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AVIAN PROTEOMICS: POTENTIAL TOOL FOR PRE- AND POST-SLAUGHTER POULTRY MUSCLE QUALITY EVALUATION – A REVIEW

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Abstract

A discipline within functional genomics, proteomics represents the method of analysis which allows a cell or a tissue's proteins (proteome) to be analyzed under certain given conditions. Functional proteomics allows scientists to address the interactions of different proteins in order to better understand the consequences of these interactions, and further on, to acquire knowledge on different specific areas, such as poultry breeding and quality of meat. Proteomics could help to characterize pathogen-host interactions in the diseases of livestock, assess the reproductive health and evaluate the muscle growth dynamics. Furthermore, it can be successfully applied in assessing the specific chemical reactions of rigor mortis (muscle quality), as well as evaluating the indicators of farm animal welfare, such as heat-induced stress.

Key words*: proteomics, muscle quality, poultry welfare.*

INTRODUCTION

Proteomics is the „analysis of the full complement of proteins of a cell or tissue under given conditions (it involves identifying and cataloguing proteins in a cell and identifying relative changes in populations between two or more states, physiological/diseases induced ones)" (Doherty et al., 2007; Kunec and Burgess, 2015).

It is a discipline within functional genomics, the context-defined analysis of complete complements of proteins, creating a bridge on „sequence-to-phenotype gap" (Burgess, 2004).

Proteomics allows the study of proteins present in a given tissue (the proteome). Its limitations are: cost, lack of good genomic data from many species of interest, a lack of awareness of the potential of this technology by animal scientists (de Almeida et al., 2015).

Proteomics offers a powerful new way to characterize the protein component of foods. It not only reveals which proteins are expressed in each tissue type, it also allows the investigation of differences in the protein composition of different tissues, and has the power to track the proteome of tissues before and after slaughter and evaluate the effect of downstream treatments such as cooking or curing (Clerens et al., 2012).

This serves as an important tool when looking for candidate genes that could influence the subcutaneous fat deposition in pigs (as an example), and since these traits are complex and even mutagenic in nature, the characterization at a molecular level would provide great benefits when correlating the proteome and transcriptome analysis (Bendixen et al., 2011).

MATERIALS AND METHODS

Proteomics studies are usually divided in three main areas:

(A) Qualitative proteomics - protein identifycation and characterization includes the analysis of the proteome relying on one or several separation steps followed by MS analysis. It involves:

- PMF (peptide-mass fingerprinting) uses two-dimensional gel electrophoresis (2-DE) to isolated an unknown protein, which is then enzymatically digested into peptides and subjected to MS.

- PFF (peptide-fragmentation fingerprinting) uses tandem MS (MS/MS) to produce fragment-ion data from one or more peptides from the protein to identify the protein unambiguously.

This approach requires the corresponding protein to be present in the database; if not, the

best match will probably be the entry with the closest homology, usually a related protein from a related species (Gallardo et al., 2013; Ortea et al., 2016).

(B) Differential proteomics shows relative abundance of a specific protein among different samples or the absolute amount of the protein, using three different methodologies:

(1) gel based – comparing the signal of an electrophoretically-isolated spot among different samples;

(2) label-based – proteins or peptides are labeled using a mass tag that is introduced metabolically, enzymatically or chemically, and relative quantification is obtained from the MS read-out (then quantification is based on the ration of heavy/light peptide pairs)

(3) label-free – avoid the labelling with stable isotopes, so protein amount is calculated based on the MS-derived ion-current signal of the peptides or proteins or on the number of identified MS/MS spectra (spectral counts) for the protein.

(C) Functional proteomics, which addresses the integrated analysis of the functional interactions among different proteins and the networks thereof, as well as the consequences of these interactions (Gallardo et al., 2013; Ortea et al., 2016).

Sentandreu et al. (2010) have recently developed a methodology for detecting the presence of chicken meat in other meat species through the identification of species-specific peptide biomarkers. This procedure combines protein enrichment strategies, identification and discriminating sequence from selected peptides by mass spectrometry. It might constitute an alternative to the current methods used for meat speciation such as immunoassays or DNA-based assays.

By implementing an OFFGEL enrichment fractionation step and MS detection by conventional LC-ion trap-MS/MS, it was possible to detect as low as 0,5 % w/v contaminating chicken in pork meat with high confidence due to acquisition of discriminating sequence information (Piras et al., 2016; Sentandreu et al., 2010). The discriminating power of this approach is based on detection of chicken-specific peptides originated from trypsin digestion of previously enriched myosin light chain 3, being comparable to methods based on DNA analysis. This proteomic approach displays more robustness in addressing some of the major limitations of DNA-based methods, such as optimization of the extraction procedures according to the different matrices and recovery of samples with high DNA quality. From this perspective, the primary amino acid sequence of the key peptide biomarkers used here will be considerably more resistant to food processing than DNA sequences (Sentandreu et al., 2010).

RESULTS AND DISCUSSIONS

Different results have emerged, considering the application of proteomics in poultry science, several of them applying in steps which precede the slaughter, while others have applied successfully in post-slaughter stages. In the latter case, the implications go through to food science, more specifically in muscle quality.

(1) Pre-slaughter application of proteomics in poultry

Among the uses of proteomics in poultry breeding, we can enumerate:

- characterization of pathogen-host interaction in the disease of production animals;
- assessment of reproductive health status;
- evaluation of the muscle growth dynamics (de Almeida et al., 2015; Doherty et al., 2004).

Pre-slaughter, proteome-based biomarkers are considered important for early diagnostics in veterinary medicine, so dedicated studies on farm animals could be performed to monitor the health and welfare as well as investigating the type and state of a disease (through analyses of serum and plasma, as well as milk – cattle) (Bendixen et al., 2011).

Agudo et al. (2005) studied the proteins from the stage 29 chicken embryos, identifying proteins implicated in the formation of various organs, CNS development and specific embryonic development – potential biomarkers for embryonic development.

In a global analysis of the chicken embryo liver proteome resulted in identification of proteins that were differentially expressed between two chicken lines that differed in hepatic lipid metabolism and fat deposition (Huang et al., 2010). Comparative analysis of the identified

liver proteins showed that proteins involved in gluconeogenesis, cholesterol metabolism and fatty acid oxidation were expressed earlier and more abundantly in the liver of lean-line of chickens.

During chicken myogenesis, in chicken thigh, hierarchical clustering analysis revealed the expression of 168 proteins. Their expression was affected by the developmental stage. Based on the expression profile, these were classified in 9 clusters, among which 2 were specific for the myogenesis: one which contained proteins showing increasing abundance (A) and another one the opposite (B). The proteins included in (A) were involved in the energy metabolism (α and β-enolase, creatine kinase, NADH dehydrogenase, cytochrome C reductase, succinyl CoA ligase), lipid transport (apolipoprotein B, fatty acid-binding protein 3) or calcium signaling (sarcalumenin). The expression of β-enolase and creatine kinase increased during postnatal muscle growth. Some of the proteins were expressed during embryogenesis, but not after hatch: alpha-fetoprotein, regucalcin, proteasome 26S subunit (early stages of myogenesis, day 7 *in ovo*).

Regucalcin regulates intracellular Ca^{2+} homeostatis by regulating Ca^{2+} transport systems in liver and renal cells (Picard et al., 2010).

HSP27 expression was at peak at 18 days *in ovo*, corresponding to sustained fibre differentiation or maturation; this may suggest that HSP27 plays a major role in the transition between the proliferation of myoblasts and the differentiation and/or maturation of myofibre in the different species, possibly through its anti-apoptotic actions or through its stabilising action on myofibrils.

There was also a high abundance of ApoA1 during the early stages of myogenesis, with a decrease in the last stages; ApoA1 is known for its involvement in cholesterol transport and lipid metabolism (Picard et al., 2010).

Proteomic characterization of the sarcoplasmic proteins in the *pectoralis major* and *supracoracoides* breast muscles was conducted for two different chicken genotypes, which included Ross 708 commercial broilers and Leghorn chicks, Hyline W-36. Results suggested that glycogen phosphorylase, enolase, elongation factor 1, creatine kinase, fructose-bisphosphatealdolase and glyceraldehyde 3-phosphate-dehydrogenase were different in the two strains during breast muscle growth (Schilling et al., 2017).

Tanaka et al. (1995) observed a dominance of alpha-enolase mRNA in the embryonic chick, while the expression of the beta-enolase mRNA is almost exclusive in mature muscle.

Han et al. (2005) analyzed the chicken gonadal primordial germ cells to probe the molecular and physiological mechanisms underlying avian germ cell development; among the identified proteins, vimentin (member of the intermediate filament protein family – valuable in the diagnosis of undifferentiated neoplasms), a specific tissue, developmentally regulated protein was highly expressed in these cells; in addition, albumin (and other proteins related to it) was also present, being attributed to a role in maintaining homeostasis of the cells.

Parada et al. (2005) studied the embryonic cerebrospinal fluid (ECSF), which is known to have a higher protein concentration (stages 18 to 30), higher than that of adult CSF. The ECSF plays the role of CNS development, expansion of cephalic cavities and in the survival, proliferation and differentiation of neuroectodermal stem cells, in collaboration with known organizing centres; at stage 24 can be registered the greatest rate of neural stem cell proliferation (time of beginning for the neurogenesis process). Proteins identified in this study (apolipoproteins, retinol, vitamin D carriers, proteins related to quiescence and cell death) may be important in the capacity of ECSF to contribute to the neurogenesis, via the exertion of a trophic role on the ectoderm.

McCarthy et al. (2006) analyzed the proteomes of the supporting stromal and B cells isolated from the chicken bursa of Fabricius (common experimental system for B-cell development). Proteins were isolated from the two major functional cell types of bursa by a sequential detergent extraction procedure that increased proteome coverage and helped to localize known and previous by unknown proteins to different cellular compartments. The analysis identified 5198 proteins in bursa, and of these, 1753 were B-cell specific, 1972 were stroma specific, and 1473 proteins were identified in both cell types. Functional modelling of the identified proteins provided insights about

signaling pathways involved in programmed cell death, proliferation and differentiation.

Korte et al. (2013) showed that enzymes of the retinoic acid metabolism play a crucial role in the early development of the primary avian B-cell organ. Similar observations were done in mammals, where vitamin A plays a similarly important role in the development of secondary lymphoid organs (van de Pavert et al., 2009)

Parada et al. (2006) characterized through proteomics the chicken embryonic cerebrospinal fluid (CSF), showing that 14 of the proteins are also present in human CSF, while 12 of them are altered in neurodegenerative diseases and/or neurological disorders (Table 1).

Table 1. Studies on potential pre-slaughter applications of proteomics in poultry science

Type of research, application and potential use	Reference
Proteins involved in the formation of various organs can be potential biomarkers for embryonic development.	Agudo et al. (2005)
Proteins involved in gluconeogenesis, cholesterol metabolism and fatty acid oxidation were expressed earlier in the chicken embryo liver proteome of the lean lines.	Huang et al. (2010)
It was observed that from the chicken proteome, β-enolase and creatine kinase increased during the postnatal muscle growth, thus being revealed their rule in myogenesis.	Picard et al. (2010)
Alpha-enolase mRNA is dominant in the embryonic chick, while β-enolase mRNA is almost exclusive in the mature muscle.	Tanaka et al. (1995)
Vimentin was highly expressed in the chicken gonadal primordial germ cells, while albumin was present too, with a potential role in maintaining the homeostasis of these cells.	Han et al. (2005)
Functional modelling of the proteins identified in chicken bursa of Fabricius may offer insights into the signaling pathways involved in programmed cell death, proliferation and differentiation.	McCarthy et al. (2006)
Out of the chicken embryonic cerebrospinal fluid proteins identified so far, 14 are also present in human CSF, while 12 of these are altered in neurodegenerative diseases and/or neurological disorders.	Parada et al. (2006)

In a study aiming to evaluate the supplementation of creatine pyruvate on the lipid and protein metabolism, by using a proteomic approach 32 liver mitochondrial proteins with differential profile changes were identified, some of these being involved in lipid and protein metabolism (Chen et al., 2012):

(1) ADRP[1] has the main established role of limiting the interaction of lipases with neutral lipids, therefore promoting the accumulation of the latter; the expression of ADRP was decreased with the creatine-pyruvate group, suggesting that this compound reduces the fatty acid accumulation and fat deposition;

(2) CETP[2] has a role in the transport of excess cholesterol from peripheral tissues to the liver, mediating the transfer of cholesteryl esters from HDL or LDL into triglyceride-rich lipoproteins, thereby stimulating reverse cholesterol transport; creatine-pyruvate determines a down-regulation of the CETP expression, its inhibition increasing the HDL-C levels and decreasing the triglyceride levels (Chen et al., 2012);

(3) FACS[3] plays a role in the fatty acid activation, catalyzing a two-step reaction, regulated by Mg^{2+}, to produce fatty acyl CoA esters (these esters being targeted by carnitinepalmitoyltransferase I for conversion to their acylcarnitine derivatives and subsequentsly being transported into mitochondria where they are subjected to β-oxidation); carnitine-pyruvate group manifested an up regulating of FACS, promoting the activation of fatty acids to fatty acyl CoA esters, which were then transported to the mitochondria;

(4) eIF2 and eIF2B[4] have a regulation role in the protein synthesis; eIF2B controls the activity of eIF2, more precisely its return to GTP bound-form, through nucleotide exchange (eIF2B promotes release of GDP from eIF2, by acting as a GDP-dissociation stimulator protein) - > a higher eIF2B expression level (creatine and creatine pyruvate groups) could lead to an increased eIF2 activation, enhancing the protein synthesis.

The conclusion is that creatine pyruvate reduces fat deposition by promoting the β-oxidation and triglyceride hydrolysis (Chen et al., 2012).

Doherty et al. (2004) focused on the changes in the most abundant proteins in the low-salt,

[1]adipose differentiation-related protein
[2]cholesteryl ester transfer protein
[3]fatty acyl-CoA synthetase
[4]eukaryotic initiation factor / and its exchange factor

water soluble component of the chicken skeletal (*pectoralis*) muscle. It seems that there are dramatic changes in protein expression from hatching to maturity. At one day post-hatch, the identified proteins are structural ones (actin, myosin light chain), proteins involved in the synthesis and modification of new proteins (elongation factor 2) and glycolytic proteins (enolase, triose-phosphate isomerase). At nine days post-hatch, the protein profile becomes simpler and more specialized, with an expansion of the glycolytic enzymes (greatest expansion being observed for GAPDH, while haemoglobin and ovotransferrin decreased to trace levels). In broiler chickens, the change in global protein expression occurs earlier than in layers, indicating that the muscle maturation occurs more rapidly in the developing broiler chicks.

After a comparison of the *pectoralis* proteomes of animals with different growth rates and different water holding capacity, within the same genotype, a total of 22 protein spots were found to show differential expression. Also, proteins such as creatine kinase, pyruvate kinase, triosephopsphateisomerase, ubiquitin, heat shock proteins, as well as several structural and contractile proteins were identified. Several of these were proposed as markers of water-holding capacity and also of growth rate, demonstrating once again the potential of proteomics in meat authentication, specifically in the selection of quality and productive traits markers (de Almeida, 2017).

On the topic of immune response studies using proteomics, the Acute Phase Response (APR) is the early and non-specific systemic reaction of the innate immune system to homeostatic disturbances. Pro-inflammatory cytokines and chemokines released from macrophages, monocytes and infected and damaged tissues affect the synthesis and secretion of hepatocytic proteins and drastically alter the plasma protein profile (O'Reilly et al., 2012).

Plasma proteins that change concentrations as a result of an APR are called termed acute phase proteins (APP). APP concentrations can change during infectious, inflammatory, stressful, traumatic or neoplastic events, often proportionally to the severity of the event.

This study characterized APP changes that occur due to gait abnormalities in broilers by measurement of the established APPs in chicken: ceruloplasmin (Cp), PIT54 (avian haemoglobin binding protein similar to mammalian haptoglobin) and ovotransferrin (OVT), which are known to increase in response to bacterial, viral and parasitic infection (O'Reilly et al., 2012).

Birds with obvious gait defect which affected the ability to move presented the highest values of all APPs. Cp was significantly associated with weight, heavier birds tending to have higher Cp concentrations. Not necessarily linking the weight to the gait score, in heavier birds there might a series of inflammatory events present, resulting in higher Cp concentrations. There were no significant associations between the APP concentrations and broiler breed (O'Reilly et al., 2012).

(2) Post-slaughter application of proteomics in poultry

After bleeding, muscle energy metabolism is modified: nutrients and oxygen are no longer supplied to the muscle, calcium ions may move from the sarcoplasmic reticulum into the cytoplasm of the muscle cell and activate many pathways including ATPases, and metabolites accumulate (Picard et al., 2010).

The regeneration of ATP depends first on the degradation of phosphocreatine, which has a half-life of 60-80 minutes in the postmortem muscle, the production of ATP from ADP catalyzed by myokinase, and subsequently on anaerobic glycogenolysis.

All these processes lead to the accumulation of protons and lactate, leading to acidification of the muscle.

In addition to energy metabolism, proteolysis, lipolysis and oxidation also play a major role in determining the meat quality; these underlie the process of "ageing" of the meat, considered to start after death and last for several days or weeks, depending on the species.

The ageing of meat allows the tenderization and the enhancement of taste and this process has proteins as a central part of it, once because they are targets and on the other hand they are also mediators of biochemical reactions (Picard et al., 2010).

The most significant change is that muscle remains functional and metabolically active for several days after slaughter although depleted

of the circulating blood that supplies oxygen and removes metabolic end products. This results in lactic acid accumulation and consequently in declining pH, in a process termed muscle acidification (Paredi et al., 2012). The acidification causes loss in water holding capacity (WHC) as well as in calcium release, and leads to cross-bridges being formed between myosin and actin filaments. As a consequence of the slaughter, glycogen level in the muscle decreases and therefore, the energy available to keep the muscle in a relaxed form also decreases.

The combination of these factors results in the onset of rigor mortis, a state essentially characterized by significant alterations at the level of the energy metabolism that result in further pH decrease and a simultaneous decrease in muscle flexibility (Paredi et al., 2012).

Post-slaughter, the food proteins are subjected to: side-chain oxidation, cross-link formation, backbone cleavage, which leads to consequences on the food properties: shelf life, nutritional value, digestibility, health effects (Clerens et al., 2012).

In a recent study, performed in 2013, Montowska and Pospiechproved that observed inter-species differences in protein expression in raw meat were retained in thermally processed meat and ready-made products after finishing the entire technological process. The proteins formed a specific pattern on 2-DE gels, thanks to which it was possible to identify the species in the products. In this study, phosphohistidine phosphatase was likely the first time identified in the chicken *pectoral* muscle. The identified proteins with species-specific electrophoretic mobility are the proteins of the largest amounts which can be found in the muscle tissue.

In livestock production is very important to understand how heat stress can prevent the muscle growth. Road transport induced-stress is known to increase the levels of corticosterone, whilst decreasing the abundance of several hexose phosphates, overall affecting the cytoskeleton structure and carbohydrate metabolism. Also, it was found that induced stress led to a repression of glycogenolysis and glycolysis and an alteration of the myofibrillar protein profile (Marco-Ramell et al., 2016). Following the heat stress, several proteins involved in glycolysis, glycogenesis and glycogenolysis were increased or modified, indicating enhanced glycolytic capacity in response to it.

For evaluating the indicators of farm animal welfare, in chickens, a study was conducted by applying a 2h restraint to chickens which were later euthanized and the tensor *fascia latae* and *biceps femoris* muscles were used to perform the proteomics analysis (Table 2). A total of 29 proteins were found to have differential expression, 37 % of which with a function in glycolysis and 14 % in cell structure. The conclusion was that the restraint period resulted in a repression of glycogenolysis and glycolysis in the thigh muscle (Paredi et al., 2013).

Table 2. Research on potential post-slaughter applications of proteomics in poultry science

Field of application	Type of research, application and potential use	Reference
Heat-induced stress	Following a period of heat stress, several proteins will go through changes (either increases or decreases), which indicates that there is an enhanced glycolytic activity associated to this stress.	Marco-Ramell et al. (2016)
	Chicken restraint may result in a repression of glycogenolysis and glycolysis in the thigh muscle.	Paredi et al. (2013)
	Heat stress may result in a proteomic response involving marked cellular changes in carbohydrate metabolism, structure and antioxidant processes in the skeletal muscle.	Cruzen et al. (2015)
Muscle quality	In comparison to commercial broiler chicken meat, the Thai chicken meat is firmer in texture and with an improved flavor, due to the expression and activity of glycolytic enzymes.	D'Alessandro and Zolla (2012b)
	Parvalbumin is overly expressed in *peroneus longus* muscle, in comparison to *pectoralis* muscle, showing that the first is more frequently used.	Jung *et al.* (2007)
	Correlations have been found between some key regulators of the cellular redox balance and the level of lipid oxidation during ageing as well as cooking.	Gobert et al. (2014)
	An overabundance of proteins involved in glycolytic pathways, muscle contraction,	Nair et al. (2017)

	proteolysis, ATP regeneration and energy metabolism in PSE breast might be related to quality differences between this type of meat and normal one.	
Adulteration	Separation of intact high and low abundance proteins from both hand-deboned and mechanically recovered meat.	Surowiec et al. (2011)
	The amount of haemoglobin can be used as a marker to differentiate mechanically recovered chicken meat from deboned chicken meat.	Schilling et al. (2017)
	Discrimination of meat species from fresh meat and meat mixtures is possible by comparing the electrophoretic mobility and by immunoblotting and LC-MS/MS analysis.	Kim et al. (2017)
Metabolic changes due to diet	Three of the 190 individual proteins identified in the *pectoralis major* muscle tissue correlated with methionine deficiency in the diet of broiler chicken.	Corzo et al. (2006)

Cruzen et al. (2015) examined the proteome response of skeletal muscle to acute (short duration and high intensity) heat stress. In the white fiber type portions, heat stress decreased abundance of tubulines and soluble actin and increased phosphorylated cofilin 2 abundance, indicating a loss of microtubule structure and a likely increase in stable actin microfilaments; an antioxidant response was observed, manifested through increasing the manganese superoxide dismutase abundance, decreasing at the same time the peroxiredoxin 2 abundance, the proteomic response to heat stress suggesting marked cellular changes in carbohydrate metabolism, structure and antioxidant processes in the skeletal muscle (Cruzen et al., 2015).

A few proteome studies have been aimed at characterizing protein markers that can assess development of tenderness during post-mortem storage of the carcass (Bendixen et al., 2011).

(a) Muscle quality

Muscle importance derives not only from its obvious physiological relevance, associated to specific diseases and metabolic conditionings, but also from its production-associated aspects such as the transformation of muscle to meat. When characterizing the muscle proteome of farm animals, two-dimensional electrophoresis separation is still the most widely used approach. Due to low sensitivity and lack of reproductibility of these systems, they have been replaced over the last years with differential proteomic assays based on fluorescent dyes, namely DIGE (Soares et al., 2012).

Proteomics may help understand biochemical mechanisms underlying meat quality in order to control or predict them. A useful approach is the comparison of proteomes between animals showing relatively high or low values for a specific trait within a population.

Other studies have been using proteomic analyses to understand better the processes underlying tenderness or to provide protein markers predicting tenderness which is one of the most important characteristic for consumer satisfaction.

Proteomics have also been used to understand better or further characterize various meat quality defects. A well-known defect is a pale, soft and exudative appearance of meat (PSE), which usually occurs in pig and poultry species, which have relatively high proportions of fast-twitch glycolytic muscle fibers (Picard et al., 2010).

Post mortem conversion of muscle into meat is a significant series of events connected with protein modification and breakdown, receiving substantial attention in the last years, which is now extended to changes occurring in cooked/cured products. Also, protein marker discovery for various meat quality attributes is another important area (Clerens et al., 2012).

Nakamura et al. (2010) and Soares et al. (2012) examined the allergenicity of meat obtained from transgenic chicken. They identified five IgE-binding protens, which through 2D-DIGE[5] proved to not be significantly changed when comparing non-GM with GM chicken.

Doherty et al. (2004) reported that the weight of *pectoralis* muscle increased approximately 44-fold from day 1 to day 27; since there is very large amount of energy derived from the glycolytic enzymes is required to maintain this mass of tissue, chickens showed a dramatic change in the relative expression levels of glycolytic enzyme, in particular to enolase isoforms, triosophosphate isoforms, creatine kinase isoforms and tubulin isoforms, along with their post-translational modifications. In a comparison between the Thai chicken meat and

[5]bidimensional (2D) difference gel eletrophoresis

the commercial broiler chicken meat, the Thai chicken type proved to be firmer in texture and had an improved flavor, largely attributed to the expression and activity of glycolytic enzymes (D'Alessandro and Zolla, 2012b).

Sentandreu et al. (2010) developed the method for extracting the myofibrillar proteins, subsequently enriching the target proteins using OFFGEL isoelectric focusing. Further on, LC-MS/MS[6] was used for the identification step.

Jung et al. (2007) aimed to investigate the differentially expressed muscle proteins between Cornish and White Leghorn breeds in order to address the possibility of improving meat quantity and quality in chicken. The skeletal muscles were removed and frozen at -80°C (chicken breast – *pectoralis* muscle and drum – *peroneus longus* muscle). The steps were: (a) preparation of the sample – centrifugation – obtaining the protein pellet; (b) electrophoresis; (c) image analysis and gel digestion; (d) characterization of protein spots.

The results showed that more than 300 protein spots were detected, of which 25 common proteins were identified, both appearing in both *pectoralis* and *peroneus longus* muscles of Cornish and White Leghorn breeds. When comparing the *pectoralis* with *peroneus longus* muscles, the parvalbumin protein was over expressed in the latter, in both Cornish as well as White Leghorn. In the muscle tissue, pravalbumin is the high affinity Ca^{2+} binding protein and plays a crucial role in the muscle contraction by translocation Ca^{2+} from the myofibril to the sarcoplasmic reticulum (the conclusion being that the *peroneus longus* is more frequently used than the *pectoralis* muscle) (Jung et al., 2007).

Proteomics has been also used to identify specific markers for predicting the susceptibility of meat to oxidation. In a recent study, the scientists aimed to identify specific markers of lipid oxidation generated in meat during refrigerated storage and cooking. For this purpose, the early post-mortem sarcoplasmic proteome was analyzed and correlated to the level of lipid oxidation. Many proteins could be potential markers of lipid oxidation during meat ageing or cooking, but none of them can serve as marker throughout the complete meat process (from one day to four days of ageing and from raw to cooked). For example, among the three spots of albumin correlated to lipid oxidation during ageing or after cooking, two of them were negatively correlated while the third one was positively correlated.

Significant correlations were found between some proteins that are key regulators of the cellular redox balance (peroxiredoxin and thioredoxin) and the level of lipid oxidation either during ageing or after cooking (Gobert et al., 2014).

Considering the muscle food color, application of mass spectrometry (MS) and proteomics could be related to characterization of myoglobin's primary structure. In order to determine the exact molecular mass of myoglobin and to differentiate meat species, MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry) and electrospray MS are successfully used. Mass spectrometric approach to determine the exact molecular mass of globin polypeptide chain has been applied to differentiate heme pigments from farm animals and for meat species identification. Mammalian and avian myoglobins are comprised of 153 aminoacids and the primary sequence of the myoglobin depends upon species. Avian myoglobins were established to be different then their mammalian counterparts in amino acid sequence. The molecular masses of avian myoglobins are, in general, 300-400 Da greater than those of livestock myoglobins (Poulson et al., 2015).

PSE meat tissue has poor protein functionality and is dry and tough after cooking, leading to economic loss for the poultry industry. A comparison between normal and PSE broiler breast meat tissue revealed 15 differentially abundant proteins. PSE broiler breast had abundance of: actin alpha, myosin heavy chain, phosphoglycerate kinase, creatine kinase M type, β-enolase, carbonic anhydrase 2, proteasome subunit alpha, pyruvate kinase, malate dehydrogenase. Normal broiler breast had an abundance of: phosphoglycerate mutase-1, α-enolase, ATP-dependent 6-phosphofructokinase, fructose 1, 6-bisphosphatase. The overabundance of proteins involved in glycolytic pathways, muscle contraction, proteolysis, ATP regeneration and

[6]liquid chromatography – mass spectrometry

energy metabolism in PSE breast could be related to the quality differences between normal and PSE meat (Nair et al., 2017).

It was demonstrated that 15 different proteins were differentially expressed between PSE and normal breast meat. In the study which proved this, male Hubbard x Cobb 500 broilers were raised *ad libitum* feed and water for 8 weeks. The normal breast samples where characterized by a 24h postomortem pH of 5.8-6.2, while PSE breast samples had 5.4-5.7 (Schilling *et al*., 2017). Phosphoglycerate kinase and beta-enolase where over-abundant in PSE meat, indicating an increased glycolytic activity and lower pH. Pyruvate kinase M type was over-abundant in PSE meat, being negatively correlated with the pH (24 h).

Another defect is the woody breast. This has decreased yields due to reduced water holding capacity, being pale, less red and more yellow than normal breast meat. In addition, it was characterized by a greater weight and a larger cross-sectional area. It typically has increased degenerative and atrophic fibers, mononuclear cell infiltration, lipidosis, and interstitial inflammation. In comparison to normal breast muscle, the woody breast contains an overabundance of myosin regulatory light chain 2 and 14-3-3 protein gamma. Also, increased abundance of serum albumin indicates increased oxidative stress in muscle cells from woody breast meat. Protein deglycase-DJ-1 was overabundant as well in the woody breast meat, this indicating both an increased need for deglycation of amino acids and oxidative stress. Phosphoglyceratemutase 1 was six times as abundant in woody breast meat in comparison to normal breast meat. This enzyme converts 3-phosphoglycerate into 2-phosphoglycerate within the glycolysis pathway (Schilling et al., 2017).

(b) Adulteration

The application of proteomics on contaminated meat is not only a matter of microorganisms or adulteration with meat/gelling agents from other species, but it also involves detection of performance enhancing agents, which are illegally used in different livestock, with the purpose of increasing food conversion and lean meat production (D'Alessandro and Zolla, 2012a). Besides chromatographic, electrophoretic and mass spectrometric approaches, it has been observed a differential protein pattern in meat from different animals, which allows easing the task of those agencies targeting adulteration frauds.

Meat proteins applications using proteomics can be:

(a) Detection of mechanically recovered meat;
(b) Detection of turkey meat in beef and pork;
(c) Detection of chicken meat in raw meat mixtures (burgers, sausages) (Kvasnicka, 2003).

Mechanically recovered meat is cheaper than raw meat and is been usually incorporated into many meat-derived products. EU regulations exclude mechanically recovered meat from the definition of meat, therefore analytical procedures are needed to differentiate it from hand-deboned meat.

In a study, Surowiec et al. (2011) showed that it is possible to separate intact high and low abundance proteins from both hand-deboned and mechanically recovered meat, which can be identified by LC-MS/MS. The methodology makes it possible to identify proteins that could serve as MRM biomarkers in food products. As an example of such proteins detection of potential chicken MRM markers – hemoglobin subunits and those similar to myosin-binding protein C were found. The next step will include validation of results in meat samples obtained from different producers and application of the methodology for detection of MRM in meat mixtures. The advantage of this approach compared to those previously used, based on SDS-PAGE separation or capillary electrophoresis is that OFF-GEL fractionation is fast and repeatable and fractions can be directly analyzed by quantitative LC-MS/MS techniques, resulting in higher selectivity, accuracy and ease of use. A proteomic approach was used for the identification of chicken meat that was mechanically recovered and hand deboned. Results suggested that the amount of haemoglobin can be used as a marker to differentiate mechanically recovered chicken meat from deboned chicken meat (Schilling et al., 2017).

TM1 (one of the muscle fiber type-related proteins) was observed as a commonly distributed protein in bovine, pork, chicken and duck meat. Using this as a marker, LC-MS/MS

spectra allows the identification of each species from fresh meat as well as meat mixtures. Better biomarkers yet were the proteins identified as TnI, enolase 3, LDH and TPI. These were found in all four types of meat samples and could be used as discrimination of mammals from poultry. Moreover, species-specific spectra from these proteins can be peptide markers for the identification of each species following additional analysis by LC-MS/MS. In conclusion, discrimination of meat species from fresh meat and their mixtures is possible by comparing the electrophoretic mobility and through immunoblotting and LC-MS/MS analysis, and we confirmed that commonly distributed proteins in all species can also be candidates for meat discrimination (Kim et al., 2017).

(c) Metabolic changes due to diet

On broiler chickens' meat, proteomic tools were helpful in evaluating the effects of dietary methionine on breast meat accretion and protein expression in the skeletal muscles of broiler chickens. From the 190 individual proteins which were identified in the *pectoralis major* muscle tissue, three correlated with methionine deficiency in the diet (Corzo et al., 2006).

CONCLUSIONS

Proteomics has the potential to offer many details about not only the development of the chicken embryo, but also about the undergoing processes of muscle development during the breeding cycle. Also, it can be successfully used in muscle quality evaluation, either when identifying the underlying causes of defects, as well as the evaluation of colour or tenderness. Furthermore, proteomic analysis has been successfully used in investigating metabolic modifications on muscle level due to diet changes, as well as identifying cases of adulteration, when poultry meat is found mixed with other types of meat, in ready to eat food products.

From these points on, many directions can be further approached considering the undergoing research, while also taking into consideration the existent ones, which can still be explored while applied on the other poultry species.

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