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2019

Serra, A., Gallart-Palau, X., Koh, W. Y., Chua, Z. J. Y., Guo, X., Chow, C. J. J., Chen, W. M., Park, J. E., Li, T., Tam, J. P. & Sze, S. K. (2019). Prooxidant modifications in the cryptome of beef jerky, the deleterious post-digestion composition of processed meat snacks. Food Research International, 125, 108569-. https://dx.doi.org/10.1016/j.foodres.2019.108569

https://hdl.handle.net/10356/149185

https://doi.org/10.1016/j.foodres.2019.108569

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Prooxidant modifications in the cryptome of beef jerky, the deleterious post-digestion composition of processed meat snacks

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Key Words: proteomics, jerky, beef, post-translational modification, advanced-glycation end products, lipoxidation, DNA-binding.

Abstract

Snacking has traditionally been associated with consumption of foods rich in fats and carbohydrates. However, new dietary trends switched to consumption of protein-rich foods. This study investigates the impact of food processing on the cryptome of one of the most widely consumed meat snacks, beef jerky. We have performed discovery-driven proteome-wide analyses, which identified a significantly elevated presence of reactive prooxidant post-translational modifications in jerky. We also found that these protein decorations impact an important subset of in-silico predicted DNA binding cryptides. Furthermore, we observed cell-dependent reduction in cell viability after prolonged treatments with endogenous-like jerky digests. Collectively these findings uncover the presence of prooxidant modifications in processed dried beef snacks and associate their presence with cytotoxicity. Thus, the findings reported here can pave the way for future studies aimed to establish appropriate dietary recommendations on snacking trends.

1. Introduction

Snacking is a dietary habit influenced by complex biopsychosocial factors estimated to contribute to almost one third of dietary intake (Njike, Smith, Shuval, Shuval, Edshteyn, Kalantari, et al., 2016). Although snacks have traditionally been associated with consumption of foods rich in fats and carbohydrates with low nutritional profiles, inclusion of whole foods and foods rich in proteins has changed the social perception and outcomes of this habit (Farajian, Katsagani, & Zampelas, 2010).

Snacks rich in proteins tend to increase satiety, balance energy consumption and reduce obesity among other significant health benefits (Chapelot, 2011; Marmonier, Chapelot, & Louis-Sylvestre, 2000). However, potential effects of long-term consumption of processed protein-rich snacks on human health remain poorly defined. Proteins are an essential macronutrient required to sustain endogenous protein synthesis and muscle mass (Phillips & Van Loon, 2011). Notwithstanding, the influence of proteins and of their derivatives extend well beyond basic nutritional values (Galvez & de Lumen, 1999; Koldovsky, 1989; Shah, 2000). Food bioactive proteinaceous range from two amino acids to long peptides, and in the human body are known to modulate hypertension, angiogenesis, diabetes, inflammation, immunity and opioid synaptic synergy, to mention just a few (Chatterjee, Gleddie, & Xiao, 2018). Although bioactive proteinaceous are naturally present in foods, a clear distinction between protein molecules naturally present in foods and the cryptome must be performed (Kitts & Weiler, 2003). Indeed, the cryptome consists in a subset of latent functional units that are liberated from protein sequences under certain conditions, such as proteolytic activity, which generates a novel cluster of bioactive peptides commonly dubbed as cryptides (J Autelitano, Rajic, Ian Smith, Berndt, Ilag, & Vadas, 2006; Samir & Link, 2011).

All food molecules, including proteins, are required to pass through a digestive process in the human body, and thus bioactivity of food proteinaceous becomes determined by the molecular composition of the cryptome, as previously revised (Samir & Link, 2011). Endogenous enzymes in the digestive system generate a vast array of multi-length proteolysed peptides that in some instances can become hard to link to the parent protein, and that may possess bioactive abilities that surpass those of the molecules naturally present in foods (Autelitano, Rajic, Smith, Berndt, Ilag, & Vadas,

2006). Thus, proteome-wide definition of cryptides and the cryptome of consumed foods has been described in recent studies (Federica Iavarone, Claudia Desiderio, Alberto Vitali, Irene Messana, Claudia Martelli, Massimo Castagnola, et al., 2018). Although it is known that biological variables are able to modify the complex molecular composition of the cryptome, and in turn to create a very singular bioactivity profile of each consumed food in each individual (Samir & Link, 2011); other variables, such as industrial processing and food composition seem to play fundamental roles in the molecular composition and biofunctional outcomes of the cryptome. Industrial processing is largely proven to alter the molecular composition and profile(s) of functional molecules in foods (Capanoglu, Beekwilder, Boyacioglu, De Vos, & Hall, 2010; Escobedo-Avellaneda, Moure, Chotyakul, Torres, Welti-Chanes, & Lamela, 2011). Similarly, food composition refers to presence of components, such as lipids or sugars, in the food composition, which alters the molecular profile(s) of functional molecules in foods by addition of protein posttranslational modifications (PTMs) (A. Serra, Gallart-Palau, See-Toh, Hemu, Tam, & Sze, 2016). Oxidative degradation of both lipids and sugars generates highly reactive dicarbonyls and other molecules that promote apparition of advanced glycoxidation end products (AGEs), and lipoxidation end products (ALEs) (Vistoli, De Maddis, Cipak, Zarkovic, Carini, & Aldini, 2013). These toxic protein decorations have been identified in food-derived nutraceuticals and bioactive peptides as consequence of food processing (A. Serra, Gallart-Palau, See-Toh, Hemu, Tam, & Sze, 2016). However, whether industrial processing and molecular composition affect the profile(s) and biofunctional outcomes cryptome-wide remains poorly uncovered.

As recently unveiled, the raise in consumption of meat snacks has already overpassed that of potato chips in United States (Nielsen, 2017). The main benefits of these snacks, in which beef jerky is the most widely consumed (NHCS, 2018) are mainly promoted on the basis of their high level of protein and their low level of fats. To further investigate the impact that food processing and food composition exert on the cryptome of meat snacks, we have characterized in this study the molecular composition of cryptides from beef jerky and unprocessed beef meat by mass spectrometry (MS)-based cryptomics (Samir & Link, 2011).

2. *Materials and methods*

2.1. Reagents and chemicals

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Water and acetonitrile (ACN) used for mass MS analysis were of high-performance liquid chromatography (HPLC) grade from Fisher Scientific Inc. (U.S.A.). Complete Protease Inhibitor cocktail tablets were obtained from Roche (Basel, Switzerland).

2.2. Protein extraction from meat samples for shotgun proteomics study

Lean raw beef cube steak (unprocessed meat) and beef jerky were purchased from a local supermarket in Singapore. Ingredients listed on beef jerky label are: beef, sugar, dextrose, maltodextrin, salt, soy protein, vegetable powders, species, flovour, acidity regulator (331), vegetable oil, antioxidant (316), spice extracts, preservative (250). Both products were of Australian origin. Homogenization of meat samples (~250 mg/sample) was performed with 2% sodium dodecyl sulfate (SDS), 100mM ammonium bicarbonate (ABB) and protease inhibitor (1:50 v/w)) (SDS extraction buffer) using a ultrasonic processor with a 3 mm microtip probe (Sonics & Materials, U.S.A.) at a 40% amplitude for 5-second pulses for five minutes, followed by 5-second cool-down interval. Homogenization was performed on ice. After homogenization samples were centrifuged at 10,000 *x g* for 10 minutes at 4°C and the supernatants were collected. Pellets were further homogenized twice as described above and supernatants were combined. Proteins were then precipitated and fat was removed from 1 mL of meat homogenized supernatant by liquid/liquid extraction adding 5 mL of 1:1 methanol/chloroform (v/v), vortexing and allowing to stand for an hour. The mixture was then centrifuged at 5000 *x g* for 10 minutes at 4°C and liquids decanted. Pellets were dissolved in a 1% sodium deoxycholate (SDC), 100mM ABB solution. The experiment was done per triplicate using three samples obtained from three packets of beef jerky or unprocessed meat.

2.3. In-solution tryptic digestion of jerky and unprocessed meat proteins for shotgun proteomics study

Digestion of proteins from jerky and unprocessed meat samples was performed as previously described (Gallart-Palau, Serra, Hase, Tan, Chen, Kalaria, et al., 2019; Aida Serra, Gallart-Palau, Wei, & Sze, 2016; A. Serra, Zhu, Gallart-Palau, Park, Ho,

Tam, et al., 2016). Briefly, reduction of disulfide bonds was performed with 10 mM dithiothreitol (DTT) at 60°C for 30 minutes, alkylation of cysteines was then performed by adding 20 mM iodoacetamide and incubating at 37°C for 30 minutes. Samples were then diluted 2.5-fold with 100 mM ABB containing 10 mM DTT and incubated for 30 min at 37°C. Protein concentration was determined with a bicinchoninic acid assay, and 3 mg of protein from each sample were digested with trypsin at 1:20 enzyme-to-protein ratio overnight at 37°C. Trypsin digestion was quenched and SDC was precipitated by acidification to a final concentration of 0.5% formic acid (FA). SDC was pelleted by centrifugation at 12,000 *x g* for 10 minutes at 4°C. The pellet was re-suspended in 100mM ABB for further peptide recovery, and a second round of SDC precipitation was performed. The supernatant from both rounds of centrifugation were collected and combined. The peptides were desalted using a 1g Sep-pack C18 cartridge (Waters, U.S.A.). The eluted peptides were dried completely in a vacuum concentrator (Eppendorf, Germany).

2.4. High-pressure liquid chromatography fractionation of jerky and unprocessed meat peptides

Desalted peptides were fractionated by high-pressure liquid chromatography (HPLC) as previously described (Gallart-Palau, Serra, Lee, Guo, & Sze, 2017), with minor modifications. Peptides were reconstituted in 200 µL of mobile phase A (85% ACN, 0.1% acetic acid) and fractionated using a PolyWAX LP 3.5 µm 4.6 × 250 mm column (PolyLC, U.S.A.) with a Shimadzu Prominence UFLC system (Dionex, USA) with UV monitoring of peptide intensities at 280 nm. Peptides were separated in a 70-min gradient as follows: 0% B (0.1% FA) for 10 min, 0–20% B for 30 min, 20–65% B for 10 min, 65-100% B for 1 min, 100% B for 8 min, 100-0% B for 1 min, and 0% B for 10 min. Fractions were collected at 1 min intervals and combined in a total of 18 fractions per sample according to the peak elution profile. Combined fractions were dried completely in a vacuum concentrator.

2.5. Liquid chromatography-mass spectrometry shotgun proteomics of jerky and unprocessed meat peptides

Dried fractionated peptide samples were reconstituted in mobile phase A (3% ACN, 0.1% FA). LC-MS/MS analysis of peptides was performed using a Dionex UltiMate 3000 UHPLC system coupled with an Orbitrap Elite mass spectrometer (Thermo

Fisher Inc., Bremen, Germany) as previously described with minor modifications (Gallart-Palau, Serra, Qian, Chen, Kalaria, & Sze, 2015; Gallart-Palau, Serra, & Sze, 2016; Gallart-Palau, Serra, Wong, Sandin, Lai, Chen, et al., 2015; Aida Serra, Xavier Gallart-Palau, Bamaprasad Dutta, & Siu Kwan Sze, 2018). Spray was generated using a Michrom Thermo CaptiveSpray nanoelectrospray ion source (Bruker-Michrom Inc., Auburn, USA) working at 1.5 kV. Peptide separation was performed using a reverse-phase Acclaim PepMap RSL column (75 µm ID × 15 cm, 2 µm particle size, Thermo Scientific Inc.) maintained at 35°C and working at 300 nL/min. Eluents A (0.1% FA) and B (90% ACN, 0.1% FA) were used to establish the following 60-min gradient at a flow rate of 300 nL/min over a 60 minute period: 3% B for 1 min, 0–35% B for 47 min, 35–50% B for 4 min, 50–80% B in 5 seconds, 80% B for 78 sec, 80–3% B in 6 seconds, 3% B for 6.5 min. Orbitrap Elite mass spectrometer was set to positive mode for data acquisition with Xcalibur 2.2 SP1.48 software (Thermo Fisher Scientific Inc., Bremen, Germany) software alternating between full Fourier transform-mass spectrometry (FT-MS) (350−2000 m/z, resolution 60000, with 1 µscan per spectrum) and FT-MS/MS (150−2000 m/z, resolution 30000, with 1 µscan per spectrum). Fragmentation of the 10 most intense precursors with charge >+2 and isolated within a 2 Da window was performed using high-energy collisional dissociation (HCD) mode using 32% normalized collision energy. A threshold of 500 counts was enabled. For Full FT-MS and FT-MS/MS automatic gain control was set to 1×10^6.

2.6. Protein extraction from meat samples for cell viability assay

Proteins from 50 g of unprocessed meat or jerky were extracted with 200 mL of SDS extraction buffer as detailed previously with minor modifications (A. Serra, X. Gallart-Palau, B. Dutta, & S. K. Sze, 2018). Homogenization was performed with the ultrasonic processor with a 6 mm probe (Sonics & Materials). Proteins were precipitated and fats removed with 200 mL of 1:1 methanol:chloroform (v/v) as previously detailed. Pelleted proteins (~1 g/sample) were dissolved in 8 mL of 4 mM hydrochloric acid (pH 2), and homogenized with the ultrasonic processor using the 3 mm probe (40% amplitude, 10-second pulses for 10 minutes, 10-second cool down, on ice). The meat samples were further homogenized in a bullet blender (Next Advance, U.S.A.) with metallic beads (1mm; Next Advance) at maximum speed for 30 minutes at 4°C. Samples were centrifuged at 10,000 *x g* for 10 minutes at 4°C, and supernatants were collected.

2.7. Preparation of endogenous-like unprocessed meat and jerky digests for cell viability assay

To simulate the sequential endogenous stomach, and duodenum human digestion, pepsin and trypsin proteases were used in a static *in vitro* digestion strategy as previously described (Nguyen, Bhandari, Cichero, & Prakash, 2015), with minor modifications. Reduction and alkylation of unprocessed meat or jerky proteins were performed as previously described for the shotgun proteomics study. Reduced and alkylated proteins were digested with pepsin (1:20 w/w) at pH 2 and 37°C overnight. Pepsin was inactivated by increasing the pH to 8 with ABB. Overnight digestion was then performed with trypsin (1:40 w/w) at 37°C, and inactivated by adjusting to pH 3 with acetic acid. Peptides were then desalted using a 1g Sep-pack C18 cartridge (Waters, U.S.A.). The eluted peptides were dried completely in a vacuum concentrator (Eppendorf, Germany).

2.8. Cell viability assay

Cell viability was measured with a colourimetric tetrazolium dye, thiazolyl blue tetrazolium bromide (MTT) assay. NIH 3T3 fibroblast and HCT116 cells (American Type Culture Collection (ATCC), U.S.A.) were seeded per well in 96-well plates and incubated overnight in Dulbecco's Modified Eagle's Medium (DMEM, GE Healthcare Life Sciences, U.S.A.) supplemented with 10% fetal bovine serum. The endogenous-like unprocessed meat or jerky protein digests were dissolved in 1 mL of 1x PBS, diluted with DMEM and filtered with a 0.22 µm polyethersulfone filter (Sartorius AG, Germany). The media was then exchanged with fresh media containing 0.25 to 1 mg/mL of endogenous-like digests and cell viability was tested via an MTT assay at 24, 48 and 72 hours according to manufacturer’s protocol. Cells treated with DMEM were considered as control. Absorbance was measured at 570nm and untreated controls were set to 100% viability. The experiments were performed per triplicate.

2.9. In-silico identification of cryptides with DNA-binding capacity

For the in-silico characterization of cryptides with DNA-binding capacity the list of unique peptides identified by shotgun proteomics after tryptic digestion of jerky and unprocessed meat were converted to FASTA format (including post-translationally modified and unmodified peptides) using an in-house program. Subsequently, the lists were submitted to the DNA binding protein prediction (DNA-Binding) online platform

(Szilagyi & Skolnick, 2006). In-silico DNA binding capacity prediction was done from peptide sequence at 15% false discovery rate (Szilagyi & Skolnick, 2006). Only peptides with a prediction score confidence > 10 were considered as cryptides with DNA binding capacity. Cryptides were in-silico predicted for every replicate (n=3).

2.10. Bioinformatics and data analysis

The raw MS/MS data were de-isotoped and converted into Mascot Generic Format using the Thermo Proteome Discoverer software (version 1.4.1.14, Thermo Fisher Scientific, U.S.A.), then searched against the *Bos taurus* reference proteome and UniMod protein modification were identified using an in-house Mascot server using the Mascot error tolerance search mode (version 2.6.1; Matrix Science, U.S.A.). The search was performed with a 5 ppm precursor MS tolerance, 0.02 Da MS/MS fragment tolerance. The missed cleavage sites was determined with Mascot search results. GraphPad Prism 6 (GraphPad Software, Palo Alto, CA) was used for parametric statistical analyses and for creating data plots. Data were analyzed by Student's t-test at p-values < 0.05 and are reported as mean ± SD.

2.11. Availability of data

The data generated in this study are publicly available via the ProteomeXchange consortium through the partner repository PRIDE (Vizcaino, Csordas, Del-Toro, Dianes, Griss, Lavidas, et al., 2016).

PRIDE Accession: PXD012112

3. Results and discussion

3.1. Protein post-translational modifications in jerky

In this study we sought to investigate the presence of protein PTMs induced by food processing and food composition in beef jerky using unprocessed meat as control. Peptidome-wide characterization revealed a significantly higher number of total unique peptides in unprocessed meat samples compared to jerky (**Supplementary Figure 1 and Supplementary datasets 1 and 2**), a fact that is consistent with previous reports (Yu, Morton, Clerens, & Dyer, 2017), and that could be related with denaturation and formation of proteinaceous aggregates of low solubility in jerky during production processes. Beef jerky production process generally involves a drying process of the marinated meat at about 71.1°C (160 degrees F) prolonged up to 12 h. The duration

of the drying process depends on specific recipe. On the contrary, drying temperature of 71.1°C was recommended by the United States Department of Agriculture (USDA) to achieve an immediate lethality of *Salmonella* and ensure an adequate microbiological safety (USDA, 2007). This long drying process is often precluded by a shorter post-drying heating step at about 135°C. In the first heating step meat is cooked and microbial numbers is reduced whilst the second drying phase stabilizes organoleptically the product and prevents microbial growth.

Protein PTMs significantly elevated in jerky were essentially derived from the reaction between proteins with endogenous sugars, lipids or other components catalyzed by high temperature treatment (**Figure 1 and Supplementary Table 1**). These subset of PTMs, practically unidentifiable in unprocessed meat, included glycated –derived from hexose, glucoronyl and galactosyl and oligosaccharides- (**Figure 1A**) and lypoilated modified proteins (**Figure 1B**), as well as their degradation products, advanced glycoxidation and lipoxidation end products (AGEs and ALEs, respectively) (**Figure 1C-D**). AGEs shown as a heterogeneous mixture of PTMs in jerky (**Figure 1E**). The most abundant AGE-related PTM detected in this processed meat snack was the highly reactive AGE precursor α-dicarbonyl 3-deoxyglucosone (3-DG)(Loughlin & Artlett, 2010), which affects Arg residues, followed by the Lys modications Nε -carboxy-methyl-lysine (CML) and Nε -carboxy-ethyl-lysine (CEL) (**Figure 1E**). CML and CEL have been subject of exhaustive study due to their capacity to interact with human AGEs receptor (RAGE) (Xue, Rai, Singer, Chabierski, Xie, Reverdatto, et al.). Additionally, Arg modifying AGE-related PTMs such as the methylglyoxal-derived hydroimidazolone (MG-H1) and dihydroxyimidazolidine were also found significantly elevated in jerky (**Figure 1E**).

Accumulation of AGEs in the mammal's body leads to apparition of oxidative stress, inflammation, protein cross-linking and remains negatively associated with lifespan (Gallart-Palau, Serra, & Sze, 2015; Gallart-Palau, Tan, Serra, Gao, Ho, Richards, et al., 2019; Sharma, Kaur, Thind, Singh, & Raina, 2015). Although AGEs are endogenously produced through the sugar metabolism in all tissues and fluids of the body (Ahmed, Luthen, Haussinger, Sebekova, Schinzel, Voelker, et al., 2005), it was shown that exogenous AGEs from foods possess the same abilities as endogenous AGEs at the time to cause protein cross-linking and oxidative stress in cells (Cai, Gao, Zhu, Peppa, He, & Vlassara, 2002). Similarly, it has been shown that dietary restriction of AGEs in diabetes subjects and subjects with renal dysfunction has a significant

repercussion in lowering the circulatory levels of inflammatory markers and VCAM-1, a specific marker of endothelial dysfunction (Uribarri, Peppa, Cai, Goldberg, Lu, He, et al., 2003; Vlassara, Cai, Crandall, Goldberg, Oberstein, Dardaine, et al., 2002). As demonstrated by Cai and colleagues (Cai, He, Zhu, Chen, Striker, & Vlassara, 2008) and emphasized by Sharma *et al.* dietary reduction of AGEs in aging populations is expected to exert therapeutic effects on the prevalence of several chronic age-related diseases (Sharma, Kaur, Thind, Singh, & Raina, 2015). Presence of AGEs in snacks has been found in chips, crackers and cookies (Sharma, Kaur, Thind, Singh, & Raina, 2015; Story, Hayes, & Kalina, 1996), however, their levels were very low compared to meats (Sharma, Kaur, Thind, Singh, & Raina, 2015). Here, we demonstrate that the levels of toxic glycans in the most widely consumed meat snack largely surpass those in unprocessed meats and in other traditionally considered unhealthy snacks such as potato chips.

3.2. Food-ubiquitous bioactive motifs contain prooxidant modifications in jerky

Toxic glycans in bioactive peptides/motifs can alter their function(s) and molecular interactions (A. Serra, Gallart-Palau, See-Toh, Hemu, Tam, & Sze, 2016). Similarly, when the cryptome is affected by reactive PTMs (J Autelitano, Rajic, Ian Smith, Berndt, Ilag, & Vadas, 2006), release of cryptome-derived peptides could become hindered following endogenous enzymatic digestion (J Autelitano, Rajic, Ian Smith, Berndt, Ilag, & Vadas, 2006). Thus, since reactive PTMs were only evident in jerky, we investigated affectation of food-ubiquitous bioactive peptides by reactive PTMs in this processed food product. In depth analysis of post-translationally modified proteomes from jerky led to identification of several bioactive peptides affected by AGE-related modifications in (or surrounding) bioactive motifs as shown in **Table 1**. Of note, we found the beef myosin-derived peptide ALGTNPTNAEVKK$_{13}$ modified in jerky by CML, CEL and Hex at the residue K$_{13}$ (**Table 1**). This peptide contains the VKK region found in the antihypertensive bioactive peptide VKKVLGNP originally discovered in pork myosin (Katayama, Jamhari, Mori, Kawahara, Miake, Kodama, et al., 2007). Nevertheless, this potentially bioactive peptide found in beef jerky displayed prooxidant modifications at the bioactive motif VKK fact that, we hypothesize, may reduce or modify its bioactivity. Stoichiometry analysis of the prooxidant modifications in the motif VKK indicated that ~40% of the total peptide was modified by prooxidant modifications in

jerky. From these, ~29.3% of the peptide was modified by AGEs and ~9.6% was glycated (Hex) (**Figure 2A**). On the contrary, no modified proteoform in this motif was detected in unprocessed meat.

Similarly the biofunctional motif RPR, originally identified in pork nebulin with contrasted antihypertensive abilities (Escudero, Toldra, Sentandreu, Nishimura, & Arihara, 2012), was found directly affected by a 3-DG modification and flanked by MG-H1, 3-DG and Hex modifications in the peptide AGFAGDDAPRAVFPSIVGRPR in jerky (**Table 1**). Stoichiometry of these prooxidant modifications in RPR peptide motif indicated that 96.2% of the total peptide content was modified by AGEs and 3.8% was modified by glycation (**Figure 2B**). Furthermore, 10.45% of the peptide containing the biofunctional motif RPR presented and additional 3-DG modification in the Arg residue R_{10} (**Table 1**). None of these modifications were detected in the RPR motif in unprocessed meat.

The bioactive motif FHG, proven to possess antihypertensive, antimicrobial and cancer cytotoxic properties, was identified in jerky flanked by a CML modification in the NDMAAQYKVLGFHG peptide from jerky (**Table 1**). Stoichiometry analysis of the modified peptide indicated that 40.8% was modified by Hex and 17.5% was modified by CML in jerky (**Figure 2C**). None of these modifications were detected in the FHG motif in unprocessed meat.

We also observed that the functional peptides AGFAGDDAPRAVFPSIVGRPR and NDMAAQYKVLGFHG, while were containing glycotoxins and prooxidant modifications in jerky, occasionally presented trypsin miscleaved sites in their modified potential digestion sites. On the contrary, no miscleaved sites were detected for these peptides in unprocessed meat.

3.3. Prooxidant modifications impair the release of cryptides from jerky

Observation of missed cleavages in bioactive peptides of jerky prompted us to investigate the presence of missed cleavage sites in jerky originated by a hindering effect exerted by PTMs on normal protease activity due to their localization affecting protease digestion sites. Trypsin is the active form of trypsinogen and represents one of the main proteases in the digestive system (Antalis, Shea-Donohue, Vogel, Sears, & Fasano, 2007; Eggermont, Molla, Tytgat, & Rutgeerts, 1971), thus it was used in this study to investigate the presence of missed cleavage sites in jerky peptidomes modified and unmodified by prooxidant modifications. As shown in **Figure 3A**, 74.6%

of the total unmodified peptides did not display any missed cleavage sites, whereas the remnant ~25% was distributed in a range that included one to four missed cleavage sites per peptide (**Figure 3A**). On the contrary, the presence of missed cleavage sites in the prooxidant modified subset of peptides was significantly upregulated compared to their unmodified counterparts affecting ~95% of total modified peptides. From these, a big portion of 66.7% displayed one missed cleavage site (**Figure 3A)**. Hindering effects of PTMs on normal protease activity have been demonstrated by acetylated lysines and other modifications (Zee & Garcia, 2012). Thus, we found that presence of prooxidant PTMs in processed meat snacks significantly reduces the endogenous protease efficiency of one of the main proteases in the digestive system, which in turn affects the release and composition of the resultant jerky-derived cryptome.

3.4. Prooxidant modifications affect cryptides with DNA binding capacity in jerky

Biological functions of cryptides are incipiently uncovered and range from signaling capacities to implication in regulatory mechanisms in target cells (Ueki, Someya, Matsuo, Wakamatsu, & Mukai, 2007). These signaling capacities involve DNA interaction (Ueki, Someya, Matsuo, Wakamatsu, & Mukai, 2007) and thus, identify potential peptides with DNA binding capacity in highly consumed foods is required to define and characterize the biofunctional outcomes of cryptides from foods. In this study we have performed bioinformatics to obtain *in silico* predictions of the cryptides with DNA binding capacity from whole peptidomes identified by shotgun proteomics in jerky and unprocessed meat. DNA binding capacity is an important biological function described *in vitro* in cryptome-released peptides (H Barkhudaryan, V Hunanyan, Sarukhanyan, M Stepanyan, H Zakaryan, Grigoryan, et al., 2012; F. Iavarone, C. Desiderio, A. Vitali, I. Messana, C. Martelli, M. Castagnola, et al., 2018) that can be predicted in a peptidome-wide approach using in-silico bioinformatics strategies as previously shown (Szilagyi & Skolnick, 2006).

Use of DNAbind predict in this study (Szilagyi & Skolnick, 2006) revealed that jerky peptidomes contained a total of 553 ± 47 peptides with DNA binding capacity whereas unprocessed meat peptidomes contained 766 ± 39 peptides. These data were shown in **Figure 3B** and whole lists of potential cryptides with DNA binding capacity from jerky and unprocessed meat are included in **Supplementary dataset 3 and 4**. Further

bioinformatics analyses revealed that prooxidant modifications were affecting 11% of the total cryptides with DNA binding capacities in jerky whereas these toxic modifications only affected 0.6% of total DNA binding cryptides in unprocessed meat (**Figure 3B**).

Previous studies reported that prooxidant modifications affecting proteins with DNA binding abilities can cause DNA damage (Guerrero, Vasudevaraju, Hegde, Britton, & Rao, 2013; Padmaraju, Bhaskar, Prasada Rao, Salimath, & Rao, 2011), and it has been hypothesized that this mechanism may play a central role in the pathology of several major diseases in which cell apoptosis is crucially involved (Guerrero, Vasudevaraju, Hegde, Britton, & Rao, 2013). Thus, based on the findings of this study, further studies will be required to evaluate the abilities of the cryptome described with DNA binding capacities in jerky and how these become modified by the effects of the specific prooxidant modifications uncovered.

3.5. Modified jerky cryptides impair cell line proliferation in fibroblasts

To explore *in vitro* the findings described in the previous section and to investigate any potential cytotoxic effect(s) of prooxidant modifications in the cryptome of beef jerky, NIH3T3 fibroblasts and HCT116 cell lines were treated with endogenous-like jerky digests. Digests from unprocessed meat in all conditions were used as control. Cells were treated with concentrations of digests ranging from 0.25 to 1 mg/ml in different time points (24h, 48h and 72h), and cell proliferation was evaluated by MTT assay. Significant time and dose-dependent cytotoxic effects of endogenous-like jerky digests were strikingly observed after 72h using 1 mg/ml of jerky digest on fibroblast cells (p-value 0.049) (**Figure 4**). Rest of time-points, cell line (data not shown) and lower concentrations tested did not yield significant differences between jerky and unprocessed meat (**Supplementary Figure 2**). These findings highlight the expected cell-dependent ability of prooxidant modified jerky cryptides to exert cytotoxicity. The observed effects are apparently linked to long and abundant exposure of susceptible cells to prooxidant modified jerky cryptides, suggesting that these detrimental effects might be linked to interaction of variables like chronic/high level of consumption and aging, a hypothesis that deserves further investigation.

Dose-dependent cytotoxic effects of AGEs, in susceptible cells, were encountered several years ago by Li and colleagues (Li, Mitsuhashi, Wojciechowicz, Shimizu, Li,

Stitt, et al., 1996). The authors found that these pathogenic adducts stimulate the presence of AGE-specific receptors (RAGEs) in target cells from tissues like renal, central nervous system and vasculature (Li, et al., 1996) affecting the endothelium, a basic target in the pathogenesis of several major diseases (A. Serra, Gallart-Palau, Park, Lim, Lim, Ho, et al., 2018). Similarly, ALEs have also been proven to interact with RAGEs in e.g. to dysregulate the levels of vasculature calcification (Ott, Jacobs, Haucke, Navarrete Santos, Grune, & Simm, 2014). Prooxidant modifications are eliminated from target cells by the renal system (Ott, Jacobs, Haucke, Navarrete Santos, Grune, & Simm, 2014), and their potential implications as central and/or comorbid factors in diseases that involve chronic failure of the renal system is under intense scientific scrutiny (Rabbani & Thornalley, 2018). Although the uptake of exogenous prooxidant modifications in the digestive system is somewhat limited (Vlassara, et al., 2002), it was clearly shown that a reduction in the amount of consumed prooxidant modifications has a significant impact on the levels of inflammatory processes and protein PTMs that take place in the body (Cecil, Johnson, Rediske, Lotz, Schmidt, & Terkeltaub, 2005; Vlassara, et al., 2002). Therefore, the links between long-time consumption of foods that contain an elevated amount of cryptides modified by prooxidant modifications, aging and human diseases, have to be further explored in the biomedical and nutritional fields by crosstalk between these disciplines. Similarly, we demonstrate here that definition of the spectrum of prooxidant modifications from highly consumed foods cryptome-wide, as well as analysis of their potential detrimental outcomes, becomes preferential to progress on the knowledge and improvement of the ties linking nutrition and health.

4. Conclusions

We confirmed an abnormal presence of AGEs and ALEs in the peptidome of beef jerky snacks, and shown that these reactive and toxic protein decorations directly affect jerky-derived cryptides and modify the global composition of the jerky cryptome by interfering with endogenous protease efficiency. Furthermore, we demonstrate that endogenous-like digests from jerky significantly reduce cell viability in a cell-type and dose-dependent manner under prolonged periods of exposure. Thus, our findings indicate that current food processing procedures affect the molecular composition of largely-consumed meat snacks. Based on these findings, additional research actions will be required to establish the basis of a proper dietary recommendation on snacking

habits in line with current snacking trends, in which protein-rich snacks gradually substitute the inclusion of carbohydrate- and fat-rich snacks in the diet.

Acknowledgments

This study was supported by the National Medical Research Council of Singapore (NMRC-OF-IRG-0003-2016), the Singapore Ministry of Education (MOE2014-T2-2-043, MOE2016-T2-2-018 and MOE2016-T3-1-003), and the Research and Education Council of the Autonomous Community of Madrid (2018-T1/BIO-10633).

Competing interests

The authors declare that they have no competing interests.

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Figure captions:

Figure 1. Relative quantitation of the significantly elevated PTMs in beef jerky compared to beef unprocessed meat. A. Comparison of the total number of matched glycated peptides (glycation category includes hexosylation [+162Da at KN], hexosamine [+161 at KT], heptosylation [+192 Da at KNQRST], lactosylation [+324 Da at KR], glucuronylation [+176 Da at S] and galactosydation [+178 Da at K]); **B.** Comparison of the total number of matched lypoilated peptides [+188 Da at K]; **C.** Comparison of the total number of matched advanced lipoxidation end products (ALEs)-containing peptides. In this category the modification nonanedioic (azelaic) acid cross link in oxidized lipoproteins [+175 Da at K] was considered. **D.** Comparison of the total number of matched advanced glycoxidation end products (AGEs)-containing peptides. This category included carboxymethylation [+58 Da at CKW], carboxyethylation [+78 Da at K], dihydroxyimidazolidine [+72 Da at R], carboxymethylation [+58 Da at CKW] methylglyoxal-derived hydroimidazolone (MG-H1) [54 Da at R] and 3-deoxyglucosone [+144 Da at R]. **E.** Categorization of AGEs in jerky.

Figure 2. Stoichiometry of bioactive peptides containing prooxidant PTMs significantly elevated in jerky compared to beef unprocessed meat. A. Beef myosin-derived peptide ALGTNPTNAEVKK# overlaps with the partial sequence VKK of the bioactive peptide VKKVLGNP. The unmodified proteoform of this peptide was detected in unprocessed meat. **B.** The gamma enteric smooth muscle actin peptide AGFAGDDAPRAVFPSIVGRPR which contains the bioactive sequence RPR. This peptide was not present in unprocessed meat. **C.** The beef myoglobin-derived peptide NDMAAQYK#VLGFHG which contains the bioactive sequence FHG. This peptide was not present in unprocessed meat. # denotes the modified site. Bioactive motifs are underlined.

Figure 3. Peptidome-wide analysis of jerky and beef unprocessed meat. A. Presence of missed cleavage sites expressed in percentage in tryptic digested jerky and unprocessed meat. *means significant differences between jerky and unprocessed meat assessed by chi-square test ($p < 0.05$). **B.** Peptidome-wide characterization of prooxidant post-translationally modified peptides with DNA binding capacity. A total of 766 and 553 peptides displayed potential DNA-binding capacity in unprocessed meat and jerky, respectively. Prooxidant modified peptides represented the 11% of the total peptides that displayed DNA-binding capacity in jerky. DNA-binding capacity of tryptic peptides was predicted *in silico*.

Figure 4. MTT assay results for NIH 3T3 exposed to 1 mg/mL of endogenous-like jerky or unprocessed meat digests after 24 h, 48 h and 72 h treatment. Data are expressed as mean ± SEM. Significant differences were assessed by ANOVA with uncorrected Fisher's LSD multiple comparisons test ($p < 0.05$).

Table 1. Peptides identified in the beef jerky that contained sequences corresponding to entire or partial known bioactive peptides. Locations of the PTM have been bolded, while the sequence matching known bioactive peptides have been underlined. [b]ACE, refers to angiotensin-I converting enzyme. Reported peptides were identified in at least two replicates.

Accession number	Protein	Identified Peptide	Reported bioactive motif	PTM	Bioactivity	Reference
A0JNJ5	Myosin light chain	ALGTNPTNAE<u>VK**K**</u>	VKKVLGNP	CML	ACE[b]-inhibitory	
		ALGTNPTNAE<u>VK**K**</u>		CEL		
		ALGTNPTNAE<u>VK**K**</u>		Hexose		
F1MKC4	Actin, gamma enteric	AGFAGDDAP**R**AVFPSIVG<u>RPR</u>	RPR	MG-H1	Antihypertensive	
	smooth muscle	AGFAGDDAP**R**AVFPSIVG<u>RPR</u>		3-DG		
		AGFAGDDAP**R**AVFPSIVG<u>RPR</u>		Hexose		
		AGFAGDDAP**R**AVFPSIVG<u>RP**R**</u>		3-DG, 3-DG		
P02192	Myoglobin	NDMAAQY**K**VLG<u>FHG</u>	FHG	CML	ACE-inhibitory,	
				Hex	antimicrobial,	
					cancer cell	
					cytotoxic	