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Muscle composition slightly affects *in vitro* digestion of aged and cooked meat: Identification of associated proteomic markers

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ABSTRACT

Meat is an appropriate source of proteins and minerals for human nutrition. Technological treatments modify the physical–chemical properties of proteins, making them liable to decrease the nutritional potential of meat. To counteract this damage, antioxidants and chaperone proteins in muscle cells can prevent oxidation, restore the function of denatured proteins, and thus prevent aggregation. This study aimed to explore the impact of indoor *vs* outdoor-reared meat protein composition on digestion and to associate protein markers to *in vitro* digestion parameters. Indoor-reared meat tended to show less oxidation and denaturation than outdoor-reared meat and was characterised by an overexpression of contractile and chaperone proteins. Outdoor-reared meat showed amplification of antioxidant and detoxification metabolism defending against oxidised compounds. Impacts on digestion remained minor. Several protein markers of *in vitro* digestion parameters were found for aged and cooked meat, linked to the detoxification process and to muscle contraction.

1. Introduction

In Europe and most developed countries, animal proteins are part of the daily diet, combined with vegetable proteins, carbohydrates, lipids and fibres. Consumers have become more health-conscious, driving a trend towards lower-fat and lower-cholesterol products. However, meat is a major source of iron, zinc and vitamin B_{12} and a good source of proteins, particularly those containing amino acids essential to human health. Their high content of well-balanced essential amino acids makes proteins from meat and meat products a critical component of a proper human diet. The nutritional quality of meat has become an increasingly important issue for nutritionists, epidemiologists, meat technologists and animal producers (Biesalski, 2005; Higgs, 2000; Norat, Lukanova, Ferrari, & Riboli, 2002). However, it was recently demonstrated that technological treatments, especially cooking, can alter the *in vitro* meat protein digestion rate (Bax et al., 2012). It has been shown that technological treatments tending to enhance protein denaturation also increased the rate of protein degradation by proteases from the digestive tract, while protein oxidation and further aggregation reduced the rate of protein digestion.

Muscle proteins contain endogenous compounds that could at least partly offset the processing-induced physical–chemical modifications of proteins (Chan & Decker, 1994). Muscle proteins include antioxidant proteins such as catalase and superoxide dismutase along with the family of heat shock proteins (HSPs), or molecular chaperones, that provide the cell with tools for restoring proteins after a variety of physiological stresses such as elevated temperatures, oxidative damage or osmotic imbalance. The amount of antioxidant compounds is influenced by rearing system (Santé-Lhoutellier, Engel, Aubry & Gatellier, 2008; Young et al., 2003) as well as by fibre type conversion and its associated energy metabolism enzyme (Shibata et al., 2009). Descalzo and Sancho (2008) showed that beef cattle reared outdoors (pasture-fed) had higher SOD activity than beef animals reared indoors (grain-fed). The main role of the chaperones is to suppress protein unfolding and restore protein structure and function. Several HSPs are expressed in skeletal and cardiac muscle and their mutations can lead to diseases such as myopathy (Bova et al., 1999; Kaminska et al., 2004). They are known to bind to nascent unfolded polypeptides and ensure correct folding and transport. In skeletal muscle, HSPs help stabilize and regulate myofibrillar proteins after thermal denaturation (actin: Pivovarova et al., 2005) or muscle atrophy (tubulin: Sakurai, Fujita, Ohto, Oguro, & Atomi, 2005). This source of cellular protection could potentially offset processing-related protein denaturation and aggregation. It was shown *in vitro* that

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the presence of αB-crystallin led to a 60% lower decrease in chicken myosin ATPase activity after 30-min incubation at 43 °C, while less myosin aggregation occurred (Melkani, Cammarato, & Bernstein, 2006). It appears that αB-crystallin, which contains hydrophobic surfaces that interact with thermally unfolded myosin intermediates under thermal stress conditions, is able to prevent myosin aggregation through transient interactions. These interactions help to protect the enzymatic properties of myosin, keep it folded and prevent molecular aggregation *in vitro* (Melkani et al., 2006). Recently, Markov, Pivovarova, Chernik, Gusev, and Levitsky (2008) showed that HSP 27 effectively prevents the thermally-induced aggregation of isolated myosin head. The authors suggested that phosphorylation leading to dissociation of large HSP 27 oligomers might increase its chaperone activity with certain protein substrates. Likewise, Sayd et al. (2006) used proteomics to demonstrate reduced protein denaturation in chaperone protein-rich muscles. Here, we aimed to explore the impact of indoor *vs* outdoor-reared meat protein composition on digestion and to associate protein markers to *in vitro* digestion parameters.

2. Materials and methods

2.1. Animals and ageing

The experiment was carried out on 30 male commercial Basque pigs (6 months old) reared in a conventional indoor system (on slatted floor; group A) and in an extensive outdoor production system (group B). The pigs were slaughtered at about 100 kg live weight in a commercial slaughterhouse. After 24 h, the *Longissimus dorsi* muscle was removed from each carcass ($n = 6$ per group). One part of the muscle was frozen in liquid nitrogen and stored at −80 °C (Day 1 samples), whereas the other part was kept for 3 days at 4 °C under air-permeable film before being frozen as described above (Day 4 samples).

2.2. Sample preparation and heating procedure

The heating procedure was performed solely on Day 4 samples. Three temperatures were applied: 70 °C, 100 °C and 140 °C. These temperatures were chosen to represent cooked ham, boiled meat and grilled meat. Thawed meat samples (3 g/tube) were placed in polypropylene test tubes (inner diameter = 10 mm, thickness = 1 mm) and heated for 30 min in a digital temperature-controlled dry bath (BT3-heater, Prolabo). The heat treatment was then immediately stopped by placing the samples on ice and freezing them at −80 °C.

2.3. Proteomic analyses

2.3.1. Myofibrillar and sarcoplasmic protein extraction and electrophoresis

The method was adapted from Sayd et al. (2006). Using an MM2 glass bead agitator (Retsch, Haan, Germany), 0.3 g of *Longissimus dorsi* was homogenised in 40 mM Tris HCl (pH 8) at 4 °C at a 1:4 (w/v) ratio for 45 min. The homogenate was centrifuged at 10 °C for 30 min at 10,000*g* in low-ionic-strength buffer. The supernatant, containing soluble sarcoplasmic proteins, was then collected and stored at −80 °C. The pellet was washed five times. After the final centrifugation, the supernatant was removed and the pellet was homogenised in 7 M urea, 2 M thiourea, 4% CHAPS (w/v) and 1% DTT (dithiothreitol) (w/v) at 4 °C in the same 1:4 (w/v) ratio as was used in the first step. The homogenate was centrifuged at 10 °C for 15 min at 10,000*g*. The supernatant, forming the myofibrillar protein fraction at high-ionic-strength, was stored at −80 °C. Protein concentration was determined by RC-DC assay (Bio-Rad, Hercule, CA, USA) for myofibrillar proteins and by Bradford assay (Bio-Rad, Hercule, CA, USA) for sarcoplasmic proteins. The electrophoresis method for both proteins is described below.

First, 1 mg of myofibrillar proteins was incorporated in a buffer containing 7 M urea, 2 M thiourea, 2% CHAPS (w/v), 0.4% carrier ampholytes (v/v), 1% DTT (w/v), and bromophenol blue. Samples were loaded onto immobilised pH gradient strips (pH 3–10 NL, 17 cm, Bio-Rad, Hercule, CA, USA), and isoelectric focusing was performed using a Protean IEF cell system (Bio-Rad, Hercule, CA, USA). Gels were rehydrated passively for 16 h. Rapid voltage ramping was then applied to reach a total of 86 kVh. After IPG strip equilibration, proteins were resolved on 11% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels using a Protean II XL system (Bio-Rad, Hercule, CA, USA) for the second dimension. Gels were stained with Coomassie Blue (colloidal blue). Two gels were produced per sample, giving 48 gels in all.

For sarcoplasmic proteins, 0.9 mg was loaded onto immobilised pH gradient strips (pH 5–8, 17 cm, Bio-Rad, Hercule, CA, USA) following the same procedure as for insoluble proteins. For the second dimension, proteins were resolved on 12% SDS–PAGE. Two gels were produced per sample, giving 48 gels in all.

2.3.2. Image analysis and statistical treatment of data

Gels were visualised and analysed using Progenesis SameSpots 2-D electrophoresis (2DE) image analysis software (Nonlinear Dynamics, New-castle, UK). Aligned spots were normalised by expressing the relative quantity of each spot as the ratio of individual spot quantity to the total quantity of valid spots. For one sample and one spot, the mean of double values (corresponding to gels in duplicate) was calculated. The resulting set of average spot quantities and the physical–chemical data was tested by one-way ANOVA using the SAS software package, with group as fixed factor. Statistically significant difference was set at $p < 0.05$ in the ANOVA.

2.3.3. Protein identification by mass spectrometry

Coomassie-stained spots-of-interest were manually excised using pipette tips. These spots were then destained with 100 μL of 25 mM NH_4HCO_3 with acetonitrile 95/5 (v/v) for 30 min, followed by two washes in 100 μL of 25 mM NH_4HCO_3 with acetonitrile 50/50 (v/v) and then dehydrated in 100% acetonitrile. Gel spots were completely dried using a Speed Vac before trypsin digestion at 37 °C over 5 h with 15 μL of trypsin (10 ng/μL; V5111, Promega, Charbonnières-les-Bains, France) in 25 mM NH_4HCO_3. Peptide extraction was optimised by adding 8 μL of acetonitrile and then running a 10-min sonication.

For matrix-assisted laser desorption/ionisation–time-of-flight (MALDI-TOF) mass spectrometry analysis, 1 μL of supernatant was loaded directly onto the MALDI target. The matrix solution (5 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid) was added immediately and allowed to dry at room temperature.

The peptide mass fingerprint (PMF) of trypsin-digested spots was determined in positive-ion reflector mode using a Voyager DE Pro MALDI-TOF-MS system (Applied Biosystems, Courtaboeuf, France). External calibration was performed with a standard peptide solution (Peptide Mix 4, Proteomix, LaserBio Labs, Sophia-Antipolis, France). Peptide mass fingerprints were compared to NCBInr Sus scrofa (15012010, 22446 seq) protein sequence databases (http://www.ncbi.nlm.nih.gov/Database) using the MASCOT 2.2 search engine (http://www.matrixscience.com). The initial search parameters allowed a single missed trypsin cleavage site, partial carbamidomethylation of cysteine, partial oxidation of methionine and mass deviation lower than or equal to 25 ppm. The validations are based on the significant score given by the Mascot software, which takes into account number of matched peptides