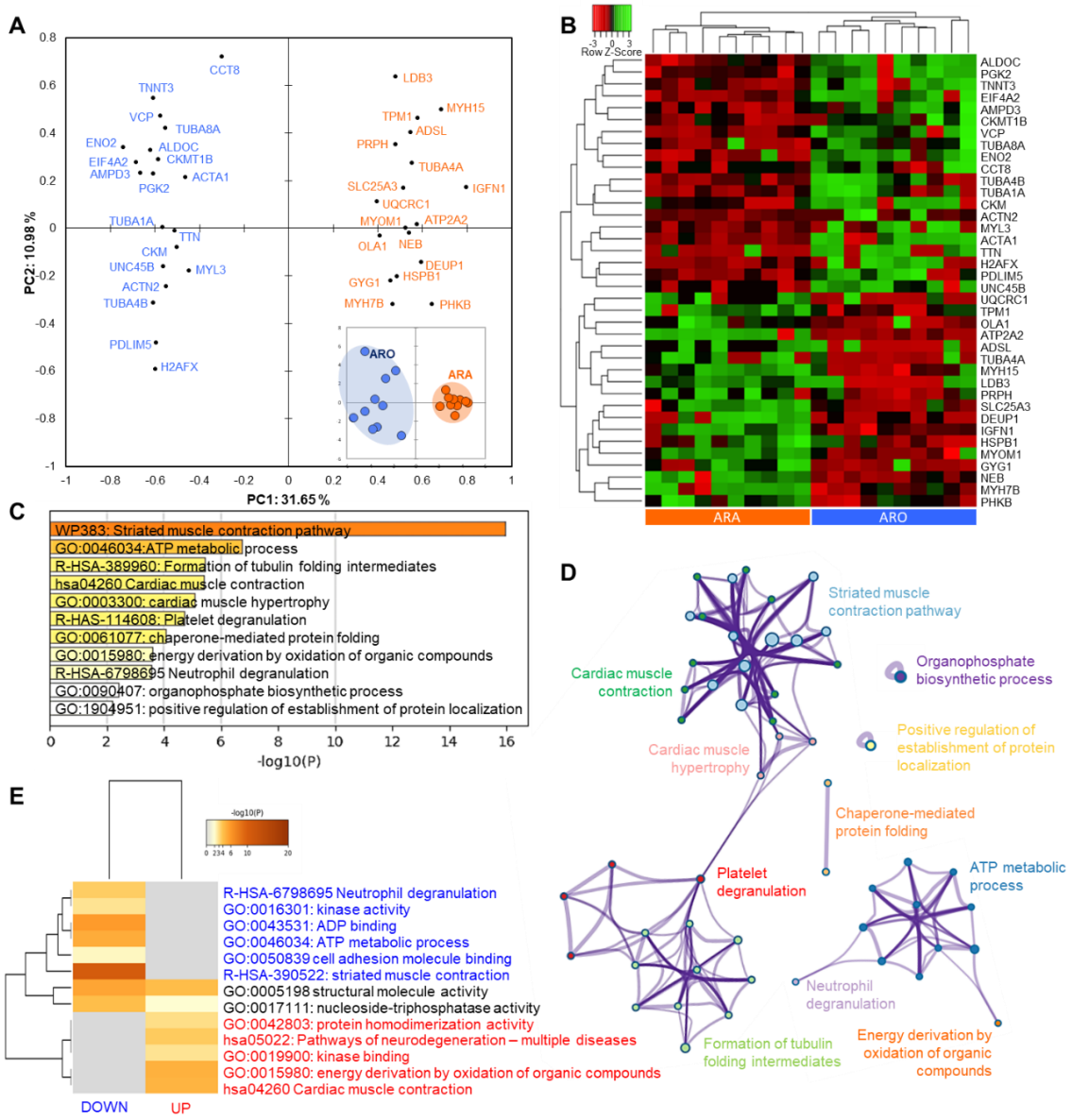

Figure 2. *Statistical and bioinformatics analyses on the differentially expressed proteins (DEPs = 38) between Ross 308 and Ranger Classic antibiotic-free chicken post-mortem muscle proteome. A) Principal Component Analysis (PCA) highlighting the distribution of the 38 DEPs and the separation of the two groups in the bi-plot in the bottom right. B) Heatmap of DEPs analyzed by hierarchical clustering. Each row represents a single protein. Each column represents an individual chicken. Protein expression values were log2-normalized and cluster analysis was performed using Z-score. Red indicates a low expression level; green indicates a high expression level. C-E) Bioinformatic enrichment analyses (Gene Ontology, KEGG, Reactome) on the 38 DEPs. C) Top enriched terms. D) Network layout based on the pathways of the 38 DEPs. Each term is represented by a circle node, where its size is proportional to the number of input genes fall under that term, and its color represent its cluster identity. Terms with a similarity score > 0.3 are linked by an edge (the thickness of the edge represents the similarity score). E) Hierarchical Heatmap clustering comparing the UP (n = 18) and DOWN (n = 20) DEPs in terms of the significant process and pathways among the top Gene Ontology terms and colored according to P-values: terms with a P-value <0.01, a minimum count of 3, and an enrichment factor > 1.5. Colors from grey to brown indicate p-values from high to low; and grey cells indicate the lack of significant enrichment. The terms in blue color are specific to down-regulated proteins, those in red are for up-regulated proteins in Ranger Classic chickens and those in black are significant and common to both protein lists.*

The comparison by means of a heatmap of the up- and down-regulated proteins in terms of enriched terms is given in Figure 2E. The common and specific biological pathways to the two protein lists are evidenced. Two enriched terms were found to be common: “structural molecule activity (GO:0005198)” and “nucleoside-triphosphatase activity (GO:0017111)”. Six pathways were specific and down-regulated in Ranger Classic: “neutrophil degranulation (R-HSA-6798695)”, “kinase activity (GO:0016301)”, “ADP binding (GO:0043531)”, “ATP metabolic process (GO:0046034)”, “cell adhesion molecule binding (GO:0050839)” and “striated muscle contraction (R-HSA-390522)”. However, five pathways were specific and up-regulated in Ranger Classic: “protein homodimerization activity (GO:0042803)”, “pathways of neurodegeneration – multiple diseases (hsa05022)”, “kinase binding (GO:0019900)”, “energy derivation by oxidation of organic compounds (GO:0015980)” and “cardiac muscle contraction (hsa04260)”.

3.2 Comparison of the muscle proteome of Ranger Classic and Ross 308 reared under organic production system

The comparison, using ANOVA test, between the two strains, Ranger Classic (ORA) versus Ross 308 (ORO) reared under organic production system, revealed 24 differentially expressed proteins (DEPs) from which 13 were up- and 11 down-regulated in ARA (Table S2). The projection of the DEPs proteins by means of a principal component analysis (PCA) revealed not a clear separation between the groups. 3 individuals of ORA and 4 of ORO can be distinguished while the other terms are overlapped (Figure 3A). This concept is further confirmed by the hierarchical clustering analysis through the heatmap using the individual abundances of the DEPs (Figure 3B).

The bioinformatics enrichment analyses on the 24 DEPs through Gene Ontology (GO), KEGG and Reactome databases are given in Figure 3C.

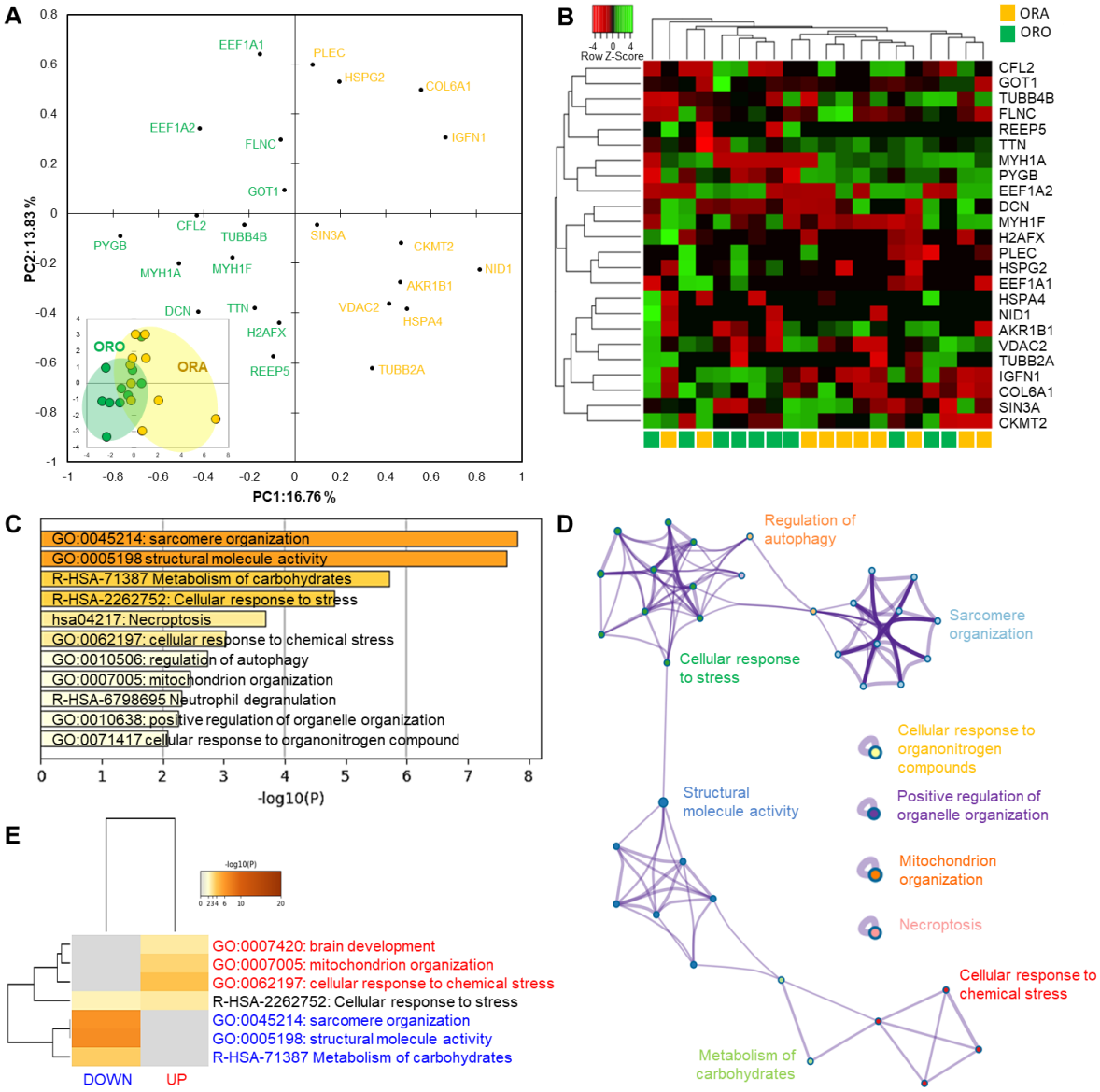

Figure 3. *Statistical and bioinformatics analyses on the differentially expressed proteins (DEPs = 24) between Ross 308 and Ranger Classic organic chicken post-mortem muscle proteome.* ***A)*** *Principal Component Analysis (PCA) highlighting the distribution of the 24 DEPs and the separation of the two groups in the bi-plot in the bottom right.* ***B)*** *Heatmap of DEPs analyzed by hierarchical clustering. Each row represents a single protein. Each column represents an individual chicken. Protein expression values were log2-normalized and cluster analysis was performed using Z-score. Red indicates a low expression level; green indicates a high expression level.* ***C-E)*** *Bioinformatic enrichment analyses (Gene Ontology, KEGG, Reactome) on the 24 DEPs.* ***C)*** *Top enriched terms.* ***D)*** *Network layout based on the pathways of the 24 DEPs. Each term is represented by a circle node, where its size is proportional to the number of input genes fall under that term, and its color represent its cluster identity. Terms with a similarity score > 0.3 are linked by an edge (the thickness of the edge represents the similarity score).* ***E)*** *Hierarchical Heatmap clustering comparing the UP (n = 13) and DOWN (n = 11) DEPs in terms of the significant process and pathways among the top Gene Ontology terms and colored according to P-values: terms with a P-value <0.01, a minimum count of 3, and an enrichment factor > 1.5. Colors from grey to brown indicate p-values from high to low; and grey cells indicate the lack of significant enrichment. The terms in blue color are specific to down-regulated proteins, those in red are for up-regulated proteins in Ranger Classic chickens and those in black are significant and common to both protein lists.*

It resulted that 11 cluster terms were significantly enriched mainly dominated by “sarcomere organization (GO:0045214)” and “structural molecule activity (GO:0005198)” followed by “metabolism of carbohydrates (R-HSA-71387)” and “cellular response to stress (R-HSA-2262752)” as the top four GO terms. These enriched cluster terms allowed to construct a process network of the pathways (Figure 3D). It confirmed the dominance of a sub-network sarcomere organization and structural molecule activity pathways and another one related to cellular response to stress processes.

The comparison by means of a heatmap of the up- and down-regulated proteins in terms of enriched terms is given in Figure 3E. The common and specific biological pathways to the two protein lists are evidenced. One enriched term was found to be common being “cellular response to stress (R-HSA-2262752)”. Three pathways were specific and down-regulated in Ranger Classic: “sarcomere organization (GO:0045214)”, “structural molecule activity (GO:0005198), and “metabolism of carbohydrates (R-HSA-71387)”. However, three pathways were specific and up-regulated in Ranger Classic: “brain development (GO:0007420)”, “mitochondrion organization (GO:0007005) and “cellular response to chemical stress (GO:0062197)”.

3.3 Comparison of the muscle proteome of Ross 308 reared under organic and antibiotic-free production systems

The comparison of Ross 308 chicken meat reared following two farming and production methods, Antibiotic-free (ARO) versus Organic (ORO), revealed 61 differentially expressed proteins (DEPs) from which 24 were up- and 37 down-regulated in ORO (Table S3). The projection of the DEPs proteins by means of a principal component analysis (PCA) allowed a clear separation of ORO from ARO (Figure 4A). This is further evidenced by the hierarchical clustering analysis through the heatmap using the individual abundances of the DEPs (Figure 4B).

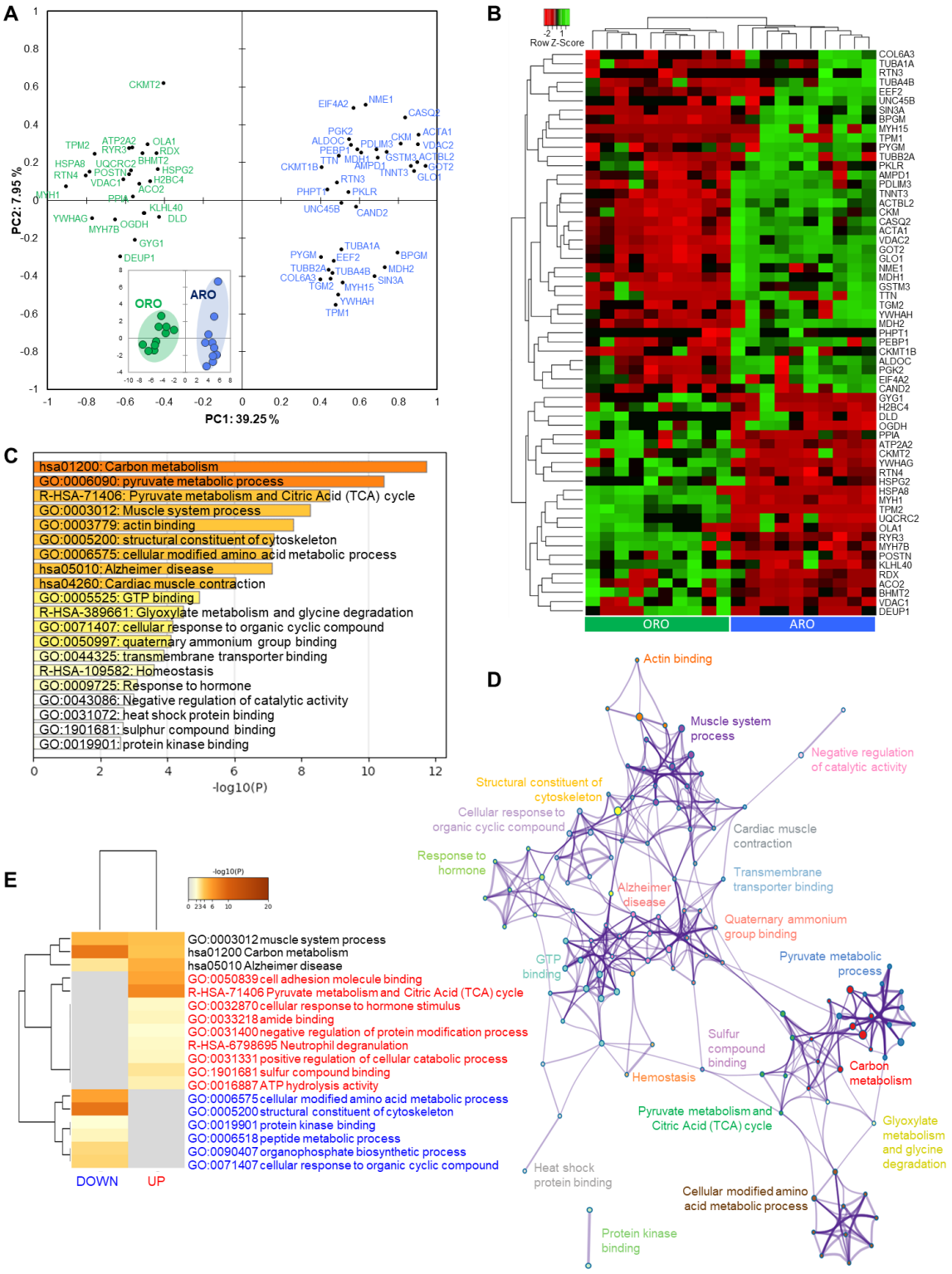

Figure 4. *Statistical and bioinformatics analyses on the differentially expressed proteins (DEPs = 61) between organic and antibiotic-free Ross 308 chicken post-mortem muscle proteome. A) Principal Component Analysis (PCA) highlighting the distribution of the 61 DEPs and the separation of the two groups in the bi-plot in the bottom left. B) Heatmap of DEPs analyzed by hierarchical clustering. Each row represents a single protein. Each column represents an individual chicken. Protein expression values were log2-normalized and cluster analysis was performed using Z-score. Red indicates a low expression level; green indicates a high expression level. C-E) Bioinformatic enrichment analyses (Gene Ontology, KEGG, Reactome) on the 61 DEPs. C) Top enriched terms. D) Network layout based on the pathways of the 61 DEPs. Each term is represented by a circle*

node, where its size is proportional to the number of input genes fall under that term, and its color represent its cluster identity. Terms with a similarity score > 0.3 are linked by an edge (the thickness of the edge represents the similarity score). E) Hierarchical Heatmap clustering comparing the UP (n = 24) and DOWN (n = 37) DEPs in terms of the significant process and pathways among the top Gene Ontology terms and colored according to P-values: terms with a P-value <0.01, a minimum count of 3, and an enrichment factor > 1.5. Colors from grey to brown indicate p-values from high to low; and grey cells indicate the lack of significant enrichment. The terms in blue color are specific to down-regulated proteins, those in red are for up-regulated proteins in organic system and those in black are significant and common to both protein lists.

The bioinformatics enrichment analyses on the 61 DEPs through Gene Ontology (GO), KEGG and Reactome databases are given in Figure 2C. It resulted that 20 cluster terms were significantly enriched mainly dominated by "carbonic metabolism (hsa01200)" and "pyruvate metabolic process (GO:0006090)" followed by "pyruvate metabolism and Citric Acid (TCA) cycle (R-HSA-71406)", "Muscle system process(GO:0003012)", "actin binding (GO:0003779)", "structural constituent of cytoskeleton (GO:0005200)", "cellular modified amino acid metabolic process (GO:0006575)", "Alzheimer disease (hsa05010)" and "Cardiac muscle contraction (hsa04260)" as the top nine GO terms. These enriched cluster terms allowed to construct a process network of the pathways (Figure 4D). It confirmed the dominance of a sub-network muscle system, structure and associated pathways and another one related to carbon, pyruvate, and other metabolic processes.

The comparison by means of a heatmap of the up- and down-regulated proteins in terms of enriched terms is given in Figure 4E. The common and specific biological pathways to the two protein lists are evidenced. Three enriched terms were found to be common: "muscle system process (GO:0003012)", "carbon metabolism (hsa01200)" and "Alzheimer disease (hsa05010)". Six pathways were specific and down-regulated in organic samples: "cellular modified amino acid metabolic process (GO:0006575)", "structural constituent of cytoskeleton (GO:0005200)", "protein kinase binding (GO:0019901)", "peptide metabolic process (GO:0006518)", "organophosphate biosynthetic process (GO:0090407)", "cellular response to organic cyclic compound (GO:0071407)". However, nine pathways were specific and up-regulated in organic meat: "cell adhesion molecule binding (GO:0050839)",

"Pyruvate metabolism and Citric Acid (TCA) cycle (R-HSA-71406)", "cellular response to hormone stimulus (GO:0032870)", "amide binding (GO:0033218)", "negative regulation of protein modification process (GO:0031400)", "Neutrophil degranulation (R-HSA-6798695)", "positive regulation of cellular catabolic process (GO:0031331)", "sulfur compound binding (GO:1901681)" and "ATP hydrolysis activity (GO:0016887)".

3.4 Comparison of the muscle proteome of Ranger Classic reared under organic and antibiotic-free production systems

The comparison of Ranger Classic chicken meat reared following two farming and production methods, Antibiotic-free (ARA) versus Organic (ORA), revealed 25 differentially expressed proteins (DEPs) from which 11 were up- and 14 down-regulated in ORA (Table S4). The projection of the DEPs proteins by means of a principal component analysis (PCA) allowed a good separation of ORA from ARA (Figure 5A). This is further evidenced by the hierarchical clustering analysis through the heatmap using the individual abundances of the DEPs (Figure 5B).

The bioinformatics enrichment analyses on the 25 DEPs through Gene Ontology (GO), KEGG and Reactome databases are given in Figure 5C. It resulted that 9 cluster terms were significantly enriched mainly dominated by "generation of precursor metabolites and energy (GO:0006091)" and "sarcomere organization (GO:0045214)" followed by "actin cytoskeleton reorganization (GO:0031532)" as the top three GO terms. These enriched cluster terms allowed to construct a process network of the pathways (Figure 5D). It confirmed the dominance of a sub-network muscle structure and associated pathways and another one related to energy metabolic processes.

The comparison by means of a heatmap of the up- and down-regulated proteins in terms of enriched terms is given in Figure 5E.

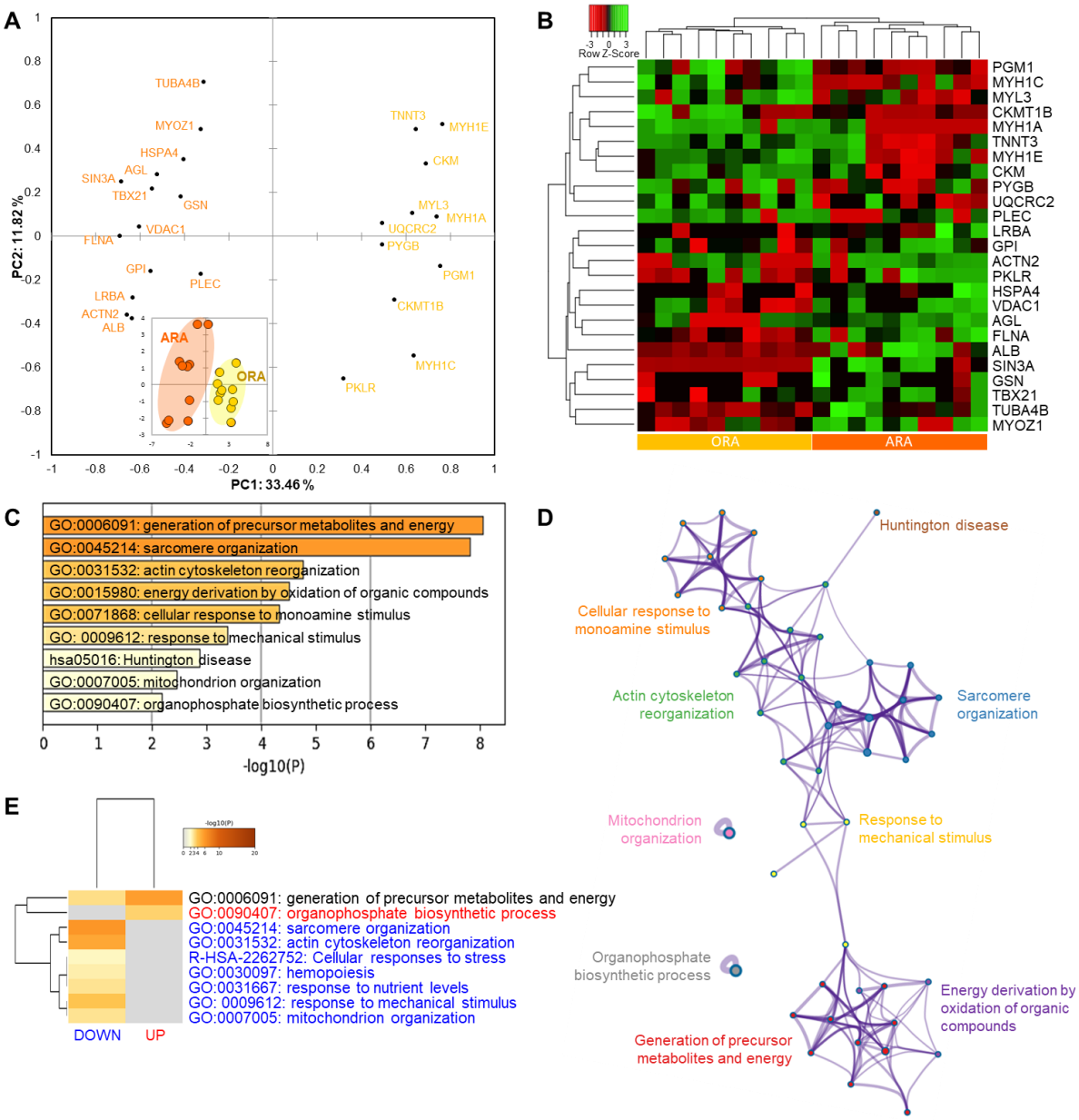

Figure 5. *Statistical and bioinformatics analyses on the differentially expressed proteins (DEPs = 25) between organic and antibiotic-free Ranger Classic chicken post-mortem muscle proteome.* ***A)*** *Principal Component Analysis (PCA) highlighting the distribution of the 25 DEPs and the separation of the two groups in the bi-plot in the bottom left.* ***B)*** *Heatmap of DEPs analyzed by hierarchical clustering. Each row represents a single protein. Each column represents an individual chicken. Protein expression values were log2-normalized and cluster analysis was performed using Z-score. Red indicates a low expression level; green indicates a high expression level.* ***C-E)*** *Bioinformatic enrichment analyses (Gene Ontology, KEGG, Reactome) on the 25 DEPs.* ***C)*** *Top enriched terms.* ***D)*** *Network layout based on the pathways of the 25 DEPs. Each term is represented by a circle node, where its size is proportional to the number of input genes fall under that term, and its color represent its cluster identity. Terms with a similarity score > 0.3 are linked by an edge (the thickness of the edge represents the similarity score).* ***E)*** *Hierarchical Heatmap clustering comparing the UP (n = 11) and DOWN (n = 14) DEPs in terms of the significant process and pathways among the top Gene Ontology terms and colored according to P-values: terms with a P-value <0.01, a minimum count of 3, and an enrichment factor > 1.5. Colors from grey to brown indicate p-values from high to low; and grey cells indicate the lack of significant enrichment. The terms in blue color are specific to down-regulated proteins, those in red are for up-regulated proteins in organic meat and those in black are significant and common to both protein lists.*

The common and specific biological pathways to the two protein lists are evidenced. One enriched term was found to be common being “generation of precursor metabolites and energy (GO:0006091)”. Seven pathways were specific and down-regulated in organic samples: “sarcomere organization (GO:0045214)”, “actin cytoskeleton reorganization (GO:0031532)”, “Cellular responses to stress (R-HSA-2262752)”, “hemopoiesis (GO:0030097)”, “response to nutrient levels (GO:0031667)”, “response to mechanical stimulus (GO: 0009612)” and “mitochondrion organization (GO:0007005)”. However, one pathway was specific and up-regulated in organic meat being “organophosphate biosynthetic process (GO:0090407)”.

3.5 Impact of production system on pectoralis major chicken meat

The comparison of chicken meat reared following two farming and production methods (Antibiotic-free versus Organic), revealed a total of 59 differentially expressed proteins (DEPs) from which 3 were in common, being titin (TTN), immunoglobulin-like and fibronectin type III domain-containing protein 1 (IGFN1) and the histone H2AFX (H2AFX) (Figure 6A). The former was reported as up-regulated in Ranger Classic when compared to Ross 308 from both production systems while IGFN1 and H2AFX were down-regulated in the same conditions.

The bioinformatics enrichment analyses on the 59 DEPs through Gene Ontology (GO), KEGG and Reactome databases are given in Figure 6B. It resulted that 20 cluster terms were significantly enriched mainly dominated by “myofibril assembly (GO:0030239)” and “structural molecule activity (GO:0005198)” followed by “metabolism of carbohydrates (R-HSA-71387)” and “phosphagen metabolic process (GO:0006599)”. These enriched cluster terms allowed to construct a process network of the pathways (Figure 6C). It confirmed the dominance of a sub-network related to structural molecules activity, muscle structure and contraction and another one related to metabolic processes and ATP hydrolysis activity.

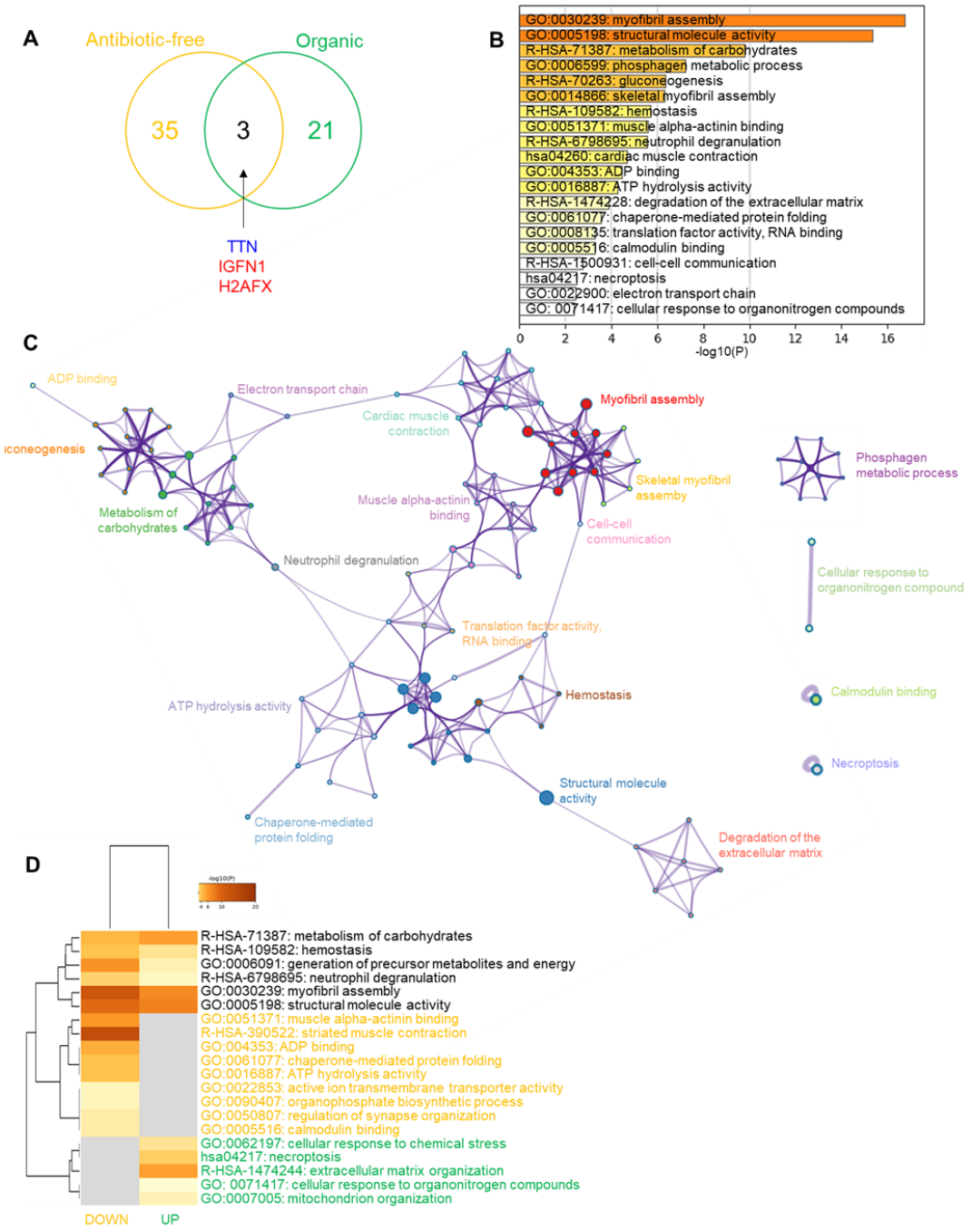

Figure 6. *Statistical and bioinformatics analyses on the differentially expressed proteins between Antibiotic-free (DEPs = 38) and Organic (DEPs=24) chicken post-mortem muscle proteome within the strains.* ***A)*** *Venn diagram highlights the number of common proteins identified. In blue down-regulated and in in red up-regulated proteins in Ranger Classic chickens respect to Ross 308.* ***B-D)*** *Bioinformatic enrichment analyses (Gene Ontology, KEGG, Reactome) on the 59 DEPs.* ***B)*** *Top enriched terms.* ***C)*** *Network layout based on the pathways of the 59 DEPs. Each term is represented by a circle node, where its size is proportional to the number of input genes fall under that term, and its color represent its cluster identity. Terms with a similarity score > 0.3 are linked by an edge (the thickness of the edge represents the similarity score).* ***D)*** *Hierarchical Heatmap clustering comparing the UP (n = 24) and DOWN (n = 38) DEPs in terms of the significant process and pathways among the top Gene Ontology terms and colored according to P-values: terms with a P-value <0.01, a minimum count of 3, and an enrichment factor > 1.5. Colors from grey to brown indicate p-values from high to low; and grey cells indicate the lack of significant enrichment. The terms in yellow color are specific to down-regulated proteins, those in green are for up-regulated proteins in organic chickens and those in black are significant and common to both protein lists.*

The comparison by means of a heatmap of the up- and down-regulated proteins in terms of enriched terms is given in Figure 6D. The common and specific biological pathways to the two protein lists are evidenced. Six enriched terms were found to be common being "metabolism of carbohydrates (R-HSA-71387)", "hemostasis (R-HSA-109582)", "generation of precursor metabolites and energy (GO:0006091)", "neutrophil degranulation (R-HSA-6798695)", "myofibril assembly (GO:0030239)" and "structural molecule activity (GO:0005198)". Nine pathways were specific and down-regulated in organic samples: "muscle alpha-actinin binding (GO:0051371)", "striated muscle contraction (R-HSA-390522)", "ADP binding (GO:004353)", "chaperone-mediated protein folding (GO:0061077)", "ATP hydrolysis activity (GO:0016887)", "active ion transmembrane transporter activity (GO:0022853)", "organophosphate biosynthetic process (GO:0090407)", "regulation of synapse organization (GO:0050807)" and "calmodulin binding (GO:0005516)". However, five pathways were specific and up-regulated in organic meat being "cellular response to chemical stress (GO:0062197)", "necroptosis (hsa04217)", "extracellular matrix organization (R-HSA-1474244)", "cellular response to organonitrogen compounds (GO: 0071417)" and "mitochondrion organization (GO:0007005)"

3.6 Impact of chicken strain on pectoralis major meat

The comparison of chicken meat from two different chicken strains (Ranger Classic versus Ross 308), revealed a total of 61 differentially expressed proteins (DEPs) from which 8 were in common, being paired amphipathic helix protein Sin3a (SIN3A), putative tubulin-like protein alpha-4B (TUBA4B), cytochrome b-c1 complex subunit 2 (UQCRC2), creatine kinase M-type (CKM), voltage-dependent anion-selective channel protein 1 (VDAC1), pyruvate kinase (PKLR), creatine kinase U-type (CKMT1B) and troponin T (TNNT3) (Figure 7A).

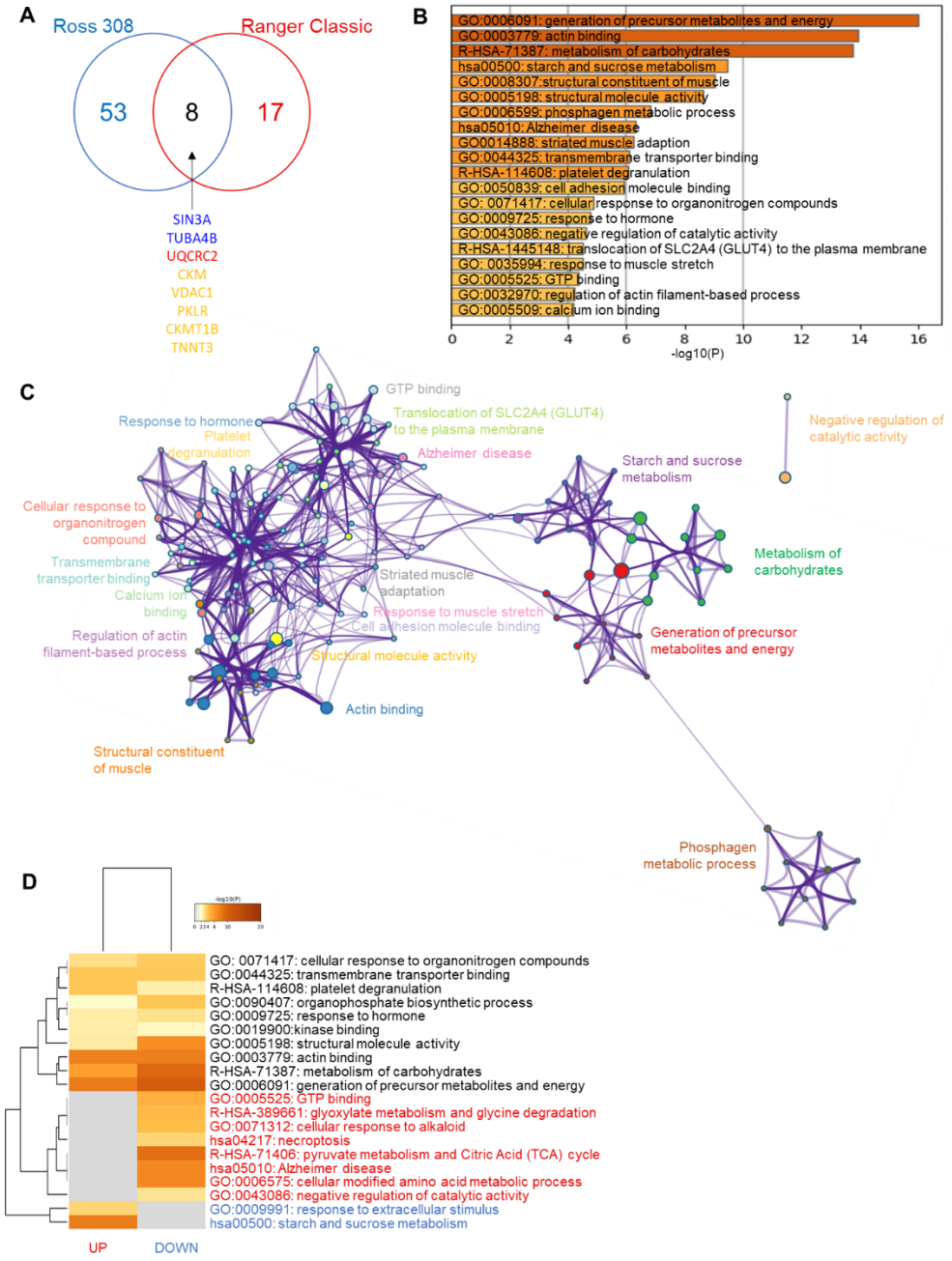

Figure 7. *Statistical and bioinformatics analyses on the differentially expressed proteins between Ross 308 (DEPs = 61) and Ranger Classic (DEPs=25) chicken post-mortem muscle proteome within the farming methods.* ***A)*** *Venn diagram highlights the number of common proteins identified. Gene names highlighted in blue are down-regulated and in red are up-regulated in organic samples respect to antibiotic-free, while proteins in yellow follow different directions.* ***B-D)*** *Bioinformatic enrichment analyses (Gene Ontology, KEGG, Reactome) on the 78 DEPs.* ***B)*** *Top enriched terms.* ***C)*** *Network layout based on the pathways of the 78 DEPs. Each term is represented by a circle node, where its size is proportional to the number of input genes fall under that term, and its color represent its cluster identity. Terms with a similarity score > 0.3 are linked by an edge (the thickness of the edge represents the similarity score).* ***D)*** *Hierarchical Heatmap clustering comparing the UP (n = 34) and DOWN (n = 49) DEPs in terms of the significant process and pathways among the top Gene Ontology terms and colored according to P-values: terms with a P-value <0.01, a minimum count of 3, and an enrichment factor > 1.5. Colors from grey to brown indicate p-values from high to low; and grey cells indicate the lack of significant enrichment. The terms in blue color are specific to down-regulated proteins, those in red are for up-regulated proteins in Ranger Classic samples and those in black are significant and common to both protein lists*

SIN3A and TUBA4B were reported as up-regulated while UQCRC2 was down-regulated in organic samples, the other reported different trends depending on production system.

The bioinformatics enrichment analyses on the 61 DEPs through Gene Ontology (GO), KEGG and Reactome databases are given in Figure 7B. It resulted that 20 cluster terms were significantly enriched mainly dominated by "generation of precursor metabolites and energy (GO:0006091)", "actin binding (GO:0003779)" and metabolism of carbohydrates (R-HSA-71387) followed by "starch and sucrose metabolism (hsa00500)", structural constituent of muscle (GO:0008307)", "structural molecule activity (GO:0005198)" and "phosphagen metabolic process (GO:0006599)" as top 7 terms. These enriched cluster terms allowed to construct a process network of the pathways (Figure 7C). It confirmed the dominance of a sub-network related to metabolic processes, structural molecules, and actin binding activities.

The comparison by means of a heatmap of the up- and down-regulated proteins in terms of enriched terms is given in Figure 7D. The common and specific biological pathways to the two protein lists are evidenced. Ten enriched terms were found to be common being "cellular response to organonitrogen compounds (GO: 0071417)", "transmembrane transported binding (GO:0044325)", "platelet degranulation (R-HAS-114608)", "organophosphate biosynthetic process (GO:0090407)", "response to hormone (GO:0009725)", "kinase binding (GO:0019900)", "structural molecule activity (GO:0005198)", "actin binding (GO:0003779)", "metabolism of carbohydrates (R-HSA-71387)" and "generation of precursor metabolites and energy (GO:0006091)". Eight pathways were specific and up-regulated in Ranger Classic meat being "GTP binding (GO: 0005525)", "glyoxylate metabolism and glycine degradation (R-HSA-389661)", " cellular response to alkaloid (GO:0071312)", "necroptosis (hsa04217)", "pyruvate metabolism Citric Acid (TCA) cycle (R-HSA-71406)", "Alzheimer disease (hsa05010)", "cellular modified amino acid metabolic

process (GO:0006575)" and "negative regulation of catalytic activity (GO:0043086)". However, two pathways were specific and down-regulated in Ranger Classic being: "response to extracellular stimulus (GO:0009991)" and "starch and sucrose metabolism (hsa00500)".

4. Discussion

In recent years and as stated in the different sections of this thesis including the chapter 4, there has been growing interest in the discovery of biomarkers for chicken meat quality and authenticity. Biomarkers are measurable indicators that can be used to assess the quality or authenticity. In the case of chicken meat, the aim of identification of biomarkers in the context of this study is to identify specific proteins or peptides that are associated with differences in chicken farming systems or strains differences. Proteomics is confirmed in this study to be a powerful tool that allows to study the entire complement of proteins present in chicken samples. The discovery of biomarkers for chicken meat quality and authenticity has important implications for the poultry industry. By using biomarkers to assess the quality and authenticity of chicken meat, producers can ensure that their products meet certain standards and are of consistent high quality. This can help to build consumer confidence and increase demand for chicken meat products. In addition to their potential use in quality control, the biomarkers for chicken meat quality and authenticity can also be used to detect food fraud. Food fraud is the intentional misrepresentation of a food product for financial gain, and it can have serious consequences for public health and safety. By using biomarkers to authenticate chicken meat products, regulators and law enforcement officials can detect and prevent food fraud, ensuring that consumers receive what they pay for. Taken all together, the discovery of biomarkers for chicken meat quality and authenticity has the potential to improve the quality and safety of chicken meat products, increase consumer confidence, and prevent food fraud. The findings presented above, revealed new pathways

and insights thanks to the shotgun proteomics approach used to understand differences between the two production systems or the two strains.

First, the comparison of the muscle proteome of Ranger Classic (ARA) and Ross 308 (ARO), that were reared under an antibiotic-free production system revealed 38 potential biomarkers between the two strains. The principal component analysis together with a hierarchical clustering analysis using a heatmap allowed a clear separation, therefore suggesting the robustness of the biomarkers. For this list of proteins, 11 cluster terms were significantly enriched, with the "striated muscle contraction pathway" being the most dominant, followed by "ATP metabolic process" and "formation of tubulin folding intermediates." These enriched cluster terms allowed to construct the first process network of the pathways, which confirmed the dominance of a sub-network related to muscle structure, contraction, and associated pathways, as well as another one related to ATP and energy metabolic processes. These are specific molecular signatures that are worthy of validation under other sampling conditions and experimental design before the setup of a targeted proteomics for the evaluation for the list of biomarkers. Overall, the results suggest that there are significant differences in the muscle proteome between the two chicken strains, which may be related to their genetic background and rearing conditions. The results also provide insights into the potential pathways that are associated with these differences, such as muscle contraction and energy metabolism. This study may have important implications for the poultry industry, as it highlights the importance of genetic selection and management practices in improving the quality of chicken meat. However, further studies are needed to confirm these findings and explore their practical implications in more detail.

Second, the comparison of the muscle proteome of Ranger Classic (ORA) and Ross 308 (ORO), reared under an organic production system revealed 24 putative biomarkers between the two strains. However, unlike the previous comparison between the same strains reared

under an antibiotic-free production system, there was no clear separation between the two groups, with only a few individuals being distinguishable from each other. This was further confirmed by the heatmap analysis, which showed a significant overlap between the two groups. Bioinformatics enrichment analyses revealed 11 significantly enriched cluster terms, with the dominant terms being "sarcomere organization" and "structural molecule activity," followed by "metabolism of carbohydrates" and "cellular response to stress." The enriched cluster terms allowed the construction of a process network of pathways, which confirmed the dominance of a sub-network of sarcomere organization and structural molecule activity pathways and another one related to cellular response to stress processes. The comparison of the few up- and down-regulated proteins in terms of enriched terms using a heatmap revealed that one enriched term was common to both protein lists, which was "cellular response to stress." However, three pathways were specific and down-regulated in Ranger Classic, including "sarcomere organization," "structural molecule activity," and "metabolism of carbohydrates." On the other hand, three pathways were specific and up-regulated in Ranger Classic, including "brain development," "mitochondrion organization," and "cellular response to chemical stress."

Third, the comparison of the muscle proteome of Ross 308 chicken meat reared under two different farming and production methods, Antibiotic-free (ARO) and Organic (ORO) identified 61 differentially expressed proteins (DEPs) between the two groups, that allowed a clear separation of ORO from ARO. The hierarchical clustering analysis through the heatmap using the individual abundances of the DEPs further supported this finding. The top enriched cluster terms were related to “carbonic metabolism,” “pyruvate metabolic process,” “pyruvate metabolism and Citric Acid (TCA) cycle,” “Muscle system process,” “actin binding,” and “structural constituent of cytoskeleton,” among others. These enriched cluster terms allowed the construction of a process network of the pathways.

It appears in comparison to the above that the two different rearing systems (organic and antibiotic-free) led to significant differences in the microbial communities, metabolites, and proteome of the chicken meat. In terms of metabolites, the organic system led to higher levels of amino acids, while the antibiotic-free system led to higher levels of metabolites associated with stress response such as glutamate and glutamine. In terms of the proteome, the organic system led to differential expression of proteins involved in various metabolic pathways such as carbon and pyruvate metabolism, as well as pathways related to muscle system and structure. Overall, these findings suggest that different rearing systems can have a significant impact on the microbiota, metabolites, and proteome of chicken meat, which may have implications for the nutritional and sensory properties of the meat, as well as for food safety.

Finally, the comparison of the muscle proteome of Ranger Classic chicken meat reared under antibiotic-free (ARA) and organic (ORA) production systems revealed 25 putative biomarkers with an acceptable level of separation using both PCA or clustering analyses. In this comparison, the “generation of precursor metabolites and energy (GO:0006091)” and “sarcomere organization (GO:0045214)” were the most changing molecular signatures. These results suggest that the organic production system may affect the energy metabolic processes and muscle structure of Ranger Classic chickens. The process network of the pathways further confirmed the dominance of the sub-network related to muscle structure and associated pathways and another one related to energy metabolic processes. Taken together, the results suggest that the organic production system may significantly affect the muscle proteome of Ranger Classic chickens, particularly in terms of energy metabolic processes, muscle structure, and actin cytoskeleton organization. However, further studies are needed to fully understand the molecular mechanisms underlying these differences and their potential impact on meat quality and nutritional value.

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Supplementary material

Table S1. List of the muscle proteins (n = 38) identified as being significantly different among the two antibiotic-free groups and their correlation with chicken strain expressed by ANOVA test results. Ross 308 up-regulated proteins are highlighted in blue while Ranger Classic up-regulated proteins are highlighted in red.

Uniprot ID	Gene names	Protein full name	Biological pathway (GO,KEGG,Reactome)	Antibiotic-free Ross 308 (ARO)	Antibiotic-free Ranger Classic (ARA)	P-value	
P68139	ACTA1	Actin, alpha skeletal muscle	GO:0003779; GO:0005198; hsa04260; GO:0014888; GO:0014866; GO:0071417	5193.84	3720.37	0.012	DOWN
P20111	ACTN2	Alpha-actinin-2	GO:0003779; GO:0005198; R-HSA-114608; GO:0050839; GO:0071417; GO:0044325; GO:0032970	346857.00	123053.78	0.043	
P53449	ALDOC	Fructose-bisphosphate aldolase C	GO:0006091; R-HSA-71387; R-HSA-6798695	446298.41	270944.50	0.011	
F1NG97	AMPD3	AMP deaminase 3	R-HSA-71387; R-HSA-6798695; GO:0106380	258860.05	171372.65	0.002	
Q6EE31	CCT8	T-complex protein 1 subunit theta	GO:0005198; R-HSA-6798695; R-HSA-114608; GO:0050839; R-HSA-8876725; GO:0032970	592817.41	295685.72	0.041	
P00565	CKM	Creatine kinase M-type	GO:0006599; GO:0106380	685527.54	359553.83	0.033	
F1NXR0	CKMT1B	Creatine kinase U-type, mitochondrial	GO:0006599; GO:0106380	421469.41	179733.12	0.010	
Q8JFP1	EIF4A2	Eukaryotic initiation factor 4A-II	GO:0005198; R-HSA-8876725	482878.70	202148.60	0.005	
O57391	ENO2	Gamma-enolase	GO:0006091; R-HSA-71387	289222.00	181901.70	0.000	
A0A1L1RNY8	H2AFX	Histone H2AX	GO:0005198	442175.30	151899.10	0.010	
F1P5V6	MYL3	Myosin light chain 3	GO:0003779; GO:0005198; hsa04260; GO:0014888	522544.50	194710.90	0.019	
A0A3Q2U6G6	PDLIM5	PDZ and LIM domain protein 5	GO:0003779; GO:0005198; GO:0019900; GO:0051371; GO:0050839; R-HSA-8876725	505248.10	231951.70	0.011	
F1NU17	PGK2	Phosphoglycerate kinase 2	GO:0006091; R-HSA-71387; GO:0005198	658055.81	367274.94	0.010	
P12620	TNNT3	Troponin T, fast skeletal muscle	GO:0003779; hsa04260	462530.45	283870.40	0.018	
A6BLM8	TTN	Titin	GO:0003779; GO:0005198; GO:0019900 ; hsa04260 ; GO:0051371; R-HSA-114608; GO:0014866	610668.70	276897.40	0.032	

A0A1D5NW27	TUBA1A	Tubulin alpha-1A chain	GO:0005198; R-HSA-114608; R-HSA-8876725	466097.30	180449.70	0.012	
F1NW97	TUBA4B	Putative tubulin-like protein alpha-4B	GO:0005198	538878.60	245302.60	0.008	
A0A1D5P5Z0	TUBA8A	Tubulin alpha-8 chain	GO:0005198	369316.70	193790.88	0.038	
F1NVB3	UNC45B	Protein unc-45 homolog B	GO:0003779	726137.28	420848.14	0.013	
Q5ZMU9	VCP	Transitional endoplasmic reticulum ATPase	GO:0006091; R-HSA-71387; GO:0005198; R-HSA-6798695; GO:0050839; R-HSA-8876725; GO:0014866; GO:0044325; GO:0032970	250466.50	144055.90	0.009	
P21265	ADSL	Adenylosuccinate lyase	GO:0006091; R-HSA-71387; GO:0006107; GO:0106380	269193.20	633215.60	0.030	UP
Q03669	ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	GO:0003779; GO:0005198; hsa04260 ; GO:0051371; R-HSA-114608; R-HSA-8876725; GO:0014888; GO:0071417; GO:0044325; GO:0032970	334101.60	585537.10	0.002	
A0A1L1RWR8	DEUP1	Deuterosome assembly protein 1	GO:0003779; GO:0005198	72752.18	578477.84	0.006	
A0A1D5PAA8	GYG1	Glycogenin-1	GO:0006091; R-HSA-71387; R-HSA-6798695; R-HSA-8982491	149661.20	428108.56	0.042	
Q00649	HSPB1	Heat shock protein beta-1	GO:0005198; GO:0019900; R-HSA-8876725	161663.08	313624.30	0.018	
A0A1D5PUM7	IGFN1	Immunoglobulin-like and fibronectin type III domain-containing protein 1	GO:0050839	388195.90	601630.80	0.001	
A0A1D5P909	LDB3	LIM domain-binding protein 3	GO:0003779; GO:0019900; GO:0051371	473116.10	789218.20	0.023	
F1NM49	MYH15	Myosin-15	GO:0003779	376126.00	627529.00	0.001	
A0A1D5NZY9	MYH7B	Myosin-7B	GO:0003779	369536.55	579407.60	0.032	
A0A1D5PSF9	MYOM1	Myomesin-1	GO:0003779; GO:0005198; GO:0019900 ; R-HSA-114608	143144.77	205990.60	0.003	
	NEB	Nebulin	GO:0003779; GO:0005198; GO:0032970	369448.30	548329.56	0.018	
Q5ZM25	OLA1	Obg-like ATPase 1	R-HSA-71387; GO:0005198; R-HSA-114608; GO:0050839; R-HSA-8876725	156863.62	429237.39	0.045	
A0A1D5PA64	PHKB	Phosphorylase b kinase regulatory subunit beta	GO:0006091; R-HSA-71387; R-HSA-8982491	303674.12	595786.50	0.003	
O42281	PRPH	Peripherin-2	GO:0003779; GO:0005198	293783.59	623013.41	0.010	
A0A3S5ZPB7	SLC25A3	Phosphate carrier protein, mitochondrial	GO:0044325	196028.90	260669.00	0.042	
P04268	TPM1	Tropomyosin alpha-1 chain	GO:0003779; GO:0005198; hsa04260; GO:0032970	317166.35	610041.70	0.009	
A0A1L1RJP8	TUBA4A	Tubulin alpha-4A chain	GO:0005198; GO:0019900; R-HSA-114608	223348.62	562225.60	0.041	
F1NAC6	UQCRC1	Cytochrome b-c1 complex subunit 1, mitochondrial	GO:0006091; R-HSA-71387; GO:0005198; hsa04260; R-HSA-8876725	222294.76	511737.61	0.036	

Table S2. List of the muscle proteins (n = 24) identified as being significantly different among the two organic groups and their correlation with chicken strain expressed by ANOVA test results. Ross 308 up-regulated proteins are highlighted in blue while Ranger Classic up-regulated proteins are highlighted in red.

Uniprot ID	Gene names	Protein full name	Biological pathway (GO,KEGG,Reactome)	Organic Ross 308 (ORO)	Organic Ranger Classic (ORA)	P-value	
P21566	CFL2	Cofilin-2	GO:0003779; GO:0014888; GO:0071417; GO:0032970	538206.10	258517.80	0.004	DOWN
P20785	COL6A1	Collagen alpha-1(VI) chain	GO:0005198; GO:0071417	285044.10	139750.80	0.006	
Q5ZKU1	DCN	DCN1-like protein 1	R-HSA-71387; GO:0005198; GO:0044325; GO:0032970	341216.18	186996.06	0.037	
A0A1L1RRR1	EEF1A1	Elongation factor 1-alpha 1	GO:0005198; R-HSA-6798695; GO:0019900; R-HSA-8876725	354577.58	137053.69	0.040	
	FLNC	Filamin-C	GO:0003779; GO:0005198	489836.94	193711.73	0.015	
P00504	GOT1	Aspartate aminotransferase, cytoplasmic	R-HSA-71387; R-HSA-8982491; GO:0006107; GO:0006599; GO:0071417	656310.31	286611.80	0.004	
A0A1D5P603	MYH1F		GO:0003779	341654.55	195709.87	0.004	
A0A1D5PJ08	NID1	Nidogen-1	GO:0005198	419749.87	81170.13	0.007	
E1BSN7	PYGB	Glycogen phosphorylase, brain form	GO:0006091; R-HSA-71387; R-HSA-6798695; R-HSA-8982491; GO:0006107	544039.60	285656.40	0.011	
A0A1L1RXA5	REEP5	Receptor expression-enhancing protein 5	GO:0050839	363430.15	100133.85	0.024	
A6BLM8	TTN	Titin	GO:0003779; GO:0005198; GO:0019900 ; hsa04260 ; GO:0051371; R-HSA-114608; GO:0014866	623323.87	191846.79	0.026	
G1K338	TUBB2A	Tubulin beta-2A chain	GO:0005198; R-HSA-114608	419855.60	212957.00	0.011	
F1NYB1	TUBB4B	Tubulin beta-4B chain	GO:0005198; R-HSA-6798695; R-HSA-114608	471994.90	309486.40	0.000	
F1NT57	AKR1B1	Aldo-keto reductase family 1 member B1	GO:0006091; R-HSA-71387	127348.88	473654.25	0.005	UP
P11009	CKMT2	Creatine kinase S-type, mitochondrial	GO:0003779; GO:0006599; GO:0106380	186108.97	611575.14	0.016	
F1N9H4	EEF1A2	Elongation factor 1-alpha 2	GO:0005198; GO:0019900; R-HSA-8876725	385663.35	561549.60	0.024	
A0A1L1RNY8	H2AFX	Histone H2AX	GO:0005198	456635.90	619216.40	0.027	
A0A1D5PPF8	HSPA4	Heat shock 70 kDa protein 4	GO:0005198; R-HSA-8876725	258549.40	347831.40	0.002	
A0A1D5PF99	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein	R-HSA-71387; GO:0005198	226036.00	346709.60	0.037	
A0A1D5PUM7	IGFN1	Immunoglobulin-like and fibronectin type III domain-containing protein 1	GO:0050839	141045.74	413900.93	0.020	
A0A1D5PUR6	MYH1A		GO:0003779	206711.20	425150.10	0.036	
	PLEC	Plectin	GO:0006091; GO:0003779; GO:0005198; hsa04260; GO:0050839; GO:0014888; GO:0014866	255340.60	464978.40	0.014	

E1BX21	SIN3A	Paired amphipathic helix protein Sin3a	GO:0005198; R-HSA-114608; GO:0050839; GO:0071417; GO:0032970	431309.46	777139.10	0.000	
F6T197	VDAC2	Voltage-dependent anion-selective channel protein 2	GO:0005198; GO:0050839; GO:0032970	315665.88	668837.08	0.034	

Table S3. List of the muscle proteins (n = 61) identified as being significantly different among the two Ross 308 groups and their correlation with farming method expressed by ANOVA test results. Antibiotic-free raised chicken up-regulated proteins are highlighted in blue while organic up-regulated proteins are highlighted in red.

Uniprot ID	**Gene names**	**Protein full name**	**Biological pathway (GO,KEGG,Reactome)**	**Antibiotic-free Ross 308 (ARO)**	**Organic Ross 308 (ORO)**	**P-value**	
P68139	ACTA1	Actin, alpha skeletal muscle	GO:0003779; GO:0005198; hsa04260; GO:0014888; GO:0014866; GO:0071417	3518.70	1798.28	<0,0001	DOWN
A0A1D5NV17	ACTBL2	Beta-actin-like protein 2	GO:0003779; GO:0005198; GO:0019900	5481.60	2674.64	<0,0001	
P53449	ALDOC	Fructose-bisphosphate aldolase C	GO:0006091; R-HSA-71387; R-HSA-6798695	446298.41	311626.20	0.034	
P81073	AMPD1	AMP deaminase 1, EC 3.5.4.6	R-HSA-71387; GO:0106380	496219.30	226036.00	0.001	
Q5ZHV4	BPGM	Bisphosphoglycerate mutase	GO:0006091; R-HSA-71387	417361.70	173964.30	<0,0001	
E1BVC2	CAND2	Cullin-associated NEDD8-dissociated protein 2	GO:0050839	684903.70	443049.25	0.016	
P19204	CASQ2	Calsequestrin-2	GO:0003779; hsa04260; GO:0014866; GO:0071417; GO:0044325	459951.40	198136.67	<0,0001	
P00565	CKM	Creatine kinase M-type	GO:0006599; GO:0106380	632794.30	381735.40	0.000	
F1NXR0	CKMT1B	Creatine kinase U-type, mitochondrial	GO:0006599; GO:0106380	434811.32	208098.56	0.042	
P15989	COL6A3	Collagen alpha-3(VI) chain	GO:0003779; GO:0005198	776680.40	336684.27	0.043	
Q90705	EEF2	Elongation factor 2	GO:0005198; R-HSA-6798695; GO:0019900; GO:0050839; R-HSA-8876725	525043.60	287928.80	0.042	
Q8JFP1	EIF4A2	Eukaryotic initiation factor 4A-II	GO:0005198; R-HSA-8876725	482878.70	282627.70	0.042	
A0A1L1RZK1	GLO1	Lactoylglutathione lyase	GO:0006091; R-HSA-71387; GO:0006599	410627.08	182377.13	<0,0001	
P00508	GOT2	Aspartate aminotransferase, mitochondrial	R-HSA-71387; GO:0006107; GO:0006599	522038.10	226165.80	<0,0001	
	GSTM3	Glutathione S-transferase Mu 3	GO:0006599	317307.50	155806.10	0.001	

Q5ZME2	MDH1	Malate dehydrogenase, cytoplasmic	GO:0006091; R-HSA-71387; GO:0006107	380724.06	174383.70	0.004	
E1BVT3	MDH2	Malate dehydrogenase, mitochondrial	GO:0006091; R-HSA-71387; GO:0006107; GO:0014866	724742.40	278803.80	<0,0001	
F1NM49	MYH15	Myosin-15	GO:0003779	404473.45	1311.35	0.008	
A0A3Q2U5Z0	NME1	Nucleoside diphosphate kinase A	R-HSA-71387; GO:0005198; GO:0106380	454966.15	211673.50	0.008	
Q9PU47	PDLIM3	PDZ and LIM domain protein 3	GO:0003779; GO:0051371	540467.60	330169.60	0.001	
A0A1L1S091	PEBP1	Phosphatidylethanolamine-binding protein 1	GO:0019900	322449.91	172216.62	0.008	
F1NU17	PGK2	Phosphoglycerate kinase 2	GO:0006091; R-HSA-71387; GO:0005198	658055.81	434969.85	0.038	
A0A1D5PWG8	PHPT1	14 kDa phosphohistidine phosphatase	GO:0044325; GO:0032970	870225.82	487973.18	0.011	
A0A1D5P9V0	PKLR	Pyruvate kinase PKLR	GO:0006091; R-HSA-71387; R-HSA-8982491; GO:0106380; GO:0014866; GO:0071417	618018.80	431001.00	0.022	
	PYGM	Glycogen phosphorylase, muscle form	GO:0006091; R-HSA-71387; R-HSA-8982491; GO:0006107	674530.56	431309.46	0.049	
A0A3Q2U787	RTN3	Reticulon-3	GO:0005198	301042.65	19590.95	0.011	
E1BX21	SIN3A	Paired amphipathic helix protein Sin3a	GO:0005198; R-HSA-114608; GO:0050839; GO:0071417; GO:0032970	558553.30	119535.52	0.000	
Q01841	TGM2	Protein-glutamine gamma-glutamyltransferase 2	GO:0005198; hsa04260; GO:0106380; GO:0071417	708372.91	322517.65	0.033	
P12620	TNNT3	Troponin T, fast skeletal muscle	GO:0003779; hsa04260	549138.80	161386.11	<0,0001	
P04268	TPM1	Tropomyosin alpha-1 chain	GO:0003779; GO:0005198; hsa04260; GO:0032970	488546.06	152785.21	0.027	
A6BLM8	TTN	Titin	GO:0003779; GO:0005198; GO:0019900 ; hsa04260 ; GO:0051371; R-HSA-114608; GO:0014866	610668.70	272187.90	0.028	
A0A1D5NW27	TUBA1A	Tubulin alpha-1A chain	GO:0005198; R-HSA-114608; R-HSA-8876725	466097.30	190504.90	0.039	
F1NW97	TUBA4B	Putative tubulin-like protein alpha-4B	GO:0005198	538878.60	292744.30	0.035	
G1K338	TUBB2A	Tubulin beta-2A chain	GO:0005198; R-HSA-114608	503102.68	262372.00	0.029	
F1NVB3	UNC45B	Protein unc-45 homolog B	GO:0003779	745930.45	411252.01	0.015	
F6T197	VDAC2	Voltage-dependent anion-selective channel protein 2	GO:0005198; GO:0050839; GO:0032970	589384.80	181964.80	<0,0001	
Q5ZKJ2	YWHAH	14-3-3 protein eta	GO:0003779; GO:0005198; GO:0050839; GO:0071417; GO:0044325	562630.50	337813.90	0.033	
Q8AYI3	ACO2	Aconitate hydratase, mitochondrial	GO:0006091; R-HSA-71387; GO:0006107	173259.86	414462.56	0.048	UP
Q03669	ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	GO:0003779; GO:0005198; hsa04260 ; GO:0051371; R-HSA-114608; R-HSA-8876725; GO:0014888; GO:0071417; GO:0044325; GO:0032970	334101.60	552791.41	0.007	
E1BSH9	BHMT2	S-methylmethionine--homocysteine S-methyltransferase BHMT2	R-HSA-71387; GO:0006107; GO:0006599; GO:0106380	273228.80	472724.30	0.039	
P11009	CKMT2	Creatine kinase S-type, mitochondrial	GO:0003779; GO:0006599; GO:0106380	262859.69	529156.70	0.041	

A0A1L1RWR8	DEUP1	Deuterosome assembly protein 1	GO:0003779; GO:0005198	43697.24	439782.17	0.028
Q5ZM32	DLD	Dihydrolipoyl dehydrogenase, mitochondrial	GO:0006091; R-HSA-71387; GO:0006107; GO:0006599; GO:0106380; GO:0071417; GO:0044325	184910.10	435918.63	0.038
A0A1D5PAA8	GYG1	Glycogenin-1	GO:0006091; R-HSA-71387; R-HSA-6798695; R-HSA-8982491	126889.36	434821.44	0.045
	H2BC4	Histone H2B type 1-C/E/F/G/I	GO:0005198	230214.31	537354.10	0.025
O73885	HSPA8	Heat shock cognate 71 kDa protein	R-HSA-71387; GO:0005198; R-HSA-6798695; R-HSA-114608; GO:0050839; R-HSA-8876725; GO:0071417; GO:0032970	101761.66	572392.70	<0,0001
A0A1D5PF99	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein	R-HSA-71387; GO:0005198	292212.80	561055.20	0.044
E1C1U8	KLHL40	Kelch-like protein 40	GO:0003779; GO:0014888	230687.43	464123.79	0.048
P02565	MYH1	Myosin-1	GO:0003779	2058.47	4019.02	<0,0001
A0A1D5NZY9	MYH7B	Myosin-7B	GO:0003779	369536.55	667520.37	0.005
	OGDH	2-oxoglutarate dehydrogenase, mitochondrial	GO:0006091; R-HSA-71387; GO:0006107; GO:0006599	285429.94	583421.13	0.046
Q5ZM25	OLA1	Obg-like ATPase 1	R-HSA-71387; GO:0005198; R-HSA-114608; GO:0050839; R-HSA-8876725	179198.98	523400.29	0.018
A0A3Q2U321	POSTN	Periostin	GO:0005198; GO:0050839	192559.50	565534.50	0.006
A0A3Q3AT29	PPIA	Peptidyl-prolyl cis-trans isomerase A	R-HSA-71387; R-HSA-6798695; R-HSA-114608; GO:0050839	254474.46	613961.90	0.009
Q9PU45	RDX	Radixin	GO:0003779; GO:0005198; R-HSA-114608; GO:0050839; GO:0032970	238820.78	536190.37	0.012
A0A1D5PPG9	RTN4	Reticulon-4	GO:0003779; GO:0005198; hsa04260; R-HSA-114608; GO:0050839; R-HSA-8876725; GO:0071417; GO:0032970	357547.40	620211.50	<0,0001
A0A1D5PCL0	RYR3	Ryanodine receptor 3	GO:0003779; GO:0005198; GO:0014866; GO:0071417	308914.70	483440.20	0.008
P19352	TPM2	Tropomyosin beta chain	GO:0003779; GO:0005198; hsa04260	1488.28	262045.60	<0,0001
P22695	UQCRC2	Cytochrome b-c1 complex subunit 2, mitochondrial	GO:0006091; R-HSA-71387; GO:0005198; hsa04260	240372.58	694470.92	0.002
A0A1D5NTT4	VDAC1	Voltage-dependent anion-selective channel protein 1	GO:0006091; R-HSA-71387; GO:0005198; GO:0019900; GO:0044325; GO:0032970	164431.08	438213.48	0.009
Q5F3W6	YWHAG	14-3-3 protein gamma	GO:0005198; GO:0019900; GO:0071417	119266.54	602488.29	<0,0001

Table S4. List of the muscle proteins (n = 25) identified as being significantly different among the two Ranger Classic groups and their correlation with farming method expressed by ANOVA test results. Antibiotic-free raised chicken up-regulated proteins are highlighted in blue while organic up-regulated proteins are highlighted in red.

Uniprot ID	Gene names	Protein full name	Biological pathway (GO,KEGG,Reactome)	Antibiotic-free Ranger Classic (ARA)	Organic Ranger Classic (ORA)	P-value	
P20111	ACTN2	Alpha-actinin-2	GO:0003779; GO:0005198; R-HSA-114608; GO:0050839; GO:0071417; GO:0044325; GO:0032970	614671.10	561549.60	0.030	DOWN
A0A1D5PF87	AGL	Glycogen debranching enzyme	GO:0006091; R-HSA-71387; R-HSA-6798695; R-HSA-8982491	154445.00	88194.82	0.018	
P19121	ALB	Albumin	GO:0005198; GO:0006107; R-HSA-114608	419592.93	123622.16	0.010	
A0A3Q2TYY9	FLNA	Filamin-A	GO:0003779; GO:0005198; GO:0019900; hsa04260; R-HSA-114608; GO:0050839; GO:0071417; GO:0044325; GO:0032970	315903.69	189927.75	0.005	
A0A1L1RQ91	GPI	Glucose-6-phosphate isomerase	GO:0006091; R-HSA-71387; R-HSA-6798695; R-HSA-8982491; hsa04260; R-HSA-8876725; GO:0014866	195281.35	100072.97	0.030	
O93510	GSN	Gelsolin	GO:0003779; R-HSA-6798695; GO:0019900; hsa04260; GO:0050839; GO:0014888; GO:0014866; GO:0032970	506738.16	235510.84	0.022	
A0A1D5PPF8	HSPA4	Heat shock 70 kDa protein 4	GO:0005198; R-HSA-8876725	416126.80	105971.49	0.032	
E1C6E6	LRBA	Lipopolysaccharide-responsive and beige-like anchor protein	GO:0003779; GO:0019900; GO:0032970	254612.36	170943.39	0.049	
E1BU93	MYOZ1	Myozenin-1	GO:0003779; GO:0014888	450276.30	258517.80	0.041	
	PLEC	Plectin	GO:0006091; GO:0003779; GO:0005198; hsa04260; GO:0050839; GO:0014888; GO:0014866;	635035.42	410295.38	0.038	
E1BX21	SIN3A	Paired amphipathic helix protein Sin3a	GO:0005198; R-HSA-114608; GO:0050839; GO:0071417; GO:0032970	567472.93	91859.94	0.000	
A0A3Q3ALX3	TBX21	T-box transcription factor TBX21	GO:0050839	315995.84	188573.66	0.041	
F1NW97	TUBA4B	Putative tubulin-like protein alpha-4B	GO:0005198	366644.70	107062.70	0.007	
A0A1D5NTT4	VDAC1	Voltage-dependent anion-selective channel protein 1	GO:0006091; R-HSA-71387; GO:0005198; GO:0019900; GO:0044325; GO:0032970	804991.45	358059.12	0.005	
P00565	CKM	Creatine kinase M-type	GO:0006599; GO:0106380	359553.83	578108.90	0.020	UP
F1NXR0	CKMT1B	Creatine kinase U-type, mitochondrial	GO:0006599; GO:0106380	186094.60	450563.40	0.017	
A0A1D5PUR6	MYH1A		GO:0003779	192353.60	668837.08	0.002	

A0A1D5P525	MYH1C		GO:0003779	152646.95	517431.30	0.001
A0A1D5NYC2	MYH1E		GO:0003779	3771.40	4349.28	0.019
F1P5V6	MYL3	Myosin light chain 3	GO:0003779; GO:0005198; hsa04260; GO:0014888	194710.90	324095.50	0.015
F1NN63	PGM1	Phosphoglucomutase-1	GO:0006091; R-HSA-71387; R-HSA-6798695; R-HSA-8982491; GO:0106380	309130.80	347831.40	0.005
A0A1D5P9V0	PKLR	Pyruvate kinase PKLR	GO:0006091; R-HSA-71387; R-HSA-8982491; GO:0106380; GO:0014866; GO:0071417	475008.92	817405.73	0.047
E1BSN7	PYGB	Glycogen phosphorylase, brain form	GO:0006091; R-HSA-71387; R-HSA-6798695; R-HSA-8982491; GO:0019900	708840.70	777139.10	0.023
P12620	TNNT3	Troponin T, fast skeletal muscle	GO:0003779; hsa04260	283870.40	335053.60	0.034
P22695	UQCRC2	Cytochrome b-c1 complex subunit 2, mitochondrial	GO:0006091; R-HSA-71387; GO:0005198; hsa04260	303427.53	679848.04	0.018

SECTION C
Results of the thesis

Chemometrics multivariate tools for the discovery of proteomics biomarkers of authenticity in organic and antibiotic-free chicken meat

Publication 5

Chemometrics multivariate tools for the discovery of proteomics biomarkers of authenticity in organic and antibiotic-free chicken meat

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Article in preparation for Journal of Agricultural and Food Chemistry

Abstract

Many factors such as the production systems and pre-slaughter stress can influence physiological and metabolic functions of the animals with consequent impact on poultry meat quality. Label free shotgun proteomics was used to analyse post-mortem *Pectoralis major* muscle proteome of Ross 308 and Ranger Classic chicken meat from an organic and antibiotic-free farming systems. More than 650 quantifiable proteins were identified, and PLS-DA statistical analysis provided a clear discrimination between the groups. Chicken strains were discriminated by 73 and 62 proteins within the antibiotic-free and organic production system respectively. The expression of 71 and 52 proteins was impacted by the production system within the Ross 308 and Ranger Classic chicken strains respectively. Variable Importance in the Projection (VIP) analysis reported several putative biomarkers of meat authenticity mainly related to muscle structure and energy metabolism pathways. This study is a significant step forward in elucidating the proteomic profile of chicken meat, which may provide opportunities for assessment of chicken authenticity.

Keywords: Poultry meat proteomics, shotgun proteomics, PLS-DA, protein biomarkers, meat authenticity

1. Introduction

In recent years, consumers have developed an ever-increasing interest in having clear and reliable information about the foods they consume. This aspect particularly influences the meat and meat products market. Not only the price and quality of the product, but also consumer's lifestyle, religious and health concerns, can impact on the choice of food product (Cooreman-Algoed et al. 2022). On this trend, more and more people prefer to buy and consume food from organic farming, produced according to the European Regulation 834/2007 (Commission 834/2007; Eurostat 2022). To meet these criteria, the meat industry is facing various challenges leading to the implementation of projects mainly related to sustainability or eco-friendly production systems with claims such as "One Welfare" (Rocchi et al. 2021). Meat authenticity parameters can be divided in animal characteristics (sex, breed, meat cuts, feed intake, geographical origin, animal age) and rearing conditions such as organic versus conventional meat (Kaygisiz et al. 2019). Authentication of organic products represents a central issue for the assessment of food quality and safety as the most common falsification is labelling conventional products as organic. The identification of non-organic meat and meat products is still not possible by common food analysis. These frauds are constantly growing and can lead to illegal economic gain and health risks for the consumers (Prache et al. 2021).

While these soft claims are generally beyond the scope of analytical chemistry, developing methodologies that can identify markers and/or signatures that can help make better decisions and guarantee the intrinsic/extrinsic qualities to consumers are highly welcomed.

From an analytical standpoint, several methodologies have been used to authenticate the geographical or the species origin of animal food products, but very few focused on organic/conventional meat discrimination (Ballin 2010; Zhang et al. 2021; Gagaoua 2022). One of the main restrictions in organic meat production is the use of veterinary drugs, so

analytical methods for quantifying any drug residues in animal tissues and fluids have been applied to investigate fraud (Kelly et al. 2006; Mainero Rocca et al. 2017). However, the mere absence of antibiotics is not a guarantee of compliance with organic guidelines, so this strategy alone cannot be used to authenticate chicken meat as organic.

Meat quality parameters (tenderness, color, pH, drip loss...), fat and protein contents were compared between organic and conventional meat. Organic chickens carcasses reported heavier breast and drumstick with lower percentages of abdominal fat. Organic muscles showed also lower pH, drip loss and cooking but higher lightness, n-3 PUFA and heme iron content than conventional chicken (Castellini et al. 2002b; Castellini et al. 2002a; Brunel et al. 2006; Bogosavljevic-Boskovic et al. 2010; Michalczuk et al. 2017). Moreover, flavour, juiciness, and texture were reported to be better in organic meat, though it was less tender than the conventional one (Grashorn & Serini 2006). These strategies revealed crucial from a consumer's standpoint but not conclusive to draw up markers to authenticate chicken meat as organic. Therefore, the development of high-throughput approaches is increasingly needed to discover unambiguous biomarkers of authenticity. In recent years, fast and reliable food omics strategies such as proteomics in conjunction with multivariate and machine learning data analyses are attracting great interest to investigate the main aspects of food quality and safety, including traceability and authenticity (Herrero et al. 2012; Gagaoua & Picard 2022). Thus, the present work is the first one designed to use data-independent acquisition – mass spectrometry proteomics (SWATH-MS: sequential window acquisition of all theoretical fragment ion spectra mass spectrometry) and chemometrics for the discrimination of meat from two chicken strains and two farming systems: organic versus antibiotic-free.

2. Materials and methods

2.1 Samples

A total of forty broiler breasts were applied to proteomic analysis in this study. Twenty of them were reared and slaughtered in an antibiotic-free inside ground farm and twenty following organic regulations. The antibiotic-free system featured a standard broiler inside-ground farming system, using concentrated feed and controlled housing (artificial light and climate control, automatic water, and feed supply) according to the Directive 2007/43/EC. The organic chicken samples were reared according to Commission Regulation No 848/2018 for organic systems for poultry and livestock production, using organic feed, controlled housing, having access to an outdoor area with the presence of pasture for at least one third of their life. Within each group, there were 10 Ross 308 and 10 Ranger Classic broilers. All the samples were collected in the factories of Fileni® industry (Cingoli, Italy) and Pectoralis major muscles were sampled immediately after slaughter. From each breast, a 2 cm3 section was cut from the top right part of the muscle, and it was frozen in liquid nitrogen and stored at -80°C until extraction day.

2.2 Protein extraction and quantification

About 200 mg of muscle tissue were homogenized in 3 mL of extraction buffer containing 8.3 M urea, 2 M thiourea, 1% Dithiothreitol (DTT), 2% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) and 2% Pharmalyte® (Immobilized pH gradient (IPG) buffer pH 3–10) using a T 25 digital Ultra-Turrax® (Picard 2020; Picard & Gagaoua 2020). Protein homogenates were then incubated for 30 min on wet-ice and centrifuged for 30 min at 10000 rpm at 4°C. Supernatants were transferred into Eppendorf tubes and stored at -80 °C until protein quantification. Protein concentrations of obtained extracts were determined using the dye-binding protocol of Bradford (Bradford 1976). A

spectrophotometer (UV-1700, Pharmaspec, SHIMADZU) and bovine serum albumin (BSA) as a standard (Bio-Rad Protein Assay kit, Bio-Rad, France) were used.

2.3 Protein bands preparation for LC-MS/MS

Muscle protein extracts were diluted and denatured by 1:1 Laemmli sample buffer (Bio-Rad Laboratories, Deeside, UK). Protein extracts quality was then assessed by SDS-PAGE gel electrophoresis on 12% acrylamide gels by the presence of distinct protein bands. The denatured protein extracts were concentrated on 1D stacking gel of SDS-PAGE. 20 μg of proteins were loaded in each gel lane and electrophoresis was run for about 15 min to concentrate the proteins. Subsequently, the gels were washed with MilliQ water, stained with EZ Blue Gel staining reagent (Sigma-Aldrich, St. Louis, USA) and kept in gentle agitation for 2 h and then washed with distilled. Stacking gels protein bands were excised with a sterile disposable scalpel and transferred into Eppendorf tubes with 150 μL of 50 mM ammonium bicarbonate 50% ethanol. Then, disulfide bonds were reduced with 200 μL of 10 mM dithiothreitol (Sigma-Aldrich, St. Louis, USA) in 50 mM ammonium bicarbonate buffer for 45 min at 56°C. Proteins alkylation was carried out with 200 μL of 55 mM iodoacetamide (Sigma-Aldrich, St. Louis, USA) in 50 mM ammonium bicarbonate buffer for 30 min in darkness. Subsequently bands were destained by 200 μL of 25 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, USA), 5% acetonitrile for 30 min and washed twice with 200 μL of 25 mM ammonium bicarbonate, 50% acetonitrile for 30 min each time. Finally, bands were dehydrated with 100% acetonitrile for 10 min then the liquid was discarded. Dried protein bands were stored at -80°C until SWATH-MS analysis.

2.4 Protein identification by LC-MS/MS

In order to make global protein identification and quantification (by SWATH analysis), an equal amount of protein (40 μg) from all samples were loaded on a 10% SDS-PAGE gel. The run was stopped as soon as the front had penetrated 3 mm into the resolving gel

(Bonzon-Kulichenko et al. 2011; Perez-Hernandez et al. 2013). The protein band was detected by Sypro-Ruby fluorescent staining (Lonza, Switzerland), excised, and processed for in-gel, manual tryptic digestion as described elsewhere (Shevchenko et al. 1996). Peptides were extracted by carrying out three 20-min incubations in 40 μL of 60% acetonitrile dissolved in 0.5% HCOOH. The resulting peptide extracts were pooled, concentrated in a SpeedVac, and stored at −20 °C.

2.5 Protein quantification by SWATH (Sequential Window Acquisition of all Theoretical Mass Spectra)

2.5.1 Creation of the spectral library

To construct the MS/MS spectral libraries, the peptide solutions were analysed by a shotgun data-dependent acquisition (DDA) approach by micro-LC-MS/MS. To get a good representation of the peptides and proteins present in all samples, pooled vials of samples from each group (ORO, ORA, ARO and ARA) were prepared using equal mixtures of the original samples. 4 μL (4μg) of each pool was separated into a micro-LC system Ekspert nLC425 (Eksigen, Dublin, CA, USA) using a column Chrom XP C18 150 × 0.30 mm, 3 μm particle size and 120 Å pore size (Eksigent, SCIEX) at a flow rate of 5μL/min. Water and ACN, both containing 0.1% formic acid, were used as solvents A and B, respectively. The gradient run consisted of 5% to 95% B for 30 min, 5 min at 90% B and finally 5 min at 5% B for column equilibration, for a total run time of 40 min. When the peptides eluted, they were directly injected into a hybrid quadrupole-TOF mass spectrometer Triple TOF 6600plus (Sciex, Redwood City, CA, USA) operated with a data-dependent acquisition system in positive ion mode. A Micro source (Sciex) was used for the interface between microLC and MS, with an application of 2600 V voltage. The acquisition mode consisted of a 250 ms survey MS scan from 400 to 1250 m/z followed by an MS/MS scan from 100 to 1500 m/z (25 ms acquisition time) of the top 65 precursor ions from the survey scan, for a

total cycle time of 2.8 s. The fragmented precursors were then added to a dynamic exclusion list for 15 s; any singly charged ions were excluded from the MS/MS analysis.

The peptide and protein identifications were performed using Protein Pilot software (version 5.0.1, Sciex, Redwood City, CA, USA). Data were searched using a chicken specific Uniprot database, specifying iodoacetamide as Cys alkylation. This software uses the algorithm ParagonTM for database search and ProgroupTM for data grouping. This false discovery rate was performed using a non-linear fitting method displaying only those results that reported a 1% global false discovery rate or better for both peptides and proteins (Shilov et al. 2007). The MS/MS spectra of the identified peptides were then used to generate the spectral library for SWATH peak extraction using the add-in for PeakView Software (version 2.2, Sciex, Redwood City, CA, USA) MS/MSALL with SWATH Acquisition MicroApp (version 2.0, Sciex, Redwood City, CA, USA). Only peptides with a confidence score above 99% (as obtained from Protein Pilot database search) were included in the spectral library.

2.5.2 Relative quantification by SWATH acquisition

SWATH (Sequential Window Acquisition of all Theoretical Mass Spectra) – MS acquisition was performed on a TripleTOF® 6600plus LC-MS/MS system (AB Sciex, Redwood City, CA, USA). Samples were analysed using a data-independent acquisition (DIA) method (40 total samples). Each sample (4 μL from a mg/ml solution) was analysed using the LC-MS equipment and LC gradient described above for building the spectral library but instead using the SWATH-MS acquisition method. The method consisted of repeating a cycle that consisted of the acquisition of 100 TOF MS/MS scans (400 to 1500 m/z, high sensitivity mode, 50 ms acquisition time) of overlapping sequential precursor isolation windows of variable width (1 m/z overlap) covering the 400 to 1250 m/z mass range with a previous TOF MS scan (400 to 1500 m/z, 50 ms acquisition time) for each cycle. Total cycle time

was 6.3 s. For each sample set, the width of the 100 variable windows was optimized according to the ion density found in the DDA runs using a SWATH variable window calculator worksheet from Sciex.

2.5.3 Data analysis library free using Dia NN software

After the samples were acquired individually using the SWATH method, DIA-NN (1.8) was used to generate the protein values per samples using recommended settings. Mass ranges and charges were set appropriately: peptide length range 7 to 35, precursor charge range 2 to 5, precursor range 350 to 1400 m/z and fragment ion range 100 to 1500 m/z. For the search chicken database from Uniprot was added. Other important parameters were as neural network classifier were set as single pass mode, as quantify strategy were set Any LC (high accuracy) and Cross-run normalization was set as RT-Dependent. For the in silico predicted library search, the reduced memory option was additionally activated. Protein FDR was set to 1.0 %.

The integrated peak areas obtained from DIA NN were exported to the MarkerView software (Sciex, Redwood City, CA, USA) for relative quantitative analysis. The export generated information about individual ions, the summed intensity of different ions for a particular peptide and the summed intensity of different peptides for a particular protein. MarkerView has been used for analysis of SWATH-MS data reported in other proteomics studies (Luo et al. 2017; Meyer & Schilling 2017; Ortea et al. 2018; Tan & Chung 2018) because of its data-independent method of quantitation. MarkerView uses processing algorithms that accurately find chromatographic and spectral peaks direct from the raw SWATH data. Data alignment by MarkerView compensated for minor variations in both mass and retention time values, ensuring that identical compounds in different samples were accurately compared to one another. To control for possible uneven sample loss across the different samples during the sample preparation process, we performed a MLR global normalization. Unsupervised

multivariate statistical analysis using principal component analysis (PCA) was performed to compare the data across the samples. The average MS peak area of each protein was derived using the MarkerView from the replicates of the SWATH-MS of each sample followed by Student's t-test analysis software for comparison among the samples based on the averaged area sums of all the transitions derived for each protein. The t-test indicated how well each variable distinguishes the two groups, reported as a p-value. For each library, its set of differentially expressed proteins (p-value <0.05) with a 1.5 fold in- or decrease was selected.

2.6 Statistical analysis and bioinformatics

Chicken breast meat proteomic data were analysed using the open-source MetaboAnalyst 5.0 tool (http: //www.metaboanalyst.ca/, accessed on 8th of June 2022). Protein concentration tables were first uploaded to MetaboAnalyst, and data were log-transformed and Pareto-scaled before analysis. Partial Least Squares-Discriminant Analysis (PLS-DA) analyses were performed to investigate production system and strain effects on chicken pectoralis major post-mortem proteome. The variable importance in the projection (VIP) was used to rank the proteins based on their importance in discriminating the different groups. Proteins with the highest VIP values (>1.0) are the most powerful group discriminators. Proteomics data were further processed with bioinformatics using Metascape tool (https://metascape.org/). Pathway enrichment analyses were performed using Gene Ontology, KEGG and Reactome databases.

3. Results

Based on the shotgun-SWATH-MS proteomics analyses, 660 quantifiable proteins were identified in chicken Pectoralis major muscle extracts. Comparisons of the relative abundances of muscle proteomes were structured according to the different conditions explained above. Proteomes of meat within the strain under the same production system and within the production system under the same strain were compared. The results are reported in the following subsections.

3.1 Antibiotic-free chicken breast muscle proteome and putative strain-specific biomarkers

The PLS-DA score plot allowed visualizing the discrimination in terms of proteome among the two strains, Ranger Classic (ARA) versus Ross 308 (ARO) reared under antibiotic-free production system. A clear separation between ARA and ARO groups can be observed (Figure 1A). The VIP analysis based on the PLS-DA shows the 73 top proteins that influence the discrimination between the strains with a cut-off of 1.0 (Figure 1B). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and tropomyosin beta chain (TPM2) were the main discriminant terms with VIP scores of 4.49 and 4.09 respectively and both up-regulated in Ranger Classic. These proteins can be investigated as biomarkers to discriminate Ranger Classic from Ross 308 chicken breast meat from antibiotic-free inside ground farming. The bioinformatics enrichment analyses on the 73 VIP proteins through Gene Ontology (GO), KEGG and Reactome databases are given in Figure 1C. It resulted that 20 cluster terms were significantly enriched mainly dominated by “generation of precursor metabolites and energy (GO:0006091)” followed by “striated muscle contraction (R-HSA-390522)” and “carbohydrate metabolic process (GO:0005975)” as the top three terms.

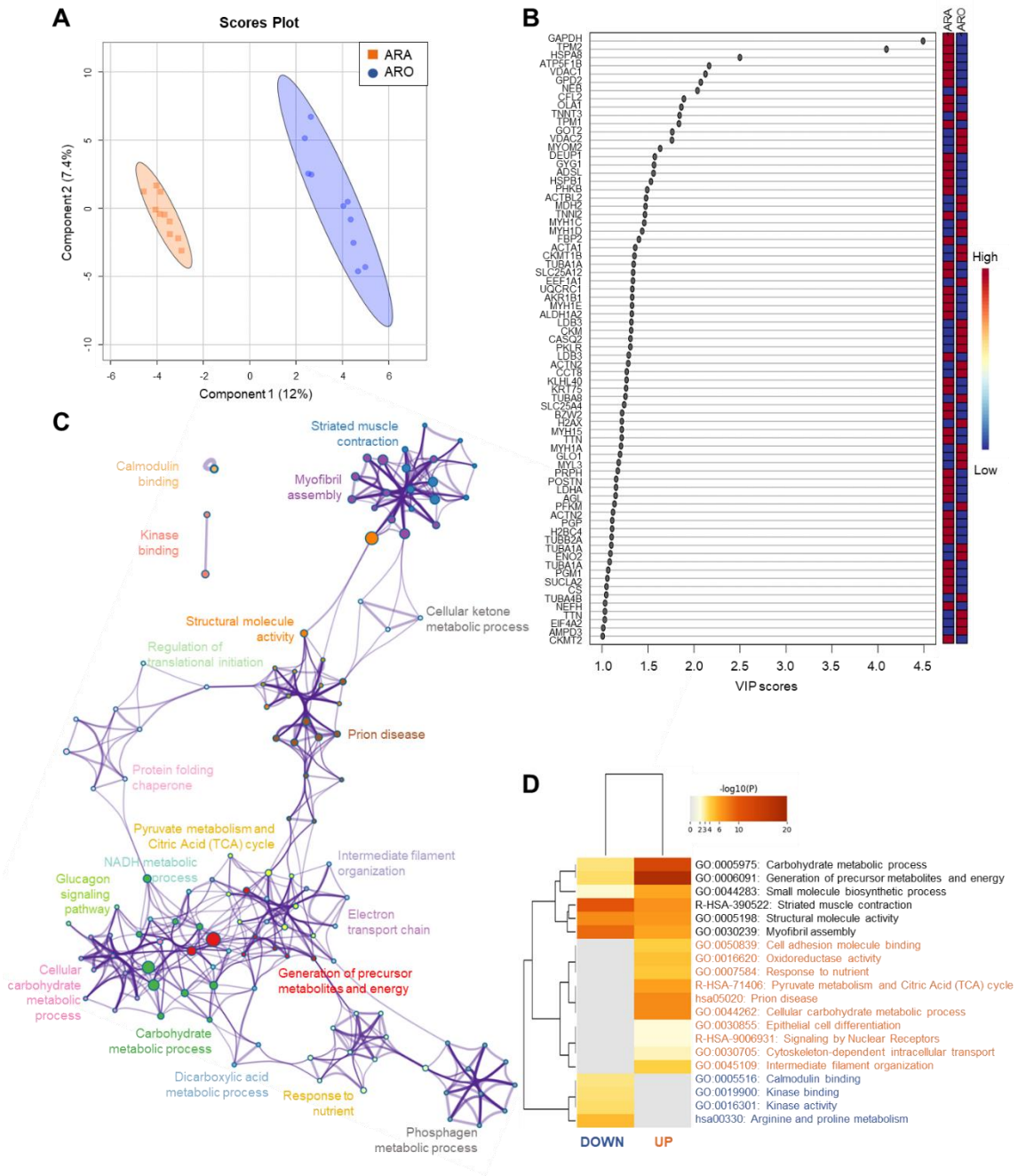

Figure 1. *Discriminant and bioinformatics analyses within the antibiotic-free chicken meat proteomes. A) Partial least squares-discriminant analysis (PLS-DA) score plot of antibiotic-free meat proteome distribution according to chicken strain, Ranger Classic (ARA) and Ross 308 (ARO). B) Variable Importance in Projection (VIP) plot values. C-D) Bioinformatics enrichment analyses (Gene Ontology, KEGG, Reactome) on the 73 discriminating proteins including the redundant proteoforms. C) Network layout based on the enriched terms of the 73 proteins. Each term is represented by a circle node, where its size is proportional to the number of input genes fall under that term, and its color represents the cluster identity. Terms with a similarity score > 0.3 are linked by an edge. D) Hierarchical Heatmap clustering comparing the up and down proteins in terms of the significant processes and pathways using the top Gene Ontology terms and colored according to P-values: terms with a P-value <0.01, a minimum count of 3, and an enrichment factor > 1.5. Colors from grey to brown indicate p-values from high to low; and grey cells indicate the lack of significant enrichment. The terms in blue color are specific to down-regulated proteins, those in red are for up-regulated proteins in Ranger Classic chickens and those in black are significant and common to both protein lists.*

To better visualise the comparison between the groups in terms of up- and down- regulated proteins, the enriched terms were reported in an heatmap where the common and specific biological pathways to the two protein lists are underlined (Figure 1D). Six enriched terms were found to be common: “carbohydrate metabolic process (GO:0005975)”, “generation of precursor metabolites and energy (GO:0006091)”, “small molecule biosynthetic process (GO:0044283)”, “striated muscle contraction (R-HSA-390522)”, “structural molecule activity (GO:0005198)” and “myofibril assembly (GO:0030239)”. Ten pathways were specific and up-regulated in Ranger Classic: “cell adhesion molecule binding (GO:0050839)”, “oxidoreductase activity (GO:0016620)”, “response to nutrient (GO:0007584)”, “pyruvate metabolism and citric acid (TCA) cycle (R-HSA-71406)”, “prion disease (hsa05020)”, “cellular carbohydrate metabolic process (GO:0044262)”, “epithelial cell differentiation (GO:0030855)”, “signalling by nuclear receptors (R-HSA-900693)”, “cytoskeleton-dependent intracellular transport (GO:0030705)” and “intermediate filament organisation (GO:0045109)”. However, four pathways were specific and down-regulated in Ranger Classic including “calmodulin binding (GO:0005516)”, “Kinase binding (GO:0019900)”, “kinase activity (GO:0016301)” and “Arginine and proline metabolism (hsa00330)”.

3.2 Organic chicken breast muscle proteome and putative strain-specific biomarkers

The PLS-DA score plot allowed visualizing the discrimination in terms of proteome among the two strains, Ranger Classic (ORA) versus Ross 308 (ORO) from organic farming and production system. A clear separation between ORA and ORO groups can be observed (Figure 2A). The VIP analysis based on the PLS-DA shows the 62 top proteins that influence the discrimination between the strains with a cut-off of 1.0 (Figure 2B).

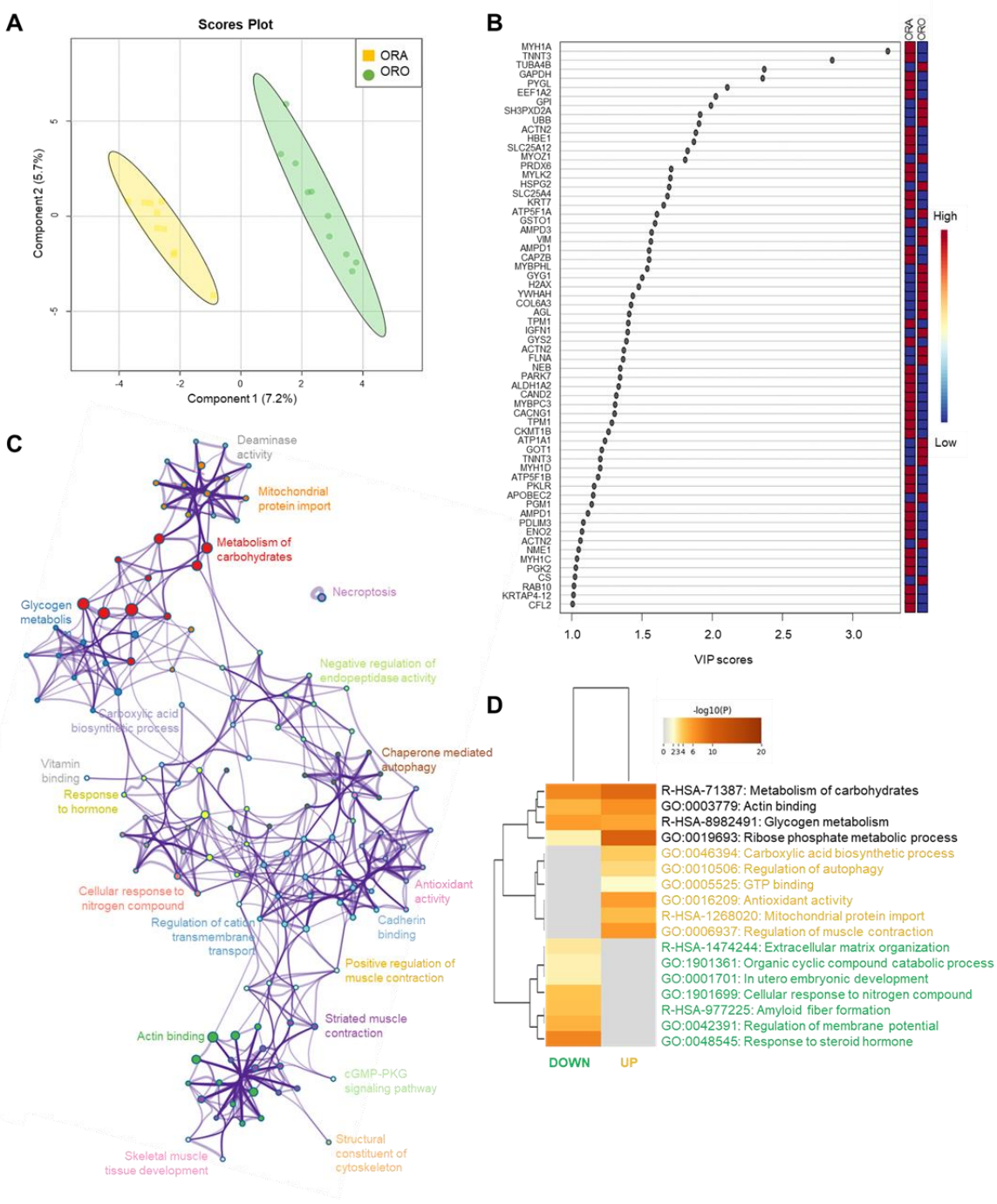

Figure 2. *Discriminant and bioinformatics analyses within the organic chicken meat proteomes. A) Partial least squares-discriminant analysis (PLS-DA) score plot of organic meat proteome distribution according to chicken strain, Ranger Classic (ORA) and Ross 308 (ORO). B) Variable Importance in Projection (VIP) plot values. C-D) Bioinformatics enrichment analyses (Gene Ontology, KEGG, Reactome) on the 62 discriminating proteins including the redundant proteoforms. C) Network layout based on the enriched terms of the 62 proteins. D) Hierarchical Heatmap clustering comparing the up and down proteins in terms of the significant processes and pathways using the top Gene Ontology terms and colored according to P-values. The terms in green color are specific to down-regulated proteins, those in yellow are for up-regulated proteins in Ranger Classic chickens and those in black are significant and common to both protein lists.*

Myosin (MYH1A) and troponin T fast skeletal muscle isoforms (TNNT3) were the main discriminant terms with VIP scores of 3.25 and 2.85 respectively and both up-regulated in Ranger Classic. These proteins can be investigated as biomarkers to discriminate Ranger Classic from Ross 308 chicken breast meat from organic production system. The bioinformatics enrichment analyses on the 62 VIP proteins through Gene Ontology (GO), KEGG and Reactome databases are given in Figure 2C. It resulted that 20 cluster terms were significantly enriched mainly dominated by "metabolism of carbohydrates (R-HSA-71387)" followed by "glycogen metabolism (R-HSA-8982491)" and "actin binding (GO:0003779)" as the top three terms. To better visualise the comparison between the groups in terms of up- and down- regulated proteins, the enriched terms were reported in a heatmap where the common and specific biological pathways to the two protein lists are underlined (Figure 2D). Four enriched terms were found to be common: "metabolism of carbohydrates (R-HSA-71387)" "actin binding (GO:0003779)" "glycogen metabolism (R-HSA-8982491)" and "ribose phosphate metabolic process (GO:0019693)". Six pathways were specific and up-regulated in Ranger Classic: "carboxylic acid biosynthetic process (GO:0046394)", "regulation of autophagy (GO:0010506)", "GTP binding (GO:0005525)", "antioxidant activity (GO:0016209)", "mitochondrial protein import (R-HSA-1268020)" and "regulation of muscle contraction (GO:0006937)". However, seven pathways were specific and down-regulated in Ranger Classic including "extracellular matrix organization (R-HSA-1474244)", "organic cyclic compound catabolic process (GO:1901361)", "in utero embryonic development (GO:0001701)", "cellular response to nitrogen compound (GO:1901699)", "amyloid fiber formation (R-HSA-977225)", "regulation of membrane potential (GO:0042391)" and "response to steroid hormone (GO:0048545)".

3.3 Ross 308 chicken breast muscle proteome and putative biomarkers related to production system

The PLS-DA score plot allowed visualizing the discrimination in terms of proteome among Ross 308 chicken breast muscles from antibiotic-free inside ground farming (ARO) and from organic production system (ORO). A clear separation between ARO and ORO groups can be observed (Figure 3A). The VIP analysis based on the PLS-DA shows the 71 top proteins that influence the discrimination between the strains with a cut-off of 1.0 (Figure 3B). Tropomyosin beta chain (TPM2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were the main discriminant terms with VIP scores of 4.33 and 3.11 respectively and both up-regulated in organic samples. These proteins can be investigated as biomarkers to discriminate organic Ross 308 chicken breast meat from non-organic antibiotic-free one. The bioinformatics enrichment analyses on the 71 VIP proteins through Gene Ontology (GO), KEGG and Reactome databases are given in Figure 3C. It resulted that 20 cluster terms were significantly enriched mainly dominated by “generation of precursor metabolites and energy (GO:0006091)” followed by “energy derivation by oxidation of organic compounds (GO:0015980)” and “myofibril assembly (GO:0030239)”. To better visualise the comparison between the groups in terms of up- and down- regulated proteins, the enriched terms were reported in an heatmap where the common and specific biological pathways to the two protein lists are underlined (Figure 3D). “Generation of precursor metabolites and energy (GO:0006091)” was found to be the common enriched term.

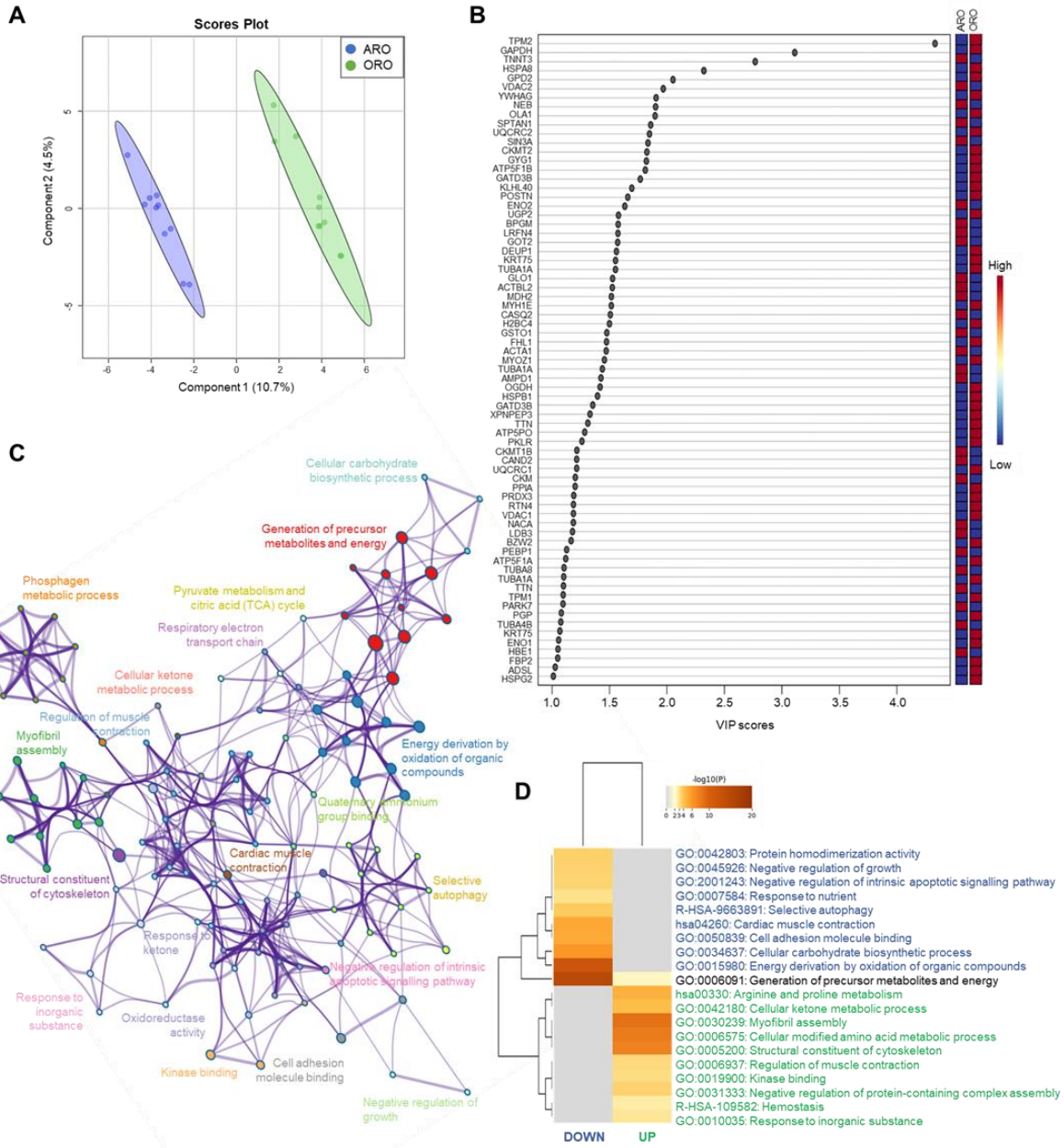

Figure 3. *Discriminant and bioinformatics analyses within the Ross 308 strain chicken meat proteome.* ***A)*** *Partial least squares-discriminant analysis (PLS-DA) score plot of Ross 308 meat proteome distribution according to production system, antibiotic-free (ARO) and organic (ORO).* ***B)*** *Variable Importance in Projection (VIP) plot values.* ***C-D)*** *Bioinformatics enrichment analyses (Gene Ontology, KEGG, Reactome) on the 71 discriminating proteins including the redundant proteoforms.* ***C)*** *Network layout based on the enriched terms of the 71 proteins.* ***D)*** *Hierarchical Heatmap clustering comparing the up and down proteins in terms of the significant processes and pathways using the top Gene Ontology terms and colored according to P-values. The terms in blue color are specific to down-regulated proteins, those in green are for up-regulated proteins in organic chickens and those in black are significant and common to both protein lists.*

Ten pathways were specific and up-regulated in organic samples: “arginine and proline metabolism (hsa00330)”, “cellular ketone metabolic process (GO:0042180)”, “myofibril assembly (GO:0030239)”, “cellular modified amino acid metabolic process (GO:0006575)”, “structural constituent of cytoskeleton (GO:0005200)”, “regulation of muscle contraction (GO:0006937)”, “kinase binding (GO:0019900)”, “negative regulation of protein-containing complex assembly (GO:0031333)”, “haemostasis (R-HSA-109582)” and “response to inorganic substance (GO:0010035)”. However, nine pathways were specific and down-regulated in organic samples including “protein homodimerization activity (GO:0042803)”, “negative regulation of growth (GO:0045926)”, “negative regulation of intrinsic apoptotic signalling pathway (GO:2001243)”, “response to nutrient (GO:0007584)”, “selective autophagy (R-HSA-9663891)”, “cardiac muscle contraction (hsa04260)”, “cell adhesion molecule binding (GO:0050839)”, “cellular carbohydrate biosynthetic process (GO:0034637)” and “energy derivation by oxidation of organic compounds (GO:0015980)”

3.4 Ranger Classic chicken breast muscle proteome and putative biomarkers related to production system

The PLS-DA score plot allowed visualizing the discrimination in terms of proteome among Ranger Classic chicken breast muscles from antibiotic-free inside ground farming (ARA) and from organic production system (ORA). A clear separation between ARA and ORA groups can be observed (Figure 4A). The VIP analysis based on the PLS-DA shows the 52 top proteins that influence the discrimination between the strains with a cut-off of 1.0 (Figure 4B). Two myosin isoforms (MYH1A and MYH1C) were the main discriminant terms with VIP scores of 3.50 and 3.34 respectively and both up-regulated in organic samples. These proteins can be investigated as biomarkers to discriminate organic Ranger Classic chicken breast meat from non-organic antibiotic-free one. The bioinformatics enrichment analyses

on the 52 VIP proteins through Gene Ontology (GO), KEGG and Reactome databases are given in Figure 4C. It resulted that 16 cluster terms were significantly enriched mainly dominated by "generation of precursor metabolites and energy (GO:0006091)" followed by "muscle contraction (GO:0006936)" and "carbon metabolism (hsa01200)" as three top terms. To better visualise the comparison between the groups in terms of up- and down-regulated proteins, the enriched terms were reported in a heatmap where the common and specific biological pathways to the two protein lists are underlined (Figure 4D). "Generation of precursor metabolites and energy (GO:0006091)", "actin binding (GO:0003779)", "muscle contraction (GO:0006936)" and "kinase binding (GO:0019900)" were found to be the four common enriched terms. Three pathways were specific and up-regulated in organic samples: "citrate cycle (TCA cycle) (hsa00020)", "phosphagen metabolic process (GO:0006599)" and "cellular amide metabolic process (GO:0043603)". However, six pathways were specific and down-regulated in organic samples including "in utero embryonic development (GO:0001701)", "response to steroid hormone (GO:0048545)", "cellular response to chemical stress (R-HSA-9711123)", "transmembrane transporter binding (GO:0044325)", "lipid binding (GO:0008289)" and "glycogen metabolism (R-HSA-8982491)".

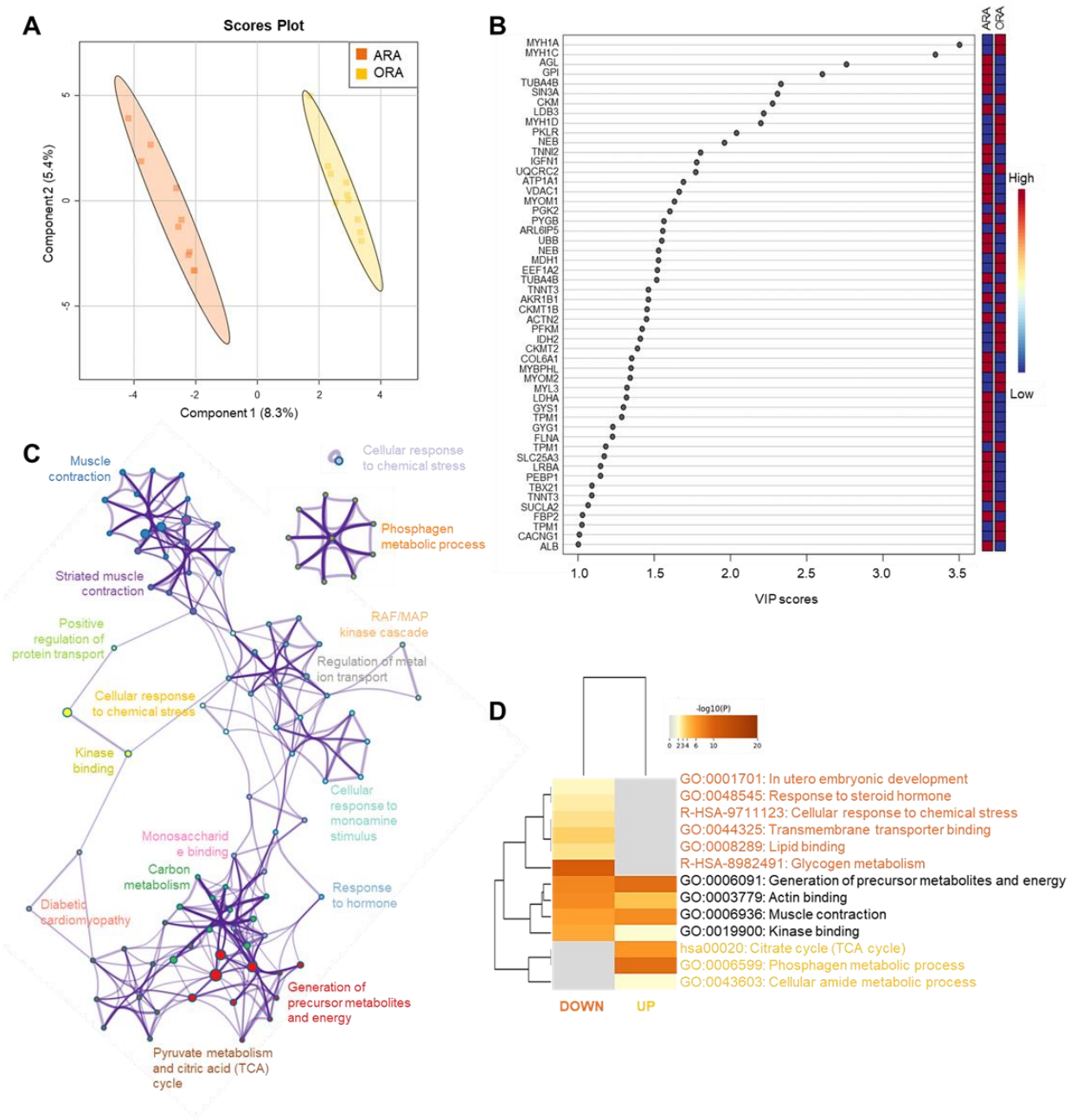

Figure 4. *Discriminant and bioinformatics analyses within the Ranger Classic strain chicken meat proteome.* ***A)*** *Partial least squares-discriminant analysis (PLS-DA) score plot of Ranger Classic meat proteome distribution according to production system, antibiotic-free (ARA) and organic (ORA).* ***B)*** *Variable Importance in Projection (VIP) plot values.* ***C-D)*** *Bioinformatics enrichment analyses (Gene Ontology, KEGG, Reactome) on the 52 discriminating proteins including the redundant proteoforms.* ***C)*** *Network layout based on the enriched terms of the 52 proteins.* ***D)*** *Hierarchical Heatmap clustering comparing the up and down proteins in terms of the significant processes and pathways using the top Gene Ontology terms and colored according to P-values. The terms in orange color are specific to down-regulated proteins, those in yellow are for up-regulated proteins in organic chickens and those in black are significant and common to both protein lists.*

3.5 Discrimination of the four groups using PLS-DA

The PLS-DA score plot allowed visualizing an overview of the discrimination in terms of proteome among the four groups of samples analysed in this study. A clear separation between antibiotic-free and organic and between Ross 308 and Ranger Classic samples can be observed (Figure 5A). The VIP analysis based on the PLS-DA shows the 48 top proteins that influence the discrimination with a cut-off of 1.0 (Figure 5B). A myosin (MYH1C) and a transcriptional factor (SIN3A) were the main discriminant terms with VIP scores of 3.60 and 2.92 respectively. The bioinformatics enrichment analyses on the 48 VIP proteins through Gene Ontology (GO), KEGG and Reactome databases are given in Figure 5C-D. It resulted that 15 cluster terms were significantly enriched mainly dominated by "generation of precursor metabolites and energy (GO:0006091)" followed by "carbohydrate catabolic process (GO:0016052)" and "muscle contraction (GO:0006936)" as three top terms. Venn diagrams report common VIP proteins in both comparisons, meat production system (Figure 6A) and chicken strains (Figure 6B).

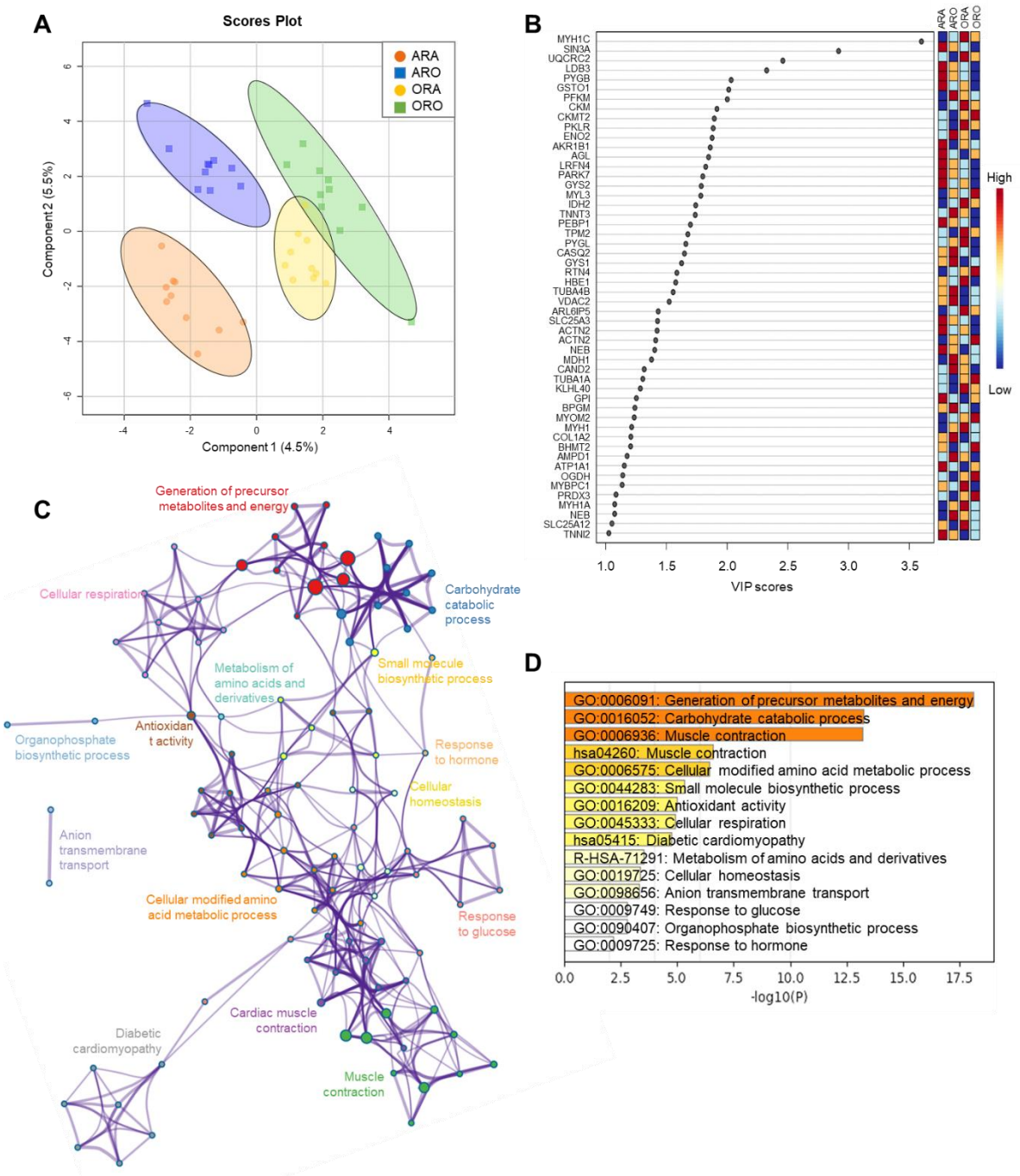

Figure 5. *Discriminant and bioinformatics analyses among the four groups.* ***A)*** *Partial least squares-discriminant analysis (PLS-DA) score plot of meat proteome distribution according to chicken strain and production system.* ***B)*** *Variable Importance in Projection (VIP) plot values separating the meat samples from ARA, ARO, ORO and ORA groups.* ***C-D)*** *Bioinformatics enrichment analyses (Gene Ontology, KEGG, Reactome) on the 48 discriminating proteins including the redundant proteoforms with VIP > 1.* ***C)*** *Network layout based on the enriched terms using the 48 proteins.* ***D)*** *Top 16 enriched terms.*

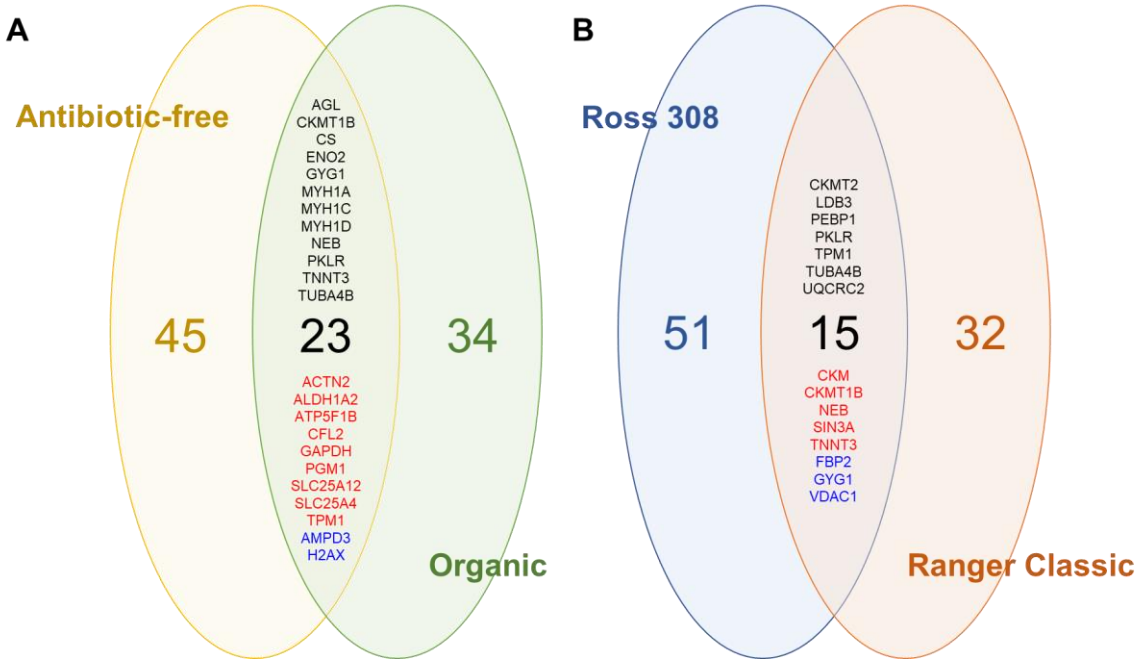

Figure 6. *Venn diagram highlighting the overlap and number of proteins identified to differ within each condition.* ***A)*** *Common differentially abundant proteins between antibiotic-free and organic chicken meat samples****.*** *In red up-regulated and in blue down-regulated in Ranger Classic samples compared to Ross 308 strain.* ***B)*** *Common differentially abundant proteins between Ross 308 strain and Ranger Classic chicken meat samples****.*** *In red up-regulated and in blue down-regulated in organic samples compared to antibiotic-free production system.*

4. Discussion

Meat industries are constantly facing challenges to meet consumer demand of more sustainable, eco-friendly and ethic products. Organic production is one of the most useful strategies to provide high quality meat with lower impact on the environment and greater attention to animal welfare. Despite the ever-increasing distribution of organic meats, from a scientific point of view, there are not many studies that investigate this topic in terms of biomarkers of authenticity. Thus, it was valuable to identify putative protein biomarkers that can discriminate between chicken meat production within the same strain and chicken strain within the production system.

This study on chicken breast meat was aimed at obtaining greater insights and proposing associations of some proteins with strains and production systems. This analysis allowed us

also to (i) use the partial least squares model to propose preliminary explanatory models of meat production system and (ii) chicken strains, (iii) increase our knowledge on the biological pathways related to farming and production conditions of broilers.

4.1 Chicken strains proteome comparison

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and tropomyosin beta chain (TPM2) were reported to be the most influencing proteins in discrimination of the two strains among antibiotic-free samples. These proteins showed VIP scores higher than 4 being up regulated in Ranger Classic meat. GAPDH plays a fundamental role in glycolytic processes, and it is related to the enriched terms of "carbohydrate metabolic process" and "pyruvate metabolism and Citric Acid (TCA) cycle". TPM2 is related to muscle structure and contraction mechanisms as troponin-mediated Ca2+-regulation governs the actin-activated myosin motor function. GAPDH was found to be overexpressed in broilers fed with the probiotic Enterococcus faecium which presented a higher pH and less yellowness (Zheng et al. 2014).

Myosin-1 (MYH1A) and troponin T (TNNT3) were reported to be the most influencing proteins in discrimination of the two strains among organic samples. These proteins showed VIP scores of 3.25 and 2.85 respectively, being up-regulated in Ranger Classic meat. These two proteins are both related to muscle structure and contraction pathways. MYH1 is highly conserved ubiquitous actin-based motor protein that drive a wide range of motile processes in eukaryotic cells while TNNT3 is the tropomyosin-binding subunit of troponin, the thin filament regulatory complex which confers calcium-sensitivity to striated muscle actomyosin ATPase activity. Both these comparisons reported muscle structure related proteins as highly influencing proteins on discrimination of chicken strains. (Sirri 2011) conducted a study on differences in meat quality of fast and slow growth-rate chickens in organic farming. They assessed that meat functional properties as well as nutritional characteristics are strongly influenced by the bird genotype. Slow growth-rate chicken meat

showed better nutritional characteristics (less fat and higher content of n-3 PUFA) and thus might better fit with the consumer's expectations of organic products. From this point of view Ranger Classic, which has a slower growth-rate than Ross 308, can be considered a better choice for organic farming. (Phongpa-Ngan et al. 2011) studied differential expressed proteins in chicken with various growth rates. TPM, keratin (KRT) and actin (ACTA) were identified as a marker of fast-growth chickens while MYH was reported to be expressed more in slow-growth rate chickens. Results of the present article agree with these assessments as statistically significant over-expression of MYH were found in Ranger Classic samples with respect to Ross 308 when both are reared following organic standards. Another study reported differential proteins between low methionine-fed chickens and high methionine fed-chickens (Zhai et al. 2012). The main differences were underlined in actin isoforms regulation. ACTA1, ACTB and ACTG expression decreased in case of increased muscle protein deposition as a subsequence of methionine-supplementation; these results were reported as indicators of a sarcoplasmic hypertrophy, and so related to chicken meat tenderness. In the present study ACTBL2, ACTA1 and ACTN2 actin isoforms were found to influence the strains comparison more than the farming and production system of chicken meat.

4.2 Chicken meat production system comparison

This study clearly demonstrated that meat production system affects post-mortem muscle proteome of broiler Pectoralis major. A myriad of biological pathways is being involved mostly related to energy metabolism and muscle contraction and structure. Tropomyosin beta chain (TPM2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were reported to be the most influencing proteins in production system discrimination of Ross 308 breasts. These proteins showed VIP scores of 4.33 and 3.11 respectively, both up-regulated in antibiotic-free samples. Myosin-1 (MYH1A and MYH1C isoforms) and glycogen

debranching enzyme (AGL) were the most influencing proteins in production system discrimination of Ranger Classic pectoralis majors. These proteins showed VIP scores higher than 2.7, the former up-regulated in organic and the second in antibiotic-free samples. AGL is a multifunctional enzyme, it plays an important role in the degradation of glycogen.

In conclusion the applied high-throughput proteomic approach coupled with chemometric multivariate tools revealed the impact of chicken genotype and production system on meat proteome and cellular pathways underlying the differences in protein expression. Obtained results could be considered as a significant step forward in elucidating the proteomic profile of chicken meat, which may provide opportunities for assessment of chicken authenticity using molecular biomarkers.

Abbreviations

ARA Antibiotic-free Ranger Classic

ARO Antibiotic-free Ross 308

ORA Organic Ranger Classic

ORO Organic Ross 308

PLS-DA Partial Least Square Discriminant Analysis

HPLC High Performance Liquid Chromatography

SWATH-MS Sequential Windowed Acquisition of all Theoretical Mass Spectra

VIP Variable Importance in Projection

Acknowledgment

The authors are grateful to Fileni S.p.a. for financially support this project and Dr. Susana Bravo López from the Proteomic Unit, Health Research Institute (IDIS) of Santiago de Compostela, Spain for support in SWATH-MS analyses.

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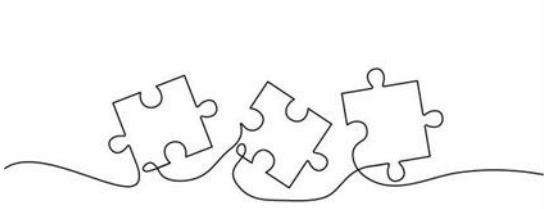

SECTION D

Conclusion and perspectives

Conclusion and perspectives

Nowadays, consumers are increasingly paying attention to the quality and origin of the food they eat. For this reason, scientists are required to focus on the food quality and safety but also on ethics, and environmental sustainability of the products in line with the farm-to-fork strategy. Two important aspects of the poultry meat supply chain have been explored in my thesis: the final packaging for placing the meat product on the market (at sale) and the production systems involving chicken farming and meat production and/or quality. The first objective was achieved in the context of the Abriopack project which involves several partners called to explore the different life stages of the new biodegradable and compostable biopackaging, from production to composting. The results obtained in experiment 1 of my thesis have led the industry to use the biodegradable and compostable packaging with similar preservation capacity as traditional plastic. This product has been used in Italian supermarkets since 2020, mainly by Fileni company replacing a large part of the previously used plastic (https://www.fileni.it/blog/fileni-bio-nuovo-pack-compostabile/).

The second objective of my thesis aimed to better evaluate the potential quality of chicken meat reared in organic and antibiotic-free farming systems. It was possible by using through different strategies, ranging from meat quality and shelf-life comparison to the investigation of the biochemical mechanisms involved in muscle to meat conversion using foodomics. Through a multidisciplinary approach, I brought new knowledge on the impact of production system on meat quality evaluation, especially meat shelf-life, and on chicken breast muscle proteome and molecular signatures discriminating/characterizing the strains and farming systems that I targeted in a co-construction manner with the partners.

Firstly, in experiment 2, important differences in preservation were found between organic and antibiotic-free chicken breast meat. Thus, the prominent differences on biogenic amines concentration allowed a clear evaluation of the shelf-life studies and led to hypothesize meaningful differences in protein expression, being biogenic amines the products of amino acid decarboxylation process during meat storage.

Based on the above, I designed proteomics studies using a new shotgun proteomics, for the first time applied in poultry research, to examine the impact of the meat farming system and chicken strain on the final breast meat product. This part of my thesis, allowed me to explore in-depth the power of omics sciences and its incredible potential in deciphering the unknowns and revealing the underlying biochemical pathways. In support of my hypothesis, the results underlined important differences in the expression/abundance of proteins related to specific molecular pathways. The application of innovative statistical and chemometric tools allowed me to further propose for the first time putative biomarkers of authenticity of chicken breast meat from organic and antibiotic-free farming systems and from divergent chicken strains, Ross 308 and Ranger Classic. Overall, the proteomics results showed the complexity of the interactions that exist between the protein biomarkers studied, their abundance and the characteristics of chicken strain, and production system. My results argue for a major role of interconnected pathways related to energy metabolism, striated muscle contraction and probably oxidative stress as underlying factors impacting chicken meat quality.

The experiment 3 paved the way for more in-depth investigations regarding the protein expression differences related to the meat samples from the two chicken strains reared according to organic and antibiotic-free farming methods.

The greatest impact of the organic farming on chicken breast meat proteome, compared to antibiotic-free or conventional farming, was assessed as a result of the quantitative proteomic

analysis carried out in experiment 4. Finally, in experiment 5, the application of statistical and chemometric tools allowed me to efficiently discriminate both the chicken strains and farming systems based on the early *post-mortem* breast muscle proteome.

A limitation of this work was the limited number of proteins reviewed for the species G*allus gallus* (2304 Swiss-prot database). This has led to the need to use *Homo sapiens* database for uncharacterizable proteoforms in *G. gallus.* In the coming years, the growing significance of omics sciences and the extensive use of databases will lead to the confirmation and reviewing of an ever-increasing number of specific proteins of different taxonomies.

The results obtained from my thesis can pave the way for further studies, especially in the frame of evaluating the potential of the putative biomarkers I discovered for the first time in the different experiments. For instance, targeted proteomics could help confirming the identified features of meat authenticity. Furthermore, the use of integrative approaches, in the frame of integromics studies, would allow the comparison of the protein biomarkers and molecular signatures I identified in my thesis to the available studies in the literature. This is one of the aspects that I launched and should be continued in the coming months using datamining and meta-analyses approaches.

Further research needs to be conducted in order to provide conclusive solutions about the correlation of the expression of specific proteins/biomarkers and meat quality traits such as drip loss or water-holding capacity, color, pH and tenderness. At the same time, it would be interesting to better assess the impact of the level of pre-slaughter stress of animals and of oxidative processes linked to the expression of certain biomarkers and their relationship with the different farming systems and applied rearing practices.

My results could further pave the way for interesting insights using other omics methods. For instance, metabolomics techniques allow the exploration of small molecules deriving

from meat cellular processes, likely those involved in catalytic and energy metabolism pathways revealed by my proteomics studies. The integration of metabolomics with the proteomics data obtained in this thesis will increase our knowledge about chicken meat determination at the molecular level and will help to have a more comprehensive view of the impact of the genotype and the type of farming on the final meat product. This should further increase the accuracy of the predictive power of the decision tools that we expect to develop.

Moreover, sampling time, stress conditions at farming and slaughter could be investigated as further variables impacting post-mortem muscle proteome. In this approach, the strategy could be the use of phosphoproteomics to analyse specifically the phosphorylated proteins. The importance of the study of phosphoproteins lies in the fact that this modification impacts not only the stability and catalytic activity of the protein, but also alters the protein/protein, protein/RNA and protein/DNA interactions, regulating a myriad of cellular processes such as the inter- and intracellular signalling pathways, protein synthesis, gene expression, cell survival and apoptosis.