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Effects of macro-nutrient, micro-nutrient composition and cooking conditions on *in vitro* digestibility of meat and aquatic dietary proteins

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ABSTRACT

Animal and aquatic meats represent important sources of dietary protein and micro-nutrients. Although red and processed meats carry some risks for human health, sensory and nutritional advantages drive meat consumption. Therefore, it is important to understand how meat processing and cooking influence healthiness. The research aim was to investigate relationships of meat composition (proximates, amino acids and minerals) and cooking conditions (raw, 90 s microwave, 200 °C oven for 10 or 30 min) on protein digestibility, for a selection of four animal (beef, chicken, pork, kangaroo) and four aquatic meats (salmon, trout, prawn, oyster). Lean meats were minced before cooking followed by *in vitro* gastro-intestinal digestion and analysed for progress of hydrolysis, and size ranges of peptides using MALDI-TOF-MS. Correlation matrix analysis between compositional and functional parameters indicated that digestibility was significantly linked with protein and metal concentrations, likely reflecting moisture-dependent solubility and inter-mixing of sarcoplasmic metallo-proteins and insoluble myofibrillar proteins.

1. Introduction

and aquatic sources provide benefits and some risks for human health (Lin et al., 2015). Benefits include the convenient supply of rich sources of high quality protein, including all of the 9 essential amino acids (Phillips et al., 2015), vitamin B12 and bioavailable iron. In addition, apart from some shellfish, meat proteins are associated with low risk of allergic reaction (Restani, Ballabio, Tripodi, & Fiocchi, 2009). On the other hand, some meats and particularly processed meat products can also contain fats and carbohydrates with high overall energy density and long-term consumption confers risk of cardiovascular disease, metabolic syndrome and obesity (Lin et al., 2015). In addition, there is now convincing evidence that excessive consumption of red and processed meats can contain increases risk of gastro-intestinal cancers (Zhu et al., 2013).

Notwithstanding the risks, sensory and nutritional advantages drive meat consumption and it is therefore desirable to understand the nature of the causative factors that confer risk, including intrinsic (composition) and manageable factors such as pre-processing, additives and cooking conditions. Meats are frequently cooked before consumption in order to inactivate pathogens and enhance sensory attributes. However, relationships between physicochemical changes in components of meat, particularly proteins, during processing and cooking and thresholds of effect on *in vivo* measures of health risk, are poorly defined.

The focus of this study is on the effects of processing and cooking on digestibility of meat proteins. Physicochemical changes in meat proteins that occur during cooking are primarily induced by heating (temperature and time) and consequences for protein denaturation and accelerating rates of chemical reactivity. For example, cooking-related temperatures promote protein denaturation and formation of intermolecular cross-links, resulting in production of large aggregates (Kaur, Maudens, Haisman, Boland, & Singh, 2014; Sante-Lhoutellier, Astrijc, Marinova, Greve, & Gatellier, 2008). Protein physical and covalent aggregation can undermine efficacy of digestive enzymes and compromise the release and bioavailability of peptides and amino acids

Abbreviations: OPA, *O*-phthaldialdehyde; MALDI-TOF-MS, Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry; AA, amino acid

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(Morzel, Gatellier, Sayd, Renerre, & Laville, 2006). Decreases in *in vitro* digestibility following cooking have been reported for pork (Wen et al., 2015), chicken (Cui, Zhou, Zhao, & Yang, 2009) and beef (Kaur et al., 2014). Similarly, pre-processing of beef by marination in acidic balsamic vinegar promoted cross-linkages that also lowered digestibility following cooking (Patel & Welham, 2013), whereas the elevation of pH associated with ageing increased beef protein digestibility (Farouk, Wu, Frost, Clerens, & Knowles, 2014).

Cooking drives the oxidation of meat proteins, which is the primary chemistry responsible for the protein aggregation and loss of digestibility. Oxidation can be mediated by disulfide-exchange between neighboring polypeptides or via reactive oxygen species (ROS, (Martinaud et al., 1997)), and is accelerated by heat and transition metals, (Estevez, 2011; Lund, Heinonen, Baron, & Estevez, 2011). Mechanisms of protein oxidation vary between amino acids with tendency for formation of carbonyls on side chains of arginine, lysine and proline whereas disulfide cross-links are favoured by S-containing amino acids (cysteine, methionine) or specific di-tyrosine cross links for tyrosine (Lund et al., 2011).

Apart from the undesirable outcome of forfeiting of amino acid absorption, compromised digestibility of proteins leads to an increased risk of immune system reactivity to large undigested peptides (York, Goldberg, Mo, & Rock, 1999) or the development of allergenic epitopes by partially-digested proteins as seen for milk, hazelnut and fish proteins (Untersmayr & Jensen-Jarolim, 2008; Untersmayr et al., 2007). Additional risk associated with incomplete protein digestion is the transfer of protein fragments to and fermentation in the colon (Blachier, Mariotti, Huneau, & Tome, 2007; Geypens et al., 1997), which is specifically associated with risk of colorectal cancer (Aune et al., 2013; Oberli et al., 2015). Protein fermentation generates potentially harmful metabolites including hydrogen disulfide, phenols, indoles, polyamines, and ammonia, that can damage the colon mucosa (Davila et al., 2013) and exert other potential toxicities in the periphery, including the brain (Galland, 2014).

It was hypothesized that *in vitro* digestibility of dietary meat proteins was related to protein primary structure and micronutrient-mediated reactivity of polypeptides, as influenced by cooking conditions, leading to altered *in vitro* digestibility of proteins. The aim of the research was to study effects of several cooking conditions and consequences for *in vitro* digestibility of proteins, across a selection of dietary meats embodying a reasonable dynamic range of chemical composition. Unlike most previous studies, meats were minced before cooking, which significantly eliminated the 'structural connectivity' of proteins during cooking and maximized dispersion of the soluble sarcoplasmic metallo-proteins. The sample preparation methods also minimized effects of secondary and higher order structures associated with protein 'type' as present in intact cuts of meat. *In vitro* digestion was monitored by curve-fitting of the digestion versus time profile and the size distribution of peptides in digestates, by MALDI-TOF-MS.

2. Materials and methods

2.1. Materials

Animal and aquatic meats (lean cuts or fillets), including beef, chicken, kangaroo, salmon, rainbow trout, prawn and oyster, were purchased in a fresh and ready to cook state, from local retail outlets in Melbourne, Australia. Porcine pancreatic pepsin (Sigma P7000, 453 U/mg solids), porcine pancreatin (Sigma, 8 × USP), bovine bile extract (Sigma, B3883), bis-tris-propane, *O*-phthaldialdehyde (OPA), dithiothreitol (DTT), trifluoro-acetic acid (TFA), sodium dodecyl sulfate (SDS), 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All other reagents and solvents were analytical grade.

2.2. Chemical analysis

Moisture analysis of freshly minced and post-treatment, of freeze-dried meat samples, was conducted using a Mettler Toledo Moisture analyser (HR73, Melbourne, Australia). Nitrogen analysis of freeze-dried samples was determined using a LECO Trumac® N analyser (LECO Corporation, Michigan, USA) and crude protein content calculated by conversion factor of 6.25. Total amino acid analysis (excluding tryptophan and cysteine) was conducted using the High Sensitivity Waters AccQTag Ultra (Milford, MA, USA) chemistry and results of duplicate analyses expressed in mg/g total nitrogen. Total ash analysis was conducted by drying to constant weight after thermal oxidation and minerals analysis was conducted by ICP. All analyses were conducted in triplicate, unless otherwise stated.

2.3. Meat sample preparation

For animal meats, skin and visible fat was removed manually before mincing under constant conditions (Breville food processer, BFP800, Sydney, Australia). Portions of minced meats (50 g) were placed into ramekins (internal diameter: 9 cm, depth: 4 cm, thickness: 0.5 cm) and pressed onto the bottoms of containers. Minced meats were cooked without any standardisation of solids or proteins. For microwave cooking, containers were sealed with cling wrap and cooked at high heat (2000W, commercial microwave) for 90 s. For oven cooking, samples were covered with foil and baked by fan-forced oven at 200 °C for 10 and 30 min, respectively. All meats appeared cooked by these methods (see Fig. 1). Raw and cooked meat samples, including juices exuded during cooking, were freeze dried, ground into powders and stored in airless plastic pouches at 4 °C until use. Freeze-drying was considered to preserve chemical interactions formed during cooking and to standardize for the status of physical and structural protein networks, so as to enable valid comparison of digestibility between proteins from different types of meat. Sample preparation was conducted in triplicate.

2.4. In vitro digestion of meat proteins

Freeze dried samples were ground to a fine powder before dispersing in simulated gastric buffer (SGB, 0.15 M NaCl, pH 2.5) at 1.25 mg/mL total nitrogen. *In vitro* digestion was conducted by enzyme hydrolysis using pepsin (pH 2.7, 60 min) followed by addition of pancreatin (210 min) and static transition to pH 7.5, in a buffer containing bile salts, as previously described (Wu et al., 2017a, b). Simulated adult digestion was conducted for each replicate meat sample in triplicate and reagent controls were conducted on each analysis day to confirm comparability of enzyme activity.

2.5. Protein hydrolysis profiling

Samples of hydrolysate were withdrawn at 15 min during pepsin digestion and 30 min during digestion in pancreatin. All aliquots were immediately heated at 100 °C for 5 (10 min for final digestate) min to inactive enzymes and stored at −20 °C before analysis. Progress of enzyme hydrolysis was monitored by OPA assay and fluorescence detection, as previously described (Wu et al., 2017a, b). Means of fluorescence readings at each time point were calculated for curve fitting analysis.

2.6. MALDI-TOF mass spectroscopy analysis of digestates

Analysis of digestate peptide masses was conducted by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) spectroscopy (UltrafleXtreme TOF-TOF spectrometer, Bruker, Bremen, Germany) after dilution in water (1/50, in duplicate), as previously described (Wu et al., 2017a,b). Peptides in the M/Z mass range: