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Omics integrating physical techniques: Aged Piedmontese meat analysis

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Abstract

Piedmontese meat tenderness becomes higher by extending the ageing period after slaughter up to 44 days. Classical physical analysis only partially explain this evidence, so in order to discover the reason of the potential beneficial effects of prolonged ageing, we performed omic analysis in the *Longissimus thoracis* muscle by examining main biochemical changes through mass spectrometry-based metabolomics and proteomics. We observed a progressive decline in myofibrillar structural integrity (underpinning meat tenderness) and impaired energy metabolism. Markers of autophagic responses (e.g. serine and glutathione metabolism) and nitrogen metabolism (urea cycle intermediates) accumulated until the end of the assayed period. Key metabolites such as glutamate, a mediator of the appreciated umami taste of the meat, were found to constantly accumulate until day 44. Finally, statistical analyses revealed that glutamate, serine and arginine could serve as good predictors of ultimate meat quality parameters, even though further studies are mandatory.
1. Introduction

The Piedmontese is a typical breed of cattle from the region of Piedmont, in north-west Italy. The breed originated when migrating Zebu (Bos indicus) cattle crossed with the autochthonous Aurochs, approximately 25 thousand years ago (Piedmontese – Origins of Breed. Breeds of Livestock, 2014). However, processes of natural selection and domestication, especially from the late nineteenth century, resulted in the selection of characteristic postpartum hypertrophic muscle growth (‘‘double muscling’’), a peculiarity that stems from the inherited inactivation of the myostatin gene and favours muscle growth in this breed (Wheeler, Shackelford, Casas, Cundiff, & Koohmaraie, 2001).

Piedmontese has been historically considered a triple feature/attribute/characteristic (meat, milk and work). In 1976 the Piedmontese breed became a specialised variety for meat. The individuals reach the average slaughter weight (males 550–650 kg, females 400–450 kg) in about 14–18 months. The Piedmontese breed is mainly known for its superior yields of lean and tender meat. In comparison to other breeds, the double-muscled Piedmontese meat is characterised by higher water and protein contents. Normally the intramuscular fat content is about 1% or lower. Consequently, the triacylglycerol content is greatly reduced, resulting in lower fat deposition, with a positive increase of the polyunsaturated/saturated fatty acid ratio (Brugiapaglia, Lussiana, & Destefanis, 2014). The meat of the hypertrophied Piedmontese animals is also very tender, because of a large reduction in muscle collagen and a lower proportion of stable non-reducible cross-links (Destefanis, Barge, and Brugiapaglia, 1993; Destefanis, Brugiapaglia, and Barge, 1993). As a result, the Piedmontese is amongst the most important Italian autochthonous beef breed, and it contributes for 37% to the beef production and for about 50% to the gross sealable product in Piedmont, with approximately 300,000 heads of cattle (Destefanis, Barge, et al., 1993; Destefanis, Brugiapaglia, et al., 1993).

Tenderness is a key factor influencing consumers’ repurchase intention, and biochemical models have been proposed over the years to describe the main events driving muscle to meat conversion and meat tenderization processes (Ouali et al., 2006; D’Alessandro & Zolla, 2013a). Overall, the complexity of the process of muscle to meat conversion process can be summarized in three main steps, with (i) a short pre-rigour phase during which muscle still remains excitable; (ii) the rigour phase, during which high energy phosphate compounds (ATP, phosphocreatine) and glycogen are exhausted, while tissue reaches its
maximum toughness; and (iii) the post-rigour tenderizing phase, largely depending on ageing duration and temperature, muscle types, individual animals and animal species (Becila et al., 2010). During phase three, tenderization is driven by the activity of proteases (calpains, cathepsins, proteasomes, caspases, serinpeptidases and metalloproteases) on skeletal muscle (Ouali et al., 2006; D’Alessandro & Zolla, 2013a). Other than proteolysis, non-enzymatic aspects such as temperature, pH, calcium concentration, sarcomere length, and connective tissue/collagen content of the muscles can all affect meat quality, as these variables have an impact on proteolytic activity in the muscle (Ouali et al., 2006; D’Alessandro & Zolla, 2013a).

During the last eight years, several Authors (Becila et al., 2010; Kemp & Parr, 2012) have produced compelling evidence about the likely involvement of apoptotic mechanisms in the processes driving meat tenderization. More recently, such theory has been further confirmed and expanded by pondering the resemblance of the biochemical environment in post mortem muscles that are induced through the process of animal’s slaughter and exsanguinations to the hypoxic/ischaemic conditions that have been investigated in other biological models, such as in the case of neuronal or cardiac ischaemia and reperfusion injury (Solaini, Baracca, Lenaz, & Sgarbi, 2010). In such cases, hypoxia is accompanied by altered mitochondrial metabolism (mitochondrial uncoupling) and production of reactive oxygen species (ROS), utterly promoting either apoptotic or autophagic events (Sierra & Oliván, 2013), and affecting meat physiological parameters, such as colour (D’Alessandro & Zolla, 2013a).

While the molecular mechanisms underlying muscle to meat conversion and meat tenderization have yet to be fully disclosed, precious insights have been gained during the last few years upon the introduction of omics technologies in the field of farm animal proteomics (D’Alessandro & Zolla, 2012; Ibáñez et al., 2013) and, in particular, in meat science (D’Alessandro & Zolla, 2013b), and their integration with standard physical assays to investigate meat quality parameters (for example, on pig and bovine meat quality (D’Alessandro, Gevi, & Zolla, 2011; D’Alessandro, Marrocco, Zolla, D’Andrea, & Zolla, 2011; D’Alessandro et al., 2012).

Biochemical evidence accumulated so far has provided a scientific rationale supporting the beneficial effects on meat tenderness of prolonged ageing for a limited time span (from 7 to 15 days, on average), while no
significant improvements are gained by further extending such period. The rationale behind such conclusion is that a series of factors end up negatively affecting protease activities in the long term, including pH lowering, altered cation homeostasis, oxidative stress and proteolytic cleavage mediated by cross-interactions (Ouali et al., 2006; D’Alessandro & Zolla, 2013a).

Nevertheless, recent empirical evidence in Piedmont farms has suggested the possibility to obtain highly marketable tender meat from cull cows simply by extending the ageing period up to more than forty days. While the producers insisted on the effectiveness of their approach, firmly supported by consumers’ appraisal of their products, no scientific experimental evidence has been produced to underpin their statements.

Therefore, we hereby investigated whether the prolonged ageing (up to 44 days at 1 _C) of Piedmontese cull cow meat was actually correlated to improved palatability (mostly affected tenderness, juiciness) and desirability (colour), as gleaned by standard biomechanical assays (Warner Bratzler Shear force measurement – Wbs; water holding capacity – WHC; Minolta values).

Physiological/mechanical assays were then supported by ‘Omic’ analyses, as to delve into the biochemical events driving muscle to meat conversion in Piedmontese Longissimus thoracis in a time course-wise fashion. Particularly, the effects of long ageing were studied in the Longissimus thoracis muscle (by monitoring 5 different time points, including day 0, 1, 10, 17 and 44) examining the main biochemical changes by means of mass spectrometry-based metabolomics and proteomics. In order to correlate and integrate ‘omics’ reading of biochemical changes regarding prolonged-stored meat with standard meat quality assays, we performed principal component analyses (PCA) and Pearson’s correlations between omics and physiological/mechanical results.

2. Materials and methods

2.1. Animals

Ten Piedmontese cull cows between 4 and 13 years old, were raised in farms belonging to Consorzio La Granda (CN, Italy), located in Piedmont, a north-west region in Italy. All the animals were slaughtered in an
industrial slaughterhouse, the carcasses were stored in a chilling room at 2 °C. Average slaughter weight of the carcasses was 389.99 ± 6.18 kg. Carcasses were transported to a meat processing plant on post-slaughter day 1. *Longissimus thoracis* (LT) muscle (that is, the thoracic region of *Longissimus dorsi*) was removed and stored in a cooler at 1°C (steady or dynamic) and a relative humidity of 78%. At 0, 1, 10, 17 and 44 days of ageing, a 10 cm section was removed from the LT muscle and used for all subsequent analyses.

2.2. Classical standard analyses

Classical standard analyses on meat samples include the measurement of microbial safety, together with physical and chemical meat parameters, in order to assess meat safety and quality.

2.2.1. Microbiological analysis

The hygienic status of meat samples were assessed through the Total Bacteria Counts (TBC), the Enterobacteriaceae counts analysis and the standardised methods ISO 4833 (2004) and AFNOR NFV08-054 (1999). For the assessment of faecal contamination, *Escherichia coli* and *Listeria monocytogenes* levels were also measured according to ISO 16649-2:2001 and ISO 11290-1:1996/Amd1:2004 (2004) procedures, respectively. Colonies with a typical *L. monocytogenes* appearance were identified using a species-specific PCR, according to D’Agostino et al. (2004).

2.2.2. Chemical and physical analysis

The evaluation of meat quality at d0 and d1 was assessed carrying out the following analysis:

– pH measurements at 1–3 h after slaughter and 24 h after slaughter, made by a Crison pH metre with an Ingold Spear electrode and automatic temperature compensator.

– Sarcomere length according to the diffraction method by Cross, West, and Dutson (1981). The diffraction patterns from muscle samples compressed between glass microscope slides were obtained using a helium–neon laser (632.8 nm) as the light source.

– Haem iron content (lg/g muscle) according to Hudzik (1990).
– Water, protein and ether extraction contents (AOAC, 1970). We applied the Kjeldhal method to achieve the determination of nitrogen using a Buchi System apparatus (Buchi Labortechnik, Flawil, Switzerland); crude protein was calculated by multiplying N X 6.25. We used a Buchi extraction system for the determination of lipids content, according to the Soxhlet method.

– Lightness (L), redness (a) and yellowness (b) on meat samples at d1, d10, d17 and d44. We used a Minolta CR-331C ChromaMeter (Minolta Camera Co., Japan) (Petracci, Betti, Bianchi, & Cavani, 2004) in the CIELAB space (CIE, 1978) calibrated on the D65 illuminant. The measures were carried out after 1 h of blooming on a 3 cm thick steak. Chroma and hue were calculated from a and b values according to expressions: 
\[ C = (a^2 + b^2)^{1/2} \] and hue \( h = \tan^{-1}(b/a) \).

– Grau and Hamm (1953). 300 mg of meat were put on the centre of a filter paper contained between two plates of Plexiglass; a constant pressure was applied for 5 min, and the areas of the meat film layer (M) and of the total surface area (T) were calculated. The M/T ratio was then calculated. At the end of each period of ageing, the aged samples were frozen at -25 °C. After a 12 h of slow thawing period at 4 °C, the following analyses were carried out:

– Thawing loss on a 3 cm thick steak was calculated as the% of weight loss before and after thawing.

– Cooking loss was measured as the percentage of weight reduction of the cooked sample (3 cm thick steak, vacuum sealed in a polyethylene bag and heated in a water bath to an internal temperature of 70 °C) compared with the raw sample (Barton-Gade et al., 1994).

– Warner Bratzler shear force (Wbs) (N) on samples of 1 X 1 X 3 cm (height X width X length), cut parallel to muscle fibres and obtained from the steaks used for the determination of cooking losses. The Wbs values were obtained through the use of an Instron Universal Testing machine (Model 5543, Instron Corp., Minnesota, USA) (Peachey, Purchas, & Duizer, 2002) equipped with a triangular Warner Bratzler blade and crosshead speed set at 200 mm/min (AMSA).

– Hardness as the force required to drive a 10 X 10 mm square compression cell on a sample of raw meat. The cell was equipped with two lateral walls to limit free strain of the sample to a direction parallel to the
myofibres (Lepetit & Culioli, 1994). Meat samples 1 X 1 X 3 cm were compressed at 200 mm/min perpendicular to the fibre axis to 20% (H20%) and 80% (H80%) of their original height.

2.2.3. 1D SDS–PAGE LC–MS/MS

Samples of LT from 10 Piedmontese cows were collected at day 0, 1, 10, 17 and 44 after slaughter. Sample preparation and solubilisation were performed by slight modification of the SWISS-2D PAGE sample preparation procedure (Hoogland, Mostaguir, Sanchez, Hochstrasser, & Appel, 2004). Frozen samples (approximately 6 mg per sample) were crushed in a mortar containing liquid nitrogen. After grinding, the samples were resuspended in an extraction buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 40 mM Tris, 0.1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.5), 2% (v/v) protease inhibitor cocktail (Sigma– Aldrich, Basle, Switzerland), 2 mM phenylmethanesulfonylfluoride (PMSF) and 1 mM dithiothreitol (DTT). After incubation with agitation at 4 °C for 60 min, samples were centrifuged at 17,000g for 20 min at 4 °C and the supernatant collected. The protein concentration of each group was determined using 2D Quant kit (GE Healthcare Life Sciences, UK) according to the manufacturer’s instructions.

1D SDS–PAGE were performed according to the Laemmli (1970) method, as previously reported (Hwang, 2004). Gels were digitalized using a Chemi-Doc imaging system (Bio-Rad Hercules, CA, USA). The densitometric image analysis was conducted by Image Lab software. Bands which were significantly different between groups (time course analysis) were excised and trypsin digested (Shevchenko, Wilm, Vorm, & Mann, 1996) and underwent subsequent identification by nano-HPLC–MS/MS. The nano-HPLC system consisted of a split-free nano-flow liquid chromatography system (EASY-nLC II, Proxeon, Odense, Denmark) coupled to a 3D-ion trap (AmaZon ETD, Bruker Daltoniks, Germany) (D’Alessandro, Gevi, et al., 2011; D’Alessandro, Marrocco, et al., 2011) equipped with an online ESI nano-sprayer (the spray capillary was a fused silica capillary, 0.090 mm o.d., 0.020 mm i.d.) (D’Alessandro, Gevi, et al., 2011; D’Alessandro, Marrocco, et al., 2011). For all experiments, a sample volume of 15 IL of tryptic digest was loaded by the autosampler onto a homemade 2 cm fused silica precolumn (100 lm I.D.; 375 lm O.D.; Reprosil C18- AQ, 5 lm, Dr. Maisch GmbH, Ammerbuch-Entringen). Sequential elution of peptides was accomplished using a flow rate of 300 nL/ min and a linear gradient from Solution A (2% acetonitrile; 0.1% formic acid) to 50% of Solution B (98% acetonitrile; 0.1% formic acid) in 40 min over the precolumn, in-line with a homemade 15
cm resolving column (75 lm I.D.; 375 lm O.D.; Reprosil C18- AQ, 3 lm, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). The acquisition parameters for the instrument were as follows: dry gas temperature, 220 °C; dry gas, 4.0 L min⁻¹; nebulizer gas, 10 psi; electrospray voltage, 4000V; high-voltage end-plate offset, _200 V; capillary exit, 140 V; trap drive: 63.2; funnel 1 in, 100 V, out 35 V and funnel 2 in, 12 V out 10 V; ICC target, 200,000; maximum accumulation time, 50 ms. The sample was measured with the “Enhanced Resolution Mode” at 8100 m/z per second (which allows monoisotopic resolution up to four charge stages), polarity positive, scan range from m/z 300 to 1500, 5 spectra averaged, and rolling average of 1. The “Smart Decomposition” was set to “auto”. Acquired ETD/CID spectra were processed in DataAnalysis 4.0, and deconvoluted spectra were further analysed with BioTools 3.2 software and submitted to Mascot search program (in-house version 2.2, Matrix Science, London, UK). The following parameters were adopted for database searches: NCBInr database (release date 22/10/2011; 15,670,865 sequences; 5,387,755.057 residues); taxonomy = Mammalia; peptide mass tolerance of ±0.3 Da; fragment mass tolerance of ±0.3 for CID ions; enzyme specificity trypsin with 2 missed cleavages considered; fixed modifications: carbamidomethyl (C); variable modifications: oxidation (M).

2.3. Metabolomics

Metabolomic analysis has been performed as previously reported, with minor modifications (D’Alessandro, Gevi, et al., 2011; D’Alessandro et al., 2012). Metabolites from each Piedmontese individual were extracted from 50 mg of meat at 0, 1, 10, 17, 44 days from slaughtering. Samples were crushed in a mortar containing liquid nitrogen and extracted in 1 ml of ice cold methanol: acetonitrile:water (50:30:20). Samples were vortexed for 30 min at max speed at 4 °C and then centrifuged at 16,000g for 15 min at 4 °C. Supernatants were thus collected for subsequent metabolomics analysis, while insoluble debris was discarded. Samples were thus loaded onto a rapid resolution HPLC system (LC Packings, DIONEX, Sunnyvale, CA, USA) as to perform chromatographic separation of hydrophilic metabolites. The system featured a binary pump and vacuum degasser, well-plate autosampler with a six-port micro-switching valve, a thermostated column compartment. A Phenomenex Luna 3 lm HILIC 200A (Torrance, CA, USA) (150 X 2.0 mm), protected by a guard column HILIC 4 X 2.0 mm ID (Phenomenex) was used to perform metabolite separation over a phase B to phase A gradient lasting 35 min. In details, we set the following LC parameters: injection volume,
20µL; column temperature, 25 °C; flow rate of 0.2 mL/min. The LC solvent gradient and timetable were identical during the whole period of the analyses. A 0–100% linear gradient of solvent B (100% acetonitrile + 10 mM ammonium acetate) to A (double distilled 18 mΩ water + 10 mM ammonium acetate) was employed over a 35 min gradient, divided into a 5 min hold in 95% B, a linear gradient to 95% A lasting 20 min, followed by a 95% A hold of 5 min, returning to 95% B in 1 min and a 5 min post-gradient solvent 95% B hold. Metabolomics analyses were performed through direct elution onto an electrospray hybrid quadrupole time-of flight mass spectrometer MicroTOF-Q (Bruker-Daltoniks, Bremen, Germany) equipped with an ESI-ion source. Instrument calibration was performed externally every day with a sodium formate solution consisting of 10 mM sodium hydroxide in 50% isopropanol:water, 0.1% formic acid. External mass scale calibration was performed twice a day through direct automated injection of the calibration solution by a 6-port divert-valve. Mass spectrometer runs were exported into mzXML files and analysed through the software MAVEN (Clasquin, Melamud, & Rabinowitz, 2012) for correct metabolite assignment, on the basis of absolute intact mass (within a 10 ppm window) against the KEGG database (Kanehisa & Goto, 2000), and expected retention times on the basis of metabolite chemical properties.

2.4. Statistics

Data elaboration was performed with Excel 2007 (Microsoft), XLStat 2013.6.3 (Addinsoft SARL) and GraphPad Prism 5.0 (Graph-Pad Software Inc). Pearson’s correlations coefficients and principal component analyses were performed via the XLStat suite on the mean results for each independent assay on each distinct animal. Results were plotted as in D’Alessandro et al. (2012). In correlation tables, absolute values close to 0 indicate low correlation, while |values| ~ 1 indicate high correlation.

3. Results and discussion

The hypothesis concerning the potential beneficial effects of prolonged ageing on meat quality (especially in terms of tenderness), as reported by the producers and underpinned by consumers’ appraisal, was tested by performing analysis for physical (Warner Bratzler shear force, myofibrillar degradation, sarcomere length, water holding capacity, Minolta values) and microbiological parameters during a carcass ageing period of 44 days at 1 °C.
3.1. Microbiological results

Extended ageing period at 4 °C might bring about concerns about the preserved safety of Piedmontese meat. Therefore, in order to test Piedmontese meat for microbial contamination, we carried out the traditional Total Bacterial Counts and Total Enterobacteriaceae Counts. Results (Supplementary Table 1) excluded any possible bacterial contamination, owing to the correct application of the Good Handling Practice (GHP) and Good Manufacturing Practice (GMP) through the cold chain. Furthermore, we have registered the total absence of *L. monocytogenes* and *E. coli* in all the tested samples during the whole ageing period (up to 44 days), which is supportive of the preserved safety of longer aged meat.

3.2. Physical results

Results for physical parameters are reported in Supplementary Tables 2–4. In terms of colour, meat was not negatively affected by the prolonged ageing (Supplementary Table 2). The overall WHC, as expected (D’Alessandro et al., 2012; Huff-Lonergan & Lonergan, 2005), underwent through a progressive decrease with ageing (Supplementary Table 2). The results of Wbs measurements indicated the improved tenderness of Piedmontese meat when subjected to ageing extension up to 44 days, thus justifying consumers’ appraisal for the resulting product. Regarding 80% compression, the absence of significant differences due to ageing was not unexpected because there is no evidence supporting influences of ageing on the mechanical strength of connective tissue, so these data do not contrast the prolonged ageing procedure. Data regarding 20% compression do not show tenderness improvement, apparently indicating a myofibrillar degradation steady-state from day 9, but the following proteomics and metabolomics data give a deeper glance into biomolecular changes driving meat ageing up to 44 days, granting compelling evidences about an ongoing myofibrillar fragmentation (and so about the consequent improved tenderness).

3.3. Proteomic results: prolonged ageing affected proteome stability starting from day 10 onwards, suggesting alterations to structural protein integrity and metabolic deregulation

One of the key events driving muscle to meat conversion and tenderization processes is related to the progressive alteration of the muscle protein stability. These alterations tend to influence both meat tenderness and its capacity to retain water moisture, thus affecting meat juiciness (D’Alessandro & Zolla, 2013a).
During the last decade, proteomics technologies have been extensively applied to investigate the influence of post mortem events on muscle proteins, as it has been extensively reviewed (D’Alessandro & Zolla, 2013b). Amongst proteomics technologies exploited in this research endeavour, gel electrophoresis has provided significant insights (Hwang, 2004). In this view, 1DE and 2DE gel electrophoresis have become mainstream approaches to the muscle proteome. However, while 2DE allows to separate thousands of protein spots on the basis of protein molecular weights and isoelectric points and 1DE only enables separation of approximately 50 bands in a 15 cm gel, 2DE gels are susceptible to technical issues related to the intrinsic difficulties to resolve high molecular weight and more hydrophobic proteins (which is often the case when dealing with structural components of muscle fibres, such as myosin heavy chains). Such inconveniences could be easily overcome with simpler and faster 1DE approaches, thus fostering a second youth of this analytical strategy during the last few years (Cottingham, 2010).

Application of 1DE, coupled to statistical analyses of digitized images, trypsin digestion of statistically significant (p < 0.05, foldchange > 1.5) differential bands and nano-LC–MS/MS-based identification yielded the individuation of six bands decreasing while four increasing thereon from post mortem day 10 up to day 44 (Fig. 1, Supplementary Table 5). Differential proteins were identified either as structural proteins (a-actinin-3, tropomyosin a-1, troponin T and I, profilin-1) or metabolic enzymes (glycogen phosphorylase, 6-phosphofructokinase, fructose-bisphosphate aldolase A, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, L-lactate dehydrogenase A, creatine kinase M-type, AMP deaminase 1), together with residual haemoglobin and small HSP (a-crystallin B chain, HSP-b5) (Supplementary Table 5). These results are consistent with proteomics literature about muscle to meat conversion events (D’Alessandro & Zolla, 2013a, 2013b), documenting changes in the sarcoplasmic proteins, myofibrillar proteins (such as partial dissociation of actomyosin, cleavage of disulphide linkages, depolymerization of F-actin filaments, cleavage of myosin filaments, disorganisation of Z-bands and troponin–tropomyosin complex) and sarcolemma. In particular, progressive decrease of structural proteins (especially from post slaughter day 10 – Supplementary Table 5) is consistent with the expected protease-mediated degradation of myofibrils (also confirmed by decreasing Wbs). This is consistent with previous proteomics reports on the Maremmana beef breed (D’Alessandro et al., 2012), where significant correlations were observed between Wbs and structural protein degradation. This holds relevant pitfalls not only in relation to tenderness, but also in terms of WHC, since failure to
degrade myofibrils post mortem after the rigour phase would result in cell shrinkage and thus reduced intra-myofibrillar space, leading to subsequent drip loss (Huff-Lonergan et al., 2005). Correlations between HSPs and bovine meat tenderness is now held as a consolidated fact (D’Alessandro et al., 2012; Guillemin et al., 2011), owing to their likely involvement in protease activity preservation, protein protection from proteolysis (D’Alessandro & Zolla, 2013b) and mediation of apoptotic cascades (Beere, 2005). This was hereby further confirmed by the alteration of α-crystallin levels in Piedmontese meat during the ageing period (Supplementary Table 5). Traces of accumulating haemoglobin (as gleaned through proteomics approaches – Supplementary Table 5) in tenderizing bovine meat have already been reported in the past in relation to accumulation of oxidative stress triggered by hemicin iron-mediated reactions (D’Alessandro et al., 2012).

The main outcome of the rapid proteomics overview gained via 1DE is that post mortem events yield the progressive increase in target key metabolic enzymes involved in glycogen mobilisation/ energy metabolism regulation (glycogen phosphorylase and AMP deaminase 1), glycolysis (6-phosphofructokinase, fructose-bisphosphate aldolase A, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, L-lactate dehydrogenase A), creatine/phosphocreatine shuttle (creatine kinase M-type). Metabolic alteration in post mortem muscles is not an unexpected finding (D’Alessandro, Gevi, et al., 2011; D’Alessandro, Marrocco, et al., 2011; D’Alessandro et al., 2012), since depletion of energy rich compounds is a hallmark of the pre-rigour to rigour stage, and impaired glycolysis ensues as pH declines below a certain threshold resulting in negative inhibitory feedback on glycolytic enzymes activities (Ouali et al., 2006; D’Alessandro & Zolla, 2013a). Of note, we observed a time-dependent decrease in the levels of glycogen phosphorylase and AMP deaminase 1, two rate-limiting enzymes of the glycolytic process as they mediate glycogen conversion to glucose 1-phosphate and AMP conversion to IMP. Indeed, depletion of AMP impairs the possibility to restore ADP reservoirs and thus hampers progression of glycolysis (Scopes, 1974). Similarly, Van Laack, Kauffman, and Greaser (2001) indicated that in pork muscle, glycogen phosphorylase activity explains 28% of the differences in pHu, while a higher amount of AMP-deaminase was associated with a 10% higher pHu in beef muscle. It is also worthwhile to stress that band J, increasing during the ageing period from post mortem day 10 onwards, was identified as glyceraldehyde 3 phosphate dehydrogenase, a 36 kDa protein, even if the band mapped at approximate 6.5 kDa apparent MW (based upon the MW ladder markers – Fig.
1. This is suggestive of the likely storage-dependent fragmentation of this key glycolytic enzyme in the LT of Piedmontese cows.

3.4. Metabolomics highlighted extended lactate accumulation in Piedmontese meat, accompanied by increased serine and nitrogen metabolism

Little is known about actual changes to the metabolome after slaughter. Metabolic changes in the post mortem muscle (D’Alessandro & Zolla, 2013b) might underpin the peculiarities related to the beneficial effects of prolonged ageing of carcasses of Piedmontese cattle on tenderization. The recent availability of analytical platforms based upon HPLC–MS instrumentation and bioinformatics tools for chromatogram analyses has paved the way for the introduction of metabolomics platforms in the field of meat science (D’Alessandro & Zolla, 2013a, 2013b). In this view, we decided to complement physical analyses with mass spectrometry- based metabolomics assays, and to correlate results through statistical interpretations. Metabolomics analyses were performed to monitor the levels of metabolites involved in energy metabolism, redox metabolism and Krebs cycle, serine and nitrogen metabolism (Fig. 2). Analyses were performed throughout the whole duration of the ageing period, at post slaughter day 0, 1, 10, 17 and 44. In living animals, skeletal muscles are amongst major oxygen consuming tissues, since muscle mitochondria show a higher respiration rate than those in liver, kidney and brain (Rolfe, Hulbert, & Brand, 1994). However, sudden blood flow arrest and exsanguinations after slaughter promote anoxia, drastically impairing energy production via oxidative metabolism in muscle cells, especially in terms of ATP production in the mitochondria (Sierra & Oliván, 2013). As a result, anaerobic glycolysis ensues, resulting in glycogen mobilisation and glucose consumption to produce ATP, while anaerobic fermentation of pyruvate results in the progressive accumulation of lactate. However, ongoing glycolysis in the muscle results in pH lowering which in turn plays an inhibitory feedback on glycolytic enzyme activity (Ohlendieck, 2010). This results in the progressive inhibition of glycolytic fluxes in the long term and pH reaching a lower threshold followed by a progressive stabilization. In the present model, we observed a time-dependent accumulation of lactate, reaching a climax between day 17 and 44 after slaughter (Fig. 2). This is suggestive of ongoing glycolysis during the first 17 days of storage, resulting in the intermediate accumulation of early glycolytic metabolites during the first two post-slaughter weeks (glucose 6-phosphate, fructose bisphosphate, glyceraldehyde
phosphate – Fig. 2). Decreased levels of these metabolites by day 44, together with the plateau reached by lactate levels, are indicative of the likely consumption of glycolytic precursors, such as through the exhaustion/impaired mobilization of glycogen (in agreement with the observed decrease in the levels of glycogen phosphorylase – Supplementary Table 5) or the reduced activity of glycolytic enzymes acting on triose phosphates (downstream to glyceraldehyde 3-phosphate and upstream to phosphoglycerate – Fig. 2) soon after post mortem day 10. In this view, it is worthwhile to recall the observed increased fragmentation of glyceraldehyde 3-phosphate dehydrogenase since ageing day 10, as gleaned via 1DE (Supplementary Table 5). Progressive depletion of glycerol phosphate (Fig. 2) might indicate a blockade in anabolic reactions, such as those involved in triglyceride biosynthesis via the generation of glycerol backbones. While glycolysis represents the main source to sustain the muscle under anaerobiosis in the long term, the phosphocreatine shuttle (fuelled by the activity of creatine kinase M, hereby progressively increasing from day 10 onwards – Fig. 1, Supplementary Table 5) sustains cell energy demands in the short term, as it relies on the accumulation of energy tokens in the form of phosphocreatine. Metabolomics results revealed that while creatine levels were apparently stable up until post-slaughter day 17, day 44 corresponded to an evident drop in the levels of this metabolite. Conversely, phosphocreatine underwent a minor increase and stabilization after post mortem day 0, while its levels dropped back below post slaughter values by day 44. These findings might suggest a progressive deregulation of the phosphocreatine shuttle. Muscle responses to anoxia also imply mitochondrial uncoupling, as the lack of oxygen results in the mitochondria-dependent promotion of reactive oxygen species (ROS) accumulation, including superoxide anion and hydrogen peroxide. This is consistent with the role of mitochondria as mediator of a wide range of other cellular processes, including signal transduction, cell cycle regulation, oxidative stress, thermogenesis and cell death (Sierra & Oliván, 2013). In previous studies, we reported increases in the levels of antioxidant enzymes, such as superoxide dismutase (SOD), and highlighted a correlation between oxidative stress (in the form of oxidised glutathione – GSSG), SOD levels and Chianina Longissimus dorsi tenderness (D’Alessandro et al., 2012). ROS generation was individuated in two main causes: (i) residual myoglobin/haemoglobin (D’Alessandro et al., 2012) (hereby haemoglobin fragments accumulated from day 10 onwards in band J – Fig. 1, Supplementary Table 5); and (ii) impaired mitochondrial respiration. The rationale underpinning this hypothesis is that inhibition or disorders of mitochondrial respiration promotes ROS production. This is
consistent with recent reinterpretation of post mortem events in the light of the role of hypoxia/anoxia on mitochondrial activity (Sierra & Oliván, 2013), in analogy to the role of these organelles in other models of prolonged exercise, starvation, hypoxia or ischaemia/reperfusion (I/R) injury (Solaini et al., 2010). ROS accumulation in the muscle might promote proteolysis on one hand, while impairing meat colour (D’Alessandro & Zolla, 2013b) by promoting carbonylation and other non-enzymatic oxidative modifications on the other (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004). In the present study, reduced glutathione levels remained stable up until storage day 17 and decreased by day 44 (Fig. 2), even if no significant alteration in terms of meat colour (especially lightness – Supplementary Table 5) was observed. Conversely, GSSG accumulated significantly soon after one day from slaughter, even if its levels did not further increase throughout storage duration (Fig. 2). As to indirectly estimate GSH biosynthesis, the levels of glutamate and its precursor glutamine were monitored throughout the ageing period, the former accumulating constantly until day 44, while the latter only spiked at day 10 and remained constant for the rest of the storage period (Fig. 2). Glutamate accumulation in stored meat might stem from advanced proteolysis, which would release aminoacids, and glutamine carboxylation. At the same time, glutamate might hold beneficial effects in terms of meat quality, since it has been indicated as a positive contributor to meat palatability as a mediator of the umami taste (Bellisle, 1999). Maintenance of GSH levels up until storage day 17 might be achieved by the observed simultaneous up-regulation of the pentose phosphate pathway (PPP) (especially phosphogluconolactone – Fig. 3), a metabolic pathway devoted to the generation of pentose phosphate compounds (e.g. ribose phosphate – Fig. 3), as to fuel nucleoside biosynthetic reactions, and the generation of NADPH, a key coenzyme promoting GSSG reduction back to GSH in glutathione homeostasis. While most of NADPH is metabolically generated via the PPP, other enzymatic reactions might contribute to its generation, such as reactions involving cytosolic version of Krebs cycle enzymes isocitrate dehydrogenase and malic enzyme, the latter producing pyruvate, CO₂ and NADPH from malate. Consistently, while Krebs intermediates succinate and fumarate accumulated progressively to storage duration, malate was progressively consumed during the first 17 days after slaughter (Fig. 3). Accumulation of Krebs cycle intermediates fumarate and succinate is also a symptom of uncoupled mitochondrial metabolism (as expected in the anoxic environment of post mortem muscle). However, this metabolic behaviour is apparently restrained by storage day 44, probably because the accumulation of lactate inhibits
the ulterior reduction of pyruvate to lactate, so hampering the oxidation of malate to give pyruvate driven by malic enzyme. It is worth noting that hypoxic environment might promote glycolytic metabolism through accumulation of Krebs cycle “oncometabolites” succinate and fumarate, which trigger succinylation of prolyl hydroxylase, the enzyme responsible for the degradation of Hypoxia Inducible Factor (HIF) 1-alpha. In turn, HIF1a promotes glycolytic metabolism and depresses shuttling of late glycolytic byproducts to the Krebs cycle, by mediating the expression of pyruvate dehydrogenase kinase. (Boulahbel, Durán, & Gottlieb, 2009; Kim, Tchernyshyov, Semenza, & Dang, 2006). Recently, biochemical events driving muscle to meat conversion and meat tenderization have been associated to mitochondrial-dependent (intrinsic) apoptosis (Ouali et al., 2006) or autophagy (Sierra & Oliván, 2013). While apoptosis promotes cell death cascades, autophagic responses imply a finely tuned process that is either committed to rescue the cell from stress or to promote cell sacrifice in the worst case scenario. Cell rescue passes through a process in which eukaryotic cells self-digest part of their cytosolic components to the end of degrading proteins and organelles in order to survive starvation, while maintaining cell homeostasis through the elimination of oxidatively-damaged, aberrant macromolecules and organelles (Sierra & Oliván, 2013). Autophagic responses are mediated by aminoacid sensing via mammalian target of rapamycin (mTOR) and AMPK activity (Kim, Kundu, Viollet, & Guan, 2011), which utterly result in the upregulation of serine biosynthetic pathways (Dando et al., 2013). Notably, precocious activation of AMPK in pigs is associated with the so-called pale soft exudative (PSE) phenotype, which is responsible for poor pig meat quality (poor WHC, pale colour, flat taste) (Shen et al., 2006). Up-regulation of serine metabolism is relevant in that it provides the substrate for glycine and cysteine biosynthesis, via one carbon metabolism and either cysteine or cystathionine synthetase activity, respectively. It is probably worthwhile to recall that, since cysteine and glycine are precursors to GSH, boosting their biosynthesis ends up promoting anti-oxidant potential of the cell as to cope with oxidative stress. Within the framework of extended ageing of meat from Piedmontese cows we expected that, whereas autophagy would be up-regulated (Sierra & Oliván, 2013), serine biosynthesis would have been boosted. As a result, metabolomics analyses outlined a progressive accumulation of serine and its precursor phosphoserine, together with increases in the levels of cysteine precursor deriving from enzyme-mediated reaction of serine with homocysteine, cystathionine (Fig. 3). Protein degradation as a byproduct of protease activity resulted in myofibrillar degradation. Myofibrillar degradation results in the accumulation of protein
fragments of variable length, which are utterly degraded into free aminoacids. In theory, this would in turn promote nitrogen metabolism and the urea cycle, with the accumulation of urea cycle intermediates arginine, ornithine and citrulline (Edmonds, Lowry, & Baker, 1987). Our results are in line with this hypothesis (Fig. 3).

3.5. Statistical analyses: principal component analysis suggested that serine, arginine and glutamate might serve as good predictors of ultimate meat tenderness and juiciness

In order to provide an additional statistical support to the reported data, we decided to perform Pearson’s correlation analysis and principal component analysis of physical and metabolomics results, in agreement with D’Alessandro et al., 2012. Layouts are reported in Table 1 (in grey are highlighted the results from Pearson’s correlations showing absolute values higher than 0.70), while principal component coordinates are summarized in Supplementary Table 4. Finally, results are graphed in Fig. 4 as to highlight group clustering on Cartesian plots for metabolites and physical parameters, including tenderness (Wbs), juiciness (WHC determined through Grau and Hamm, thawing loss and water bath loss) and ultimate pH. At a first glance, it is evident that metabolites in the first quarter (positive:positive quadrant of PC1 and PC2) are characterised by high positive correlation with Grau and Hamm values, while they are strongly negatively correlated to all the other physical parameters (Fig. 4). While Table 1 also highlights the interconnectedness of metabolic pathways (though this is not a novel finding), it also suggests the likely correlation of metabolite levels and the predictability of physical parameters, such as in the case of serine and arginine with Wbs, Grau and Hamm or water bath losses (Fig. 5). However, it is worthwhile to note that some correlations might be affected by single outliers (e.g. Fig. 5F) or rather be biased by the relatively small subset of biological samples assayed in the present study. Of note, PCA and correlations were performed on metabolic and physical assays on the same animals at the same time points, thus strong correlations throughout the whole storage period are suggestive of the likely indirect predictability of physical values through the assessment of metabolites such as serine, arginine and glutamate soon after slaughter. While these results are but preliminary, we believe that this research/applicative aspect will deserve further attention in the future.

4. Conclusions
The Piedmontese breed of cattle is one of the most valuable in Italy, owing to the high carcass yield and good quality of its meat. In the present study, *Longissimus thoracis* muscles from Piedmontese cull cows were assayed for physical, microbiological, proteomics and metabolomics parameters during a carcass ageing period of 44 days at 1 °C. The rationale behind this study was to verify, through accurate and analytical strategies, if the extension of the ageing period up to 44 days, could improve the commercial value of Piedmontese cull cows meats, actually considered less valuable. Routine practice and currently available biochemical knowledge encouraged further research, since no beneficial effects of extended ageing periods over two weeks after slaughter has been hitherto reported. However, physical analyses confirmed the empirical reports by the producer and consumers, while indicating a progressively increased tenderness and decreased water holding capacity of longer stored meat. Quality and safety of meat were confirmed by microbiological analyses. Proteomics and metabolomics confirmed a progressive decline in myofibrillar structural integrity and impaired energy metabolism. From a metabolomics standpoint, it emerged that while energy metabolism appeared to be progressively impaired after post mortem day 17, other markers of autophagic responses (e.g. aminoacid and glutathione metabolism) and nitrogen metabolism (urea cycle intermediates) accumulated proportionally to ageing until post slaughter day 44. Key metabolites such as glutamate, which is known to be related to the umami taste of the meat, were found to constantly accumulate until day 44. Statistical analyses conducted on physical and omics data showed the synergy between classical standard measurements and omics platforms in the disclosure of biochemical changes intervening in ageing meat, revealing that glutamate, together with serine and arginine, could serve as good predictors of ultimate meat quality parameters, even though further studies are mandatory.

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Policies (Ministero delle Politiche Agricole, Alimentari e Forestali). The authors would like to thank Dott. Sergio Capaldo, national coordinator of “Slow Food” for the livestock sector and president of the consortium “La Granda” for providing the sample of longissimus dorsi from Piedmontese cows.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.09.146.

References


Figure and table captions

**Fig. 1.** 1DE gel of protein extracts from the Longissimus dorsi of meat from formerly dairy Piedmontese cows at post slaughter day 0, 1, 10, 17 and 44. Statistically differential bands (p<0.05 ANOVA) are highlighted and indicated with letters from A to J, as detailed in Supplementary Table 5. Molecular weight ladder is presented on the left side of the gel.

**Fig. 2.** Absolute metabolomics quantification (arbitrary ion counts) of metabolites from glycolysis, phosphocreatine shuttle and glutathione homeostasis in the *Longissimus dorsi* of meat from formerly dairy Piedmontese cows at post slaughter day 0, 1, 10, 17 and 44. Data are presented as mean ± SD.

**Fig. 3.** Absolute metabolomics quantification (arbitrary ion counts) of metabolites from pentose phosphate pathway, Krebs cycle, serine metabolism, urea cycle, inosine and hypoxanthine in the *Longissimus dorsi* of meat from formerly dairy Piedmontese cows at post slaughter day 0, 1, 10, 17 and 44. Data are presented as mean ± SD.

**Fig. 4.** Group clustering on Cartesian plots by means of principal component analysis (PCA) for metabolites and physical parameters. PCA is based upon single measurements for physiological values and metabolite levels as variables. Variables (points) that map closely to each other in the same quadrants are highly positively correlated. Variables that map on opposed quadrants (relative distance from the origin of the axes, represented by principal components 1 and 2 – F1 and F2, respectively) are negatively correlated amongst each others. The plot shows that, for example, WBS and pH24 h map closely and are positively related to glycerol phosphate levels, while they are distant from and negatively related to metabolites in the first quadrant of the Cartesian plot, amongst which serine levels.

**Fig. 5.** Plotting of at least 8 different single measurements and interpolation for Warner Bratzler shear force (Wbs), water bath losses and water holding capacity (Grau and Hamm method) in relation to the level of three metabolites (serine, arginine and glutamate) at post slaughter day 0, 1, 10, 17 and 44 in the Longissimus dorsi of meat from formerly dairy Piedmontese cows. Interpolation curves are continuous and linear correlation coefficients (r) are reported in the right portion of the graph.
Table 1 Pearson correlation of metabolites and physical variables.\textsuperscript{a}

\textsuperscript{a}Grey cells: Pearson’s correlations P \leq 0.70.
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