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To what extent does the nitrosation of meat proteins influence their digestibility?

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ABSTRACT

Nitrosation can occur during meat digestion due to the physicochemical conditions of the stomach (low pH and reducing conditions). The aim of the present study was to elucidate the link between the nitrosation of proteins from beef meat and their digestibility by comparing cooked meat digested with and without the addition of nitrite. To do this, a dynamic *in vitro* artificial digestive computer-controlled system (DIDGI®) was used to reflect human gastro-intestinal conditions. Peptides and proteins from gastrointestinal digestion were identified by high-resolution LC-MS/MS mass spectrometry. The results showed a dynamic digestion pattern of meat proteins according to their cellular localization. A combined effect of the digestive compartment and the addition of nitrite was established for the first time on peptides profile, linking the nitrosation of proteins and their digestibility.

1. Introduction

Dietary nitrite and nitrate are considered dangerous for human health because they can react with secondary amines, resulting in the formation of mutagenic nitrosamines (Shephard, Schlatter, & Lutz, 1987). Nitrite can be provided by food directly; cured meats and some vegetables are rich in nitritesNitrite can also be produced by the endogenous reduction of nitrate in the oral cavity (Goaz & Biswell, 1961), and in the stomach (Rowland, Granli, Bockman, Key, & Massey, 1991). Some amino acids from meat proteins can be nitrosated in the presence of nitrite. Aminoacids containing secondary amine groups and free thiols can form nitroso-thiols by *S*-nitrosation and nitrosamines by *N*-nitrosation (de La Pomelie, Sante-Lhoutellier, & Gatellier, 2017; Goldstein & Czapski, 1996). In addition, protein nitrosation can occur during the meat curing process or during the cooking of cured meat at high temperature, but the level of this exogenous nitrosation remains generally low. Digestive conditions greatly favor endogenous nitrosation reactions. The main catalysts of the nitrosation process are the low pH and reducing conditions encountered in the stomach, and the free iron released all along the digestive tract by the degradation of the food matrix (de La Pomelie et al., 2017; De La Pomelie, Santé-Lhoutellier, Sayd, & Gatellier, 2018). Moreover, the hydrolysis of protein into peptides and aminoacids favors the accessibility of nitrite to its specific targets. Nitrosamines are highly unstable and they may be hydrolyzed and/or oxidized to form carcinogenic products known to be involved in oral and digestive cancers (Pegg & Shahidi, 2000; Shephard et al., 1987). Nitrosothiols have controversial effects on human health. By releasing nitric oxide in the blood flow, they participate in controlling the blood pressure, but they are also the precursors of the formation of mutagenic nitrosylheme in the gut (Kuhnle & Bingham, 2007). Nitrosylheme has been described as a very powerful oxidizing agent, linked to the risk of colon cancer through its action favoring luminal peroxidation (Bastide, Pierre, & Corpet, 2011). This gastro-intestinal reactivity led the International Agency for Research on Cancer (IARC) to classify red meat as a probable carcinogen and processed meat as a carcinogen in 2015 (Bouvard et al., 2015). Meat cooking induces protein modifications such as oxidation, aggregation, conformational changes, and decreased solubility (for a review, see (Yu, Morton, Clerens, & Dyer, 2017). The negative effect of these modifications on protein digestibility is now well known (Bax et al., 2012; Gatellier, Kondjoyan, Portanguen, & Sante-Lhoutellier, 2010; Kajak-Siemaszko et al., 2011; Promeyrat et al., 2010; Sante-Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008; Sante-Lhoutellier, Aubry, & Gatellier, 2007). On the contrary, very little is known about the link

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between the endogenous/exogenous nitrosation of proteins and their digestibility. This point is of great importance to better assess the effect of nitrite on the nutritional and health qualities of beef meat. Indeed, it has been shown that the reduced digestibility of proteins induces their accumulation in the colon where they are prone to fermentation by colonic flora, thus producing mutagenic products such as phenols and cresols (Evenepoel et al., 1998).

It has been shown previously that the intensity of proteolysis during cooked meat digestion decreases in the presence of nitrite (De La Pomelie et al., 2018). This conclusion was based on fluorescence measurements of the fluorescamine-specific labeling of N-terminal α-amino groups of peptides and free amino acids (Harkouss, Mirade, & Gatellier, 2012). However, no information was available regarding which proteins were impacted by nitrite and to what extent. In this context, the aim of the present study is to identify and quantify the digested proteins from cooked meat and the resulting peptides using label-free quantitation by high-resolution mass spectrometry.

2. Material and methods

2.1. Meat

2.1.1. Meat preparation

This study is based on the samples obtained in the same conditions as described in a previous study, and all the methodology is described by De La Pomelie et al. (2018). Briefly, the meat was obtained from a commercial Charolais heifer *semimembranosus* muscle. Muscle aging was performed under vacuum for 13 days at 4 °C. Steak samples were cut into slices 1-cm-thick and 50 ± 2 g, cooked in vacuum bags by immersion in a water bath (Polystat CC3, Huber) at 90 °C for 30 min, and cooled in ice for 10 min. These conditions reflected core temperatures for medium rare and overcooked meat respectively (Green, 2005). To simulate mastication, the cooked meat samples were ground to 8 mm using a Pro 2000 Excel (Kenwood). The *in vitro* food boluses were composed by ground meat and cooking juices, and stored at −80 °C until *in vitro* dynamic digestion.

2.1.2. In vitro dynamic digestion of meat

The *in vitro* dynamic digestion of meat was performed in triplicate using the DIDGI® system, developed by INRA (Menard et al., 2014). The DIDGI® system is composed of three consecutive compartments that simulate dynamic digestion from the stomach to the duodenum/jejunum and to the ileum part. The gastric and the duodenum/jejunum compartments were fitted with pH and temperature probes. Both pHs were measured and regulated if necessary according to the sequence parameters chosen to simulate a healthy adult digestion, using HCl in the gastric compartment and using $NaHCO_3$ in the duodenum/jejunum compartment. In the gastric compartment, two pumps added pepsin and lipase; and in the duodenum/jejunum compartment another pump added pancreatin. The entire *in vitro* dynamic system was controlled using the SToRM® software (Guillemin, Perret, Picque, Menard, & Cattenoz, 2010) which allows monitoring and regulating all these parameters.

In the gastric compartment, 150 mL of simulated gastric fluid (Minekus et al., 2014) adjusted to pH 2 were added. When 20 g of *in vitro* food boluses of cooked meat were added, the stomach pH rose to 5.5; the parameters of the digestion sequence controlled its decrease following a linear regression down to 2 in 120 min. Gastric enzymes, *i.e.* pepsin and lipase, were dissolved in simulated gastric fluid at pH 2. The pepsin flow rate was set at 520 U/min and the lipase flow rate at 20 U/min. In the intestinal compartments, pH was set at 6.5 to 7 in the duodenum/jejunum and remained constant at 7 in the ileum. In the duodenum/jejunum compartment, pancreatin was dissolved in distilled water and its flow rate was set at 1 U/min. Bile was not used in this experiment because of the interference between its pigments and nitrosamine absorbance. The transit times in both the gastric and intestinal compartments were set according to the mathematical model described by Elashoff et al. (Elashoff, Reedy, & Meyer, 1982). *In vitro* meat digestion was performed in the presence of a mixture of sodium nitrite (1 mM) and sodium ascorbate (1 mM) or in the presence of sodium ascorbate (1 mM) only, to simulate the endogenous gastro-intestinal chemical reactivity.

2.1.3. Digest sampling

Digest samples were collected to monitor the digestion kinetics in each of the three compartments, and the endpoints were kept for mass spectrometry analysis, *i.e.* at 120 min of digestion in the gastric compartment, after 150 min in the duodenum/jejunum compartment, and after 210 min in the ileum compartment. The pH was raised to 7 by adding NaOH to stop the enzymatic reactions. Proteins were precipitated using acetone, and samples were filtered through syringe filters with a 0.45 μm regenerated cellulose membrane (Interchim, Montluçon, France), and stored at −80 °C until peptide extraction.

2.2. Identification and quantitation of meat proteins and peptides by LC-MS/MS

2.2.1. Peptide extraction

Peptide extraction was performed on the gastric, duodenum/jejunum and ileum digest samples, in duplicate, using porous silica nanoparticles MCM-41 (Sigma Aldrich) according to (Tian et al., 2009) with modifications according to (Sayd, Chambon, & Sante-Lhoutellier, 2016). Briefly, 25 mg of MCM-41 nanoparticles was hydrated with 1 mL of TCA at 3%, added to 1 mL of the supernatant resulting from the TCA precipitation, and shaken for 2 h at 4 °C. The suspension was then centrifuged at 4000*g* for 15 min at 4 °C, and the supernatant was removed. Three washes with 1 mL of distilled water were performed. The peptides elution was done with 1 mL of acetonitrile at 80% and 400 μL were taken and dried using the SpeedVacuum until a volume of 30 μL was obtained. This volume was completed with 150 μL of a solution of 0.05% trifluoroacetic acid (TFA), homogenized and stored in HPLC vials at −20 °C until mass spectrometry analysis. An additional sample was obtained with 2 μL of each peptide extract. This sample, called "Mix", was used as a reference in the alignment step of the ionic maps in the LCProQI quantitation software (NonLinear Dynamics).

2.2.2. LC-MS/MS analysis

The peptides were analyzed in LC-MS/MS for protein identification and quantification. Briefly, 1 μL of peptides digest was injected into the Ultimate nano HPLC (ThermoFisher). After desalting on the load column (Pepmap C18, 300 μm, 0.5 cm) for 4 min, peptides were separated on the analytical C18 nanocolumn (Pepmap C18, Acclaim ThermoFisher, 75 μm, 15 cm) with a gradient from 10 to 40% using a solution of acetonitrile (99.9% ACN, 0.1% formic acid), for 35 min at a flow rate of 300 nL/min. The separated peptides were electrosprayed in the nanoESI source of an orbitrap mass spectrometer (LTQ Orbitrap Velos, ThermoFisher). The mass spectrometer was used in DDA mode and, each full scan MS1 (200–800 *m/z*, R60000) was followed by 15 MS/MS CID (R15000, with dynamic exclusion).

2.2.3. Label-free quantification and identification

The acquired runs were loaded into LCProQI quantitation software (NonLinear Dynamics). For the protein quantitation, the software performed run alignment and peak picking steps to obtain a single ionic map gathering all the detected ions. Detected ions having at least one MS/MS (82,373 MS/MS in total) were exported to MASCOT (v. 2.52) in .mgf input format. The query against *Bos taurus* database (23,971 sequences, 08/2017) was carried out with the following parameters: deamidation (NQ), nitrosyl (C), nitro (W, Y) and oxidation (M) as variable modifications; peptide and fragment mass tolerance set to 5 ppm and 0.5 Da respectively; and two missed cleavages were allowed. Protein identification was validated when two unique peptides of this

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