Antioxidant protection of proteins and lipids in processed pork loin chops through feed supplementation with avocado

Silvia H. Hernández-López¹ · Javier G. Rodríguez-Carpena² · Clemente Lemus-Flores² · Jorge Galindo-García³ · Mario Estévez⁴

Revised: 11 May 2016 / Accepted: 17 May 2016 / Published online: 4 July 2016
© Association of Food Scientists & Technologists (India) 2016

Abstract This study was conducted to analyze the impact of dietary avocado on the oxidative stability of lipids and proteins during pork processing. Loins from control (fed basic diet) and treated pigs (fed on avocado-supplemented diet) were roasted (102 °C/20 min) and subsequently packed in trays wrapped with oxygen-permeable films and chilled at 4 °C for 12 days. At each processing stage (raw, cooked and cooked & chilled), pork samples from both groups were analyzed for the concentration of TBARS, the loss of tryptophan and free thiols, and the formation of protein carbonyls, disulphide bonds and Schiff bases. Processing led to a depletion of tryptophan and sulfur-containing amino acids and an increase of lipid and protein oxidation products. Dietary avocado was not able to protect against the oxidation of tryptophan and thiols but cooked & chilled loins from treated pigs had significantly lower concentration of lipid and protein carbonyls than control counterparts. Likewise, dietary avocado alleviated the formation of Schiff bases during cooking. These results illustrate the benefits of dietary avocado on the oxidative stability of processed pork loins.

Keywords Avocado · Carbonylation · Protein cross-links · Pork quality · TBARS · Thiols

Introduction

Meat is especially prone to oxidation processes due to its complex structures and composition including unsaturated fatty acids, myofibrillar systems and pro-oxidants (Monahan et al. 1993). Oxidative damage to lipids and proteins is one of the primary causes for functional, sensory and nutritional quality loss in meat and meat products (Soladoye et al. 2015). Meat lipids are chemically unstable and easy to undergo oxidation, especially during post-mortem handling, cooking and storage (Mapiye et al. 2012). Changes associated to lipid oxidation include rancid odour, off-flavour development, drip losses, discoloration, loss of nutritional value, decrease in shelf life and formation of toxic compounds, which may be detrimental to the health of consumers (Mapiye et al. 2012). The oxidation of lipids has been profoundly studied for decades, whereas the occurrence and impact of protein oxidation in food systems has been regarded to play a minor role.

Most of the studies on protein oxidation have been conducted to examine the role played by oxidize proteins in human diseases (Stadtman 1990). Nowadays, protein oxidation is also of interest among food scientists owing to recent studies that highlight the influence of food protein oxidation on meat quality, human nutrition and human health (Soladoye et al. 2015; Estévez and Luna 2016). Meat processing conditions largely determine the onset, intensity and consequences of protein oxidation in meat products (Soladoye et al. 2015). In cooking procedures, the high temperature induces modifications in the structure of the meat constituents, mainly proteins, as a consequence of degradation, denaturation, oxidation and polymerization events (Gatellier et al. 2010; Utrera

✉ Mario Estévez
mariovet@unex.es

¹ Autonomous University of Nayarit, 63190 Tepic, Nayarit, Mexico

² Food Science, Faculty of Veterinary, Autonomous University of Nayarit, 63190 Tepic, Nayarit, Mexico

³ Department of Animal Production, University of Guadalajara, 44100 Guadalajara, Jalisco, Mexico

⁴ IPROCAR Research Institute, TECAL Research Group, University of Extremadura, 10003 Cáceres, Spain

et al. 2014). Manifestations of protein oxidation derived of cooking include: depletion of thiol groups and tryptophan residues, increases in carbonyl groups and formation of inter- and intra-protein cross-links (Soladoye et al. 2015). Oxidative modifications of muscle proteins are manifested as impaired meat quality, affecting the texture, color, aroma, flavor, water-holding capacity and biological functionality (Estévez 2011). Protein oxidation involves loss of nutritional value in meat including a decrease in the bioavailability of protein, change in amino acid composition, decrease in protein solubility, proteolytic activity loss and impaired protein digestibility (Soladoye et al. 2015). However, not only the quality meat deterioration is critical, oxidized proteins may also have implications on human health and safety (Estévez 2011; Soladoye et al. 2015).

Antioxidant strategies based on the usage of natural sources may be a viable option of enriching meat with health promoting bioactive compounds that would, in turn, prevent against oxidation deterioration. Antioxidant phytochemicals can be applied either through food formulation or dietary strategies (Falowo et al. 2014). Unlike the direct addition of bioactive compounds to meat products, their administration to the meat animal in the form of agricultural by-products, is profitable from a productive point of view, innocuous for consumers and beneficial for meat quality. In dietary manipulation, antioxidants are introduced into the muscle via animal feed or diet. Inclusion of natural antioxidant in animal diets has been reported by different authors with positive effect in terms of controlling oxidative processes and improving meat quality (Haak et al. 2008; Ventanas et al. 2007; Botsoglou et al. 2012).

Avocado is known for having a high amount of bioactive compounds, including tocopherols, chlorophylls and assorted phenolic compounds (Rodríguez-Carpena et al. 2011a). In line with this, avocados have been found to display the highest lipophilic total antioxidant capacity among many other fruits and vegetables (Wu et al. 2004). The direct addition of oil and bioactive compounds from avocado by-products to porcine patties was found to protect lipids and proteins against oxidation during storage and processing (Rodríguez-Carpena et al. 2012; Utrera et al. 2012). However, avocado supply in the pig feeding and its impact on oxidative stability on pork is unknown. Hence, the objective of this study was evaluating the antioxidant protection of proteins and lipids in processed pork through dietary avocado in pig feeding.

Materials and methods

Animals

This experiment was carried out with sixteen hybrids of commercial genotype pigs (50 % York–50 % Landrace)

with an initial live average weight of 53.77 kg. All procedures were performed in compliance with relevant laws and institutional guidelines and were approved by the ethical and animal well-being committees from the involved Universities. Pigs were randomly divided into two groups according to the type of feeding during the fattening period: Control group (control, $n = 8$) and Treated group (treated, $n = 8$). The composition of the control diet was as follows (Percentage in dry matter): sorghum meal 83.7, soybean meal 12.9, $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ 1.0, CaCO_3 1.2, NaCl 0.2, vitamins and trace elements 1.0. Treated diet contained (Percentage in dry matter): sorghum meal 53.7, soybean meal 12.9, avocado paste 30.0, $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ 1.0, CaCO_3 1.2, NaCl 0.2, vitamins and trace elements 1.0. The share of avocado paste in the treated diet (30 %) was optimized in a previous trial following digestibility and productivity criteria (Unpublished data). The avocado paste was made with avocado waste from packing company. When avocado reached its consumption ripeness, determined objectively by firmness with 2.41 ± 0.34 kg of force values (Stable Micro Systems Model TA. XT2, Texture Technologies Corp., Scarsdale, NT), the entire avocado fruit was ground in a screenless hammer mill until obtaining a homogeneous paste and then was mixed with the other ingredients. Pigs were in individual pens. Mixed diets and water were supplied to pigs ad libitum. Table 1 shows the chemical composition of the control and treated diet.

At the end of the fattening period at a live weight of 100 kg, pigs were stunned and slaughtered. Loins (muscle *longissimus thoracis et lumborum*; LTL) were removed from the carcasses, freed from visible fat, and kept at 4 °C during 24 h.

Samples

Raw pork: LTL muscle was cut into 2.5 cm thick chops which were analyzed at day 0 (24 h postmortem). Immediately were kept at -80 °C for further determinations (<2 weeks).

Cooked pork: LTL muscle was cut into 2.5 cm thick chops 24 h postmortem. Slices were cooked in a forced-air oven at 102 °C for 20 min (10 min each side). The cooking loss was calculated as follows: $\text{cooking loss} = [(W_b - W_a)/W_b] \times 100$, where W_b and W_a are the weights of slices before and after cooking, respectively. After cooling they were kept at -80 °C for further determinations (<2 weeks).

Cooked and chilled pork: cooked pork (produced following the aforementioned procedure) was dispensed in polystyrene trays, wrapped in oxygen-permeable film (14 μm thickness and 10,445 $\text{mL}/\text{m}^2/24$ h of oxygen permeability) and stored at 4 °C for 12 days under white fluorescent light (1620 lx), simulating retail display

Table 1 Analysis of control and treated experimental diets

	Control		Treated	
	Mean	SD	Mean	SD
Moisture (g/100 g feed)	12.64	0.36	30.54	1.44
Crude protein (g/100 g feed)	13.40	1.64	12.24	1.74
Ether extract (g/100 g feed)	0.31	0.09	3.22	0.13
Ash (g/100 g feed)	3.91	0.10	3.84	0.16
Tocopherol (mg/kg feed)	164.62	18.77	273.03	16.24
Digestibility (% dry matter)	87.33	1.81	86.25	1.85
Gross energy (kJ/g feed dry matter)	17.84	–	25.58	–

Results partially published in Hernández-López et al. (2016)

SD standard deviation

conditions. After that, samples were stored at -80°C until further determinations (<2 weeks).

Analytical methods

Chemical composition of animal's feeds

Moisture, protein, ether extractives, nitrogen free extractives (NFE) and ash were determined in feeds and muscles using official methods (AOAC 2000). Digestibility was calculated though the analysis of fecal material according to the procedure described by Paiva-Martins et al. (2009).

Chemical composition of LTL muscle

Moisture, protein and ash were determined using official methods (AOAC 2000). Intramuscular total lipids were extracted using Folch method (Folch et al. 1957). Tocopherols determination were performed on a Shimadzu “Prominence” HPLC (Shimadzu Corporation, Kyoto, Japan) equipped with a quaternary solvent delivery system (LC-20AD), DGU-20AS on-line degasser, SIL-20A auto-sampler, RF-10A XL fluorescence detector, and CBM-20A system controller. Separation was made on a reversed-phase C18 column (150 mm length $\times$ 4.6 mm i.d., 5 μm particle diameter) manufactured by Phenomenex (USA) with the mobile phase being methanol:water (97:3 v/v) at a flow rate of 1.5 mL/min, and peaks were registered at 285 and 335 nm as excitation and emission wavelength, respectively. The mobile phases were filtered by a Millipore vacuum filtration system equipped with a 0.45 μm pore size filter. IMF was dissolved in isopropanol (1:10, v/v) and then was injected 2 μL by the aid of the auto-sampler. Identification and quantification of the peaks were done by comparison with tocopherol standards. Results were expressed as $\mu\text{g/g}$ fresh loin.

Tocopherol quantification

Prior to analysis, IMF and ether extractives from feeds were dissolved in isopropanol (1:10, v/v). Tocopherol determination was performed on a Shimadzu “Prominence” HPLC (Shimadzu Corporation, Kyoto, Japan) equipped with a quaternary solvent delivery system (LC-20AD), DGU-20AS on-line degasser, SIL-20A auto-sampler, RF-10A XL fluorescence detector, and CBM-20A system controller. Separation was made on a reversed-phase C18 column (150 mm length $\times$ 4.6 mm i.d., 5 μm particle diameter) manufactured by Phenomenex (USA) with the mobile phase being methanol:water (97:3 v/v) at a flow rate of 1.5 mL/min, and peaks were registered at 285 and 335 nm as excitation and emission wavelength, respectively. The mobile phases were filtered by a Millipore vacuum filtration system equipped with a 0.45 μm pore size filter. Samples were injected (2 μL) by the aid of the auto-sampler. Identification and quantification of the peaks were done by comparison with α -tocopherol and γ -tocopherol standards (0.2–14 $\mu\text{g/mL}$). Results were expressed as μg of α - or γ -tocopherol/g fresh matter.

Thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) were assessed using the method described by Ganhão et al. (2011) with some modifications. Five g of pork loin were dispensed in cone plastic tubes and homogenized with 15 mL perchloric acid (3.86 %) and 0.5 mL BHT (4.2 % in ethanol). During homogenization, the plastic tubes were immersed in an ice bath to minimize oxidative reactions during extraction of TBARS. The mix was filtered and centrifuged (500 g for 4 min) and 2 mL aliquots were mixed with 2 mL TBA (0.02 M) in test tubes. The tubes were placed in a boiling water bath (100°C) for 45 min together with the tubes from the standard curve. After

cooling, the absorbance was measured at 532 nm. The standard curve was prepared using 1,1,3,3-tetraethoxypropane (TEP) solutions in 3.86 % perchloric acid.

Fluorescence measurements of Tryptophan

Natural fluorescence of tryptophan was assessed using fluorescence spectroscopy in accordance to the method proposed by Utrera and Estévez (2012a). One g of pork loin was ground and homogenized in 1:5 (w/v) ratio in 5 mL of 20 mM sodium phosphate buffer (pH 6.5) with urea 8 M using ultra-turrax homogenizer during 30 s. Then 0.5 mL of the homogenates was redissolved in 1.5 mL of guanidine buffer. Subsequently, 20 μ L of this homogenized was dispensed in a quartz spectrofluorometer cell and dissolved in 2 mL 20 mM sodium phosphate buffer (pH 6.5) with urea 8 M. Emission spectra of tryptophan were recorded at 360 nm with the excitation wavelength established at 283 nm (LS 55 Perkin-Elmer luminescence spectrometer). Excitation and emission slit widths were set at 10 nm and data were collected at 500 nm per minute. Tryptophan content was calculated from fluorescence intensity at 360 nm using a standard curve of *N*-acetyl-L-tryptophan amide (NATA). The linearity ($R^2 = 0.9955$; $p < 0.05$) between NATA concentration (ranged from 0.1 to 0.5 μ M) and fluorescence intensity at 360 nm was statistically significant. Results were expressed as milligrams NATA equivalents per 100 g of sample.

Determination of free thiols and disulphide bonds in proteins

Free and total thiols were determined with 4-DPS following the procedure described by Rysman et al. (2014). One g of pork was homogenized in 25 mL of 6 M guanidine hydrochloride (GuHCl) in 100 mM Tris buffer (pH 8.0) using an Ultra Turrax. The homogenates were centrifuged (20 min 5000 g and 4 °C), and supernatants consequently filtered through qualitative filter paper (11 μ m particle retention). An aliquot of 3 mL of filtrate was subjected to disulfide reduction by adding 50 μ L of 1-octanol and 100 μ L of freshly prepared 30 % (w/v) sodium borohydride in 1 M NaOH. After incubation at 50 °C for 30 min, an aliquot of 1.35 mL of 6 M HCl was added, followed by stirring for 10 min. An aliquot of 500 μ L of filtrate was mixed with 2 mL of 6 M GuHCl in 1 M citric acid buffer (pH 4.5) and 500 μ L of 4-DPS solution (4 mM 4-DPS in 12 mM HCl). The absorbance was measured at 324 nm against 6 M GuHCl in 1 M citric acid buffer (pH 4.5) before the addition of 4-DPS (Apre) and after exactly 30 min of reaction with 4-DPS in the dark at room temperature (Apost). A mixture of 2.5 mL of 6 M GuHCl in

1 M citric acid buffer (pH 4.5) and 500 μ L of 4-DPS solution was prepared as a blank sample (Ablank). The absorbance corresponding to the thiol concentration was calculated by subtracting Apre and Ablank from Apost. The thiol concentration was calculated on the basis of a standard curve ranging from 2.5 to 500 μ M L-cysteine in 6 M GuHCl in 1 M citric acid buffer (pH 4.5). The thiol content was expressed as nanomoles of thiol per milligram of protein.

Protein carbonyls quantification

Protein oxidation, as measured by the total carbonyl content, was evaluated by derivatization with dinitrophenylhydrazine (DNPH) according to the procedure described by Ganhão et al. (2010) with slight modifications. Sample loin (1 g) was minced and then homogenized 1:10 (w/v) in 20 mM sodium phosphate buffer containing 0.6 M NaCl (pH 6.5) using an ultraturax homogenizer for 30 s. Total of 2 equal aliquots of 0.15 mL were taken from the homogenates and dispensed in 2 mL eppendorf tubes. Proteins were precipitated by cold 10 % trichloroacetic acid (TCA) (1 mL) and subsequent centrifugation for 5 min at 2236 g. One pellet was treated with 1 mL 2 M HCl (protein concentration measurement) and the other with an equal volume of 0.2 % (w/v) DNPH in 2M HCl (carbonyl concentration measurement). Both samples were incubated for 1 h at room temperature. Afterwards, samples were precipitated by 10 % TCA (1 mL) and washed twice with 1 mL ethanol:ethyl acetate (1:1, v/v) to remove excess of DNPH. The pellets were carefully drained and dissolved in 1.5 mL of 20 mM sodium phosphate buffer containing 6 M guanidine HCl (pH 6.5), stirred and centrifuged for 2 min at 358 g to remove insoluble fragments. Protein concentration was calculated from absorption at 280 nm using bovine serum albumin as standard. The amount of carbonyls was expressed as nanomol of carbonyl per milligram of protein using an absorption coefficient of 21 nM^{-1}/cm at 370 nm for protein hydrazones. The same samples as used for the TBARS measurement were used.

Fluorescence measurements of Schiff base structures

Emission of fluorescence by protein oxidation products (Schiff base structures) were assessed by using fluorescence spectroscopy. One g of pork loin was ground and homogenized in 1:5 (w/v) ratio in 5 mL of 20 mM sodium phosphate buffer (pH 6.5) with urea 8 M using ultra-turrax homogenizer during 30 s. Then 0.5 mL of the homogenates was redissolved in 1.5 mL of guanidine buffer, after 50 μ L of this homogenized was dispensed in a quartz spectrofluorometer cell and dissolved in 2.5 mL 20 mM sodium

phosphate buffer (pH 6.5) with urea 8 M. Emission spectra of Schiff bases were recorded from 400 to 500 nm with the excitation wavelength set at 350 nm (LS 55 Perkin-Elmer luminescence spectrometer). Excitation and emission slit widths were set at 10 nm and data were collected at 500 nm per minute. Content of Schiff base structures was expressed as fluorescence intensity units emitted at 460 nm. These values were corrected according to the protein content of pork loin via multiplication by a correction factor ($Cf = Pt/Pp$, where Pt is the total average of the protein contents from all pork loin chops and Pp is the mean of the protein content in each kind of treatment).

Statistical analysis

To assess significant differences between control group and the treated group a Student “t” test for independent samples was used (SPSS 1999). The General Linear Model with repeated measures was used to analyze the data, lipid oxidation (TBARS), tryptophan, thiols, protein carbonyls concentration and Schiff bases over time of refrigerated storage. In the mixed statistical model, the individual pig was considered as a random effect, while the dietary treatment (control vs treated), storage time (days 0 or 12), and the Diet $\times$ Time interaction were considered as fixed effects. The Tukey’s test was used for multiple comparisons of the means. The significance level was set at $p < 0.05$.

Results and discussion

Chemical composition and cooking loss

Table 2 displays the chemical composition and cooking loss of loin chops from pigs fed a control diet (control) and pigs fed an experimental avocado diet (treated). Main meat components were not significantly affected by the finishing diet. However, treated pigs had significantly higher tocopherol content than control pigs prior to cooking. Avocado contributed large amounts of tocopherols in treated pigs since it is a fruit rich in tocopherols (Rodríguez-Carpena et al. 2011a). Pigs cannot synthesize tocopherols (Wood and Enser 1997), therefore, is logical to consider that tocopherols presents in loins are provided by diet. In fact, the higher tocopherol levels in the treated feeds compared to the control counterpart (Table 1) supports this hypothesis. These results are particularly important because tocopherols have been included in diets of several experiments showing to have beneficial effects on oxidative stability in pork (Wood and Enser 1997) and is perhaps the best known dietary strategy of improving meat quality by reducing lipid and myoglobin oxidation in fresh meat

Table 2 Chemical composition and cooking loss of loin chops from pigs fed a control diet (control) and pigs fed an experimental avocado diet (treated)

	Control		Treated		p^A
	Mean	SD	Mean	SD	
Moisture ^B	55.50	1.85	53.61	1.53	0.160
Protein ^B	21.71	2.02	23.57	0.75	0.134
IMF ^B	20.29	2.07	23.26	0.81	0.088
Ash ^B	1.49	0.49	1.07	0.47	0.291
Tocopherol ^C	7.04	0.63	8.73	0.54	0.031
Cooking loss ^D	29.40	5.50	28.33	4.48	0.425

IMF Intramuscular fat

^A Significance level in student “T”-test. Bold p values denote significant differences ($p \leq 0.05$)

^B Expressed as g/100 g cooked loin

^C Analysis in fresh loin prior to cooking, Expressed as $\mu\text{g/g}$ fresh loin

^D Expressed as percent

(Dunshea et al. 2005). Cooking loss of pork loins were not affected by the finishing diet. Similar cooking losses were reported by Dall Aaslyng et al. (2003) in pork loins cooked in oven at 90 °C. Cooking loss is mainly due to proteins denaturation during cooking of pork, which causes less water to be entrapped within the protein structures (Dall Aaslyng et al. 2003). A high cooking loss provides an expectation of less optimal eating quality as it was negatively correlated with juiciness (Toscas et al. 1999). Since cooking loss is considered a negative effect on the quality of meat, it is important to highlight that, in this study, the finishing diet did not affect this parameter in pork loins.

Impact of dietary avocado on TBARS during loin processing

The concentration of TBARS formed during processing of pork loins is shown in Fig. 1. The degree of lipid oxidation did not differ statistically between raw and cooked loins. However, cooked & chilled pork loins showed significantly higher ($p < 0.05$) TBARS values than raw and cooked samples. In addition, cooked & chilled treated loins had significantly lower TBARS than the control counterparts. This is in agreement with earlier observations made by Botsoglou et al. (2012) who studied cooked pork from animals fed on linseed oil plus olive leaves and linseed oil plus α -tocopheryl acetate as natural sources of antioxidants. On the other hand, Rodríguez-Carpena et al. (2011a) reported lower TBARS in cooked & chilled patties formulated with avocado oil than those formulated with animal fat, and suggested that natural components of

Fig. 1 TBARS concentration in raw, cooked and cooked & chilled loin chops from pigs fed a control diet (control) and pigs fed an experimental avocado diet (treated) during refrigerated storage (+4 °C/12 days). *ns* no significant differences between control and treated at a given sampling point

vegetable oils such as tocopherols, chlorophylls and phenols may have contributed antioxidant activity (Dreher and Davenport 2013). Cooked & chilled pork is highly susceptible to oxidative reactions by a combination of the effects of high temperatures during cooking (membrane disruption, pro-oxidant release and decreased antioxidant protection) and the oxygen exposure during the subsequent refrigeration (Soladoye et al. 2015). Results from the present study are particularly meaningful because tocopherols accumulated in the muscles from treated pigs had a clear protective effect against lipid oxidation during chilled storage of cooked pork. Lipid oxidation is considered an economic problem for meat industry because decreases shelf life of meat and meat products by causing serious deleterious effects on particular nutritional and sensory attributes (Falowo et al. 2014). Therefore the use of avocado via diet in pigs seems to be a good strategy to prevent the adverse effects caused by lipid oxidation generated during cooking and chilling processing.

Impact of dietary avocado on the loss of tryptophan and thiols during pork processing

Tryptophan content was determined in raw, cooked and cooked & chilled loin chops (Fig. 2a) by measuring the natural fluorescence emitted by this amino acid. Tryptophan concentration decreased 50 % on average in pork samples after cooking, and an additional 20 % during the following chilled storage. The loss of tryptophan fluorescence has been attributed to its oxidative degradation and its conversion into free radicals at an early stage and into kynurenine derivatives as final oxidation products (Giesauf et al. 1995). The analysis of tryptophan through the measurement of its fluorescence is fairly specific for meat systems given the differences between this and other aromatic amino acids for their spectral properties (Bell 1981). This phenomenon has also been reported to occur as a

Fig. 2 Depletion of TRP (a) and thiols (b) in raw, cooked and cooked & chilled loin chops from pigs fed a control diet (control) and pigs fed an experimental avocado diet (treated) during refrigerated storage (+4 °C/12 days). *ns* no significant differences between control and treated at a given sampling point

result of the oxidative cleavage of this very sensitive amino acid residue during processing of muscle foods (Soladoye et al. 2015) including cooked beef (Gatellier et al. 2010), cooked pork patties (Utrera et al. 2012) and fermented sausages (Villaverde et al. 2014). Along with tryptophan depletion, protein thiols were also found to decrease around 50 % upon cooking and remained stable during the following chilled storage (Fig. 2b). The loss of free thiols has commonly been used as indicator of the oxidative degradation of sulfur-containing amino acids which are also very sensitive to oxidative reactions (Soladoye et al. 2015). In the presence of reactive oxygen species, cysteine and methionine are readily oxidized to yield assorted oxidation products such as disulphide bonds and methionine sulfoxide (Stadtman 1990). Pork processing has previously been found to cause the oxidation of thiols and this phenomenon is commonly linked to the pro-oxidant action of hypervallent myoglobin (Frederiksen et al. 2008). Unlike the effect previously reported on lipids, feeding on avocado had no effective protection against tryptophan and thiol depletions. We may argue that avocado tocopherols may have protected muscle lipids upon deposition in avocado membranes. These natural antioxidants seemed not to be effective against tryptophan and thiols oxidation. In

agreement with the present results, Utrera et al. (2012) did not find a beneficial effect of the direct addition of tocopherols-rich avocado oil and phenolic-rich extracts against tryptophan oxidation. The depletion of tryptophan and sulfur-containing amino acids during meat processing remains a relevant nutritional loss owing to the multiple biological functions of these essential amino acids (Soladoye et al. 2015).

Impact of dietary avocado on protein carbonylation during pork processing

The extent of protein carbonylation was used as a measurement of the oxidative damage caused to muscle proteins during processing of pork loins (Fig. 3). Each processing stage led to a significant increase of protein carbonyls in control pork loins. The high concentration of protein carbonyls found in cooked & chilled loins denotes severe oxidative degradation of meat proteins. Equivalent results have been reported in cooked beef (Gatellier et al. 2010) and cooked pork (Ganhão et al. 2010; Rodríguez-Carpena et al. 2011c). The susceptibility of the cooked meats to protein oxidation may be due to the disruption of the muscular tissue as a result of high temperature that leads to the release of pro-oxidants naturally present in the muscle and allow the incorporation of oxygen to the system (Soladoye et al. 2015). In fact, non-heme iron has been recognized as one of the key elements in the carbonylation of meat proteins (Estévez and Heinonen 2010). The formation of protein carbonyls responds to the oxidative deamination of alkaline amino acids (i.e. lysine, arginine) in the presence of transition metals and reactive oxygen species (Estévez 2011).

A significant effect of dietary avocado was observed on the carbonyl level of cooked & chilled pork loins ($p < 0.05$). Protein carbonyls were significantly reduced by

Fig. 3 Protein carbonyls in raw, cooked and cooked & chilled loin chops from pigs fed a control diet (control) and pigs fed an experimental avocado diet (treated) during refrigerated storage (+4 °C/12 days). ns no significant differences between control and treated at a given sampling point

treated diet compared to the control diet. In contrast, others authors did not observe a significant effect of antioxidant-rich diets on the carbonyl level of cooked beef (Gatellier et al. 2010) and cooked pork (Haak et al. 2008; Botsoglou et al. 2012). Individual bioactive compounds of avocado such as tocopherols, chlorophylls and phenolic compounds have been found to protect proteins against carbonylation in model systems (Estévez et al. 2008; Estévez and Heinonen 2010) and processed muscle foods (Utrera et al. 2012). While it is plausible that dietary tocopherols may have contributed to protect proteins against oxidation, remains indefinite whether other bioactive compounds from dietary avocado were able to contribute to this protection upon absorption and potential accumulation in muscle tissue. Furthermore, it is a matter of further study the fact that the same dietary strategy was effective against protein carbonylation but showed no effect against the oxidation of tryptophan and thiols. It is known, however, that protein oxidation is a complex phenomenon and the chemistry that governs the oxidative degradation of each amino acid is fairly specific. It is also worth mentioning the consistent results between protein carbonylation and lipid oxidation as measured by TBARS, which is in line with previous reports (Ventanas et al. 2007; Estévez et al. 2008; Rodríguez-Carpena et al. 2011b). In view of these results, including avocado as a natural antioxidant source in the pig diet seems to be a good strategy to improve the quality of cooked pork through the inhibition of protein carbonylation and lipid oxidation.

Impact of dietary avocado on the formation of protein cross-linking during pork processing

In the present study, the formation of cross-links in processed pork was assessed through the analysis of two chemical structures: Schiff bases and disulphide bonds (Fig. 4a, b, respectively). Schiff bases are conjugated fluorophores formed as a result of the reaction between protein carbonyls and secondary amines (Estévez 2011). On the other hand, the oxidation of two cysteines leads to the formation of disulphide bonds (Soladoye et al. 2015). The formation of both protein cross-links was promoted during cooking and remained stable during the following chilled storage. Chelh et al. (2007) reported the formation of fluorescent pigments during meat cooking and linked their formation to the reaction of meat proteins with aldehydic products from lipid peroxidation. Later reports argued that the reaction between protein carbonyls and the δ -amino groups located in the side-chains of alkaline amino acids may be the more plausible formation pathway for Schiff bases in oxidized meat proteins (Estévez 2011; Utrera and Estévez 2012b). These fluorescent products are believed to contribute to the formation of insoluble protein aggregates

Fig. 4 Cross-links: Schiff bases (a) and disulphide bonds (b) in raw, cooked and cooked & chilled loin chops from pigs fed a control diet (control) and pigs fed an experimental avocado diet (treated) during refrigerated storage (+4 °C/12 days). ns no significant differences between control and treated at a given sampling point

during cooking, leading to meat shrinkage and water loss (Chelh et al. 2007; Gatellier et al. 2010). Other reports have associated Schiff bases with impaired protein functionality, altered protein digestibility and meat texture deterioration (Ganhão et al. 2010; Utrera and Estévez 2012b; Soladoye et al. 2015). Disulphide bonds are, on the other hand, contributors to myofibril shrinkage, meat toughening and hence, impaired meat tenderness (Soladoye et al. 2015). In the present study, loins from control group tended to have lower concentration of cross-links, which is in line with other protein oxidation measurements (i.e. protein carbonylation). These differences reached a significant extent between control and treated cooked loins for the Schiff bases measurement (Fig. 4a). Other authors have previously reported the impact of dietary manipulation on the extent of protein oxidation on cooked beef oxidation as measured by the formation of Schiff bases (Gatellier et al. 2010). In this experiment, supplementation with avocado led to an inhibition of Schiff bases during pork cooking through a plausible control of protein carbonylation given the aforementioned effectiveness of the dietary strategy against the formation of protein carbonyls. Given the negative effects attributed to the formation of these advanced protein oxidation products, the usage of avocado

by-products on pig feeding may have particular benefits in terms of meat quality.

Conclusion

This study provides scientific evidences on the benefits of using avocado by-products for pig feeding on the oxidative stability of processed pork. However, the inconsistency of the effects of dietary avocado on different lipid and protein oxidation measurements (1) brings into question the specific chemical nature of the bioactive compounds responsible for the observed effects (2) highlights the complex chemical mechanisms behind the interaction between these bioactive compounds and meat proteins and (3) advises towards the completion of further studies aimed to shed light on this complex chemistry and guarantee the benefits of dietary antioxidant strategies.

Acknowledgments Author Hernández-López thanks Conacyt for his research fellowship. The authors acknowledge Antonio Silva from SiPA (University of Extremadura) for his technical advice.

References

- AOAC (2000) Official methods of analysis of AOAC International, 17th ed. Association of Official Analytical Chemists, Gaithersburg, MD, USA
- Bell JE (1981) Fluorescence: solution studies. *Spectrosc Biochem I*(4):155–194
- Botsoglou E, Govaris A, Ambrosiadis L, Fletouris D (2012) Lipid and protein oxidation of α -linolenic acid-enriched pork during refrigerated storage as influenced by diet supplementation with olive leaves (*Olea europaea* L.) or α -tocopheryl acetate. *Meat Sci* 92:525–532
- Chelh I, Gatellier P, Saté-Lhoutellier V (2007) Characterisation of fluorescent Schiff bases formed during oxidation of pig myofibrils. *Meat Sci* 76:2010–2015
- Dall Aaslyng M, Bejerholm C, Ertbjerg P, Bertram HC, Andersen HJ (2003) Cooking loss and juiciness of pork in relation to raw meat quality and cooking procedure. *Food Qual Pref* 14:277–288
- Dreher LM, Davenport JA (2013) Hass avocado composition and potential health effects. *Crit Rev Food Sci Nutr* 53:738–750
- Dunshea FR, D'Souza DN, Pethick DW, Harper GS, Warner RD (2005) Effects of dietary factors and other metabolic modifiers on quality and nutritional value of meat. *Meat Sci* 71:8–38
- Estévez M (2011) Protein carbonyls in meat systems: a review. *Meat Sci* 89:259–279
- Estévez M, Heinonen M (2010) Effect of phenolic compounds on the formation of α -aminoacidic and γ -glutamic semialdehydes from myofibrillar proteins oxidized by copper, iron, and myoglobin. *J Agric Food Chem* 58:4448–4455
- Estévez M, Luna C (2016) Dietary protein oxidation: a silent threat to human health? *Crit Rev Food Sci Nutr*. doi:10.1080/10408398.2016.1165182
- Estévez M, Kylli P, Puolanne E, Kivikari R, Heinonen M (2008) Oxidation of skeletal muscle myofibrillar proteins in oil-in-water emulsions: interaction with lipids and effect of selected phenolic compounds. *J Agric Food Chem* 56:10933–10940

- Falowo BA, Fayemi OP, Muchenje V (2014) Natural antioxidants against lipid–protein oxidative deterioration in meat and meat products: a review. *Food Res Int* 64:171–181
- Folch L, Lees M, Sloane-Stanley CH (1957) A simple method for the isolation and purification of total lipid from animal tissues. *J Biol Chem* 226:497–509
- Frederiksen AM, Lund MN, Andersen ML, Skibsted LH (2008) Oxidation of porcine myosin by hypervalent myoglobin: the role of thiol groups. *J Agric Food Chem* 56:3297–3304
- Ganhão R, Morcuende D, Estévez M (2010) Protein oxidation in emulsified cooked burger patties with added fruit extracts: influence on colour and texture deterioration during chill storage. *Meat Sci* 85:402–409
- Ganhão R, Estévez M, Morcuende D (2011) Suitability of the TBA method for assessing lipid oxidation in meat system with added phenolic-rich material. *Food Chem* 126:772–778
- Gatellier P, Kondjoyan A, Portanguen S, Santé-Lhoutellier V (2010) Effect of cooling on protein oxidation in n-3 polyunsaturated fatty acids enriched beef. Implication on nutritional quality. *Meat Sci* 85:645–650
- Giessauf A, Steiner E, Esterbauer H (1995) Early destruction of tryptophan residues of apolipoprotein B is a vitamin E independent process during copper mediated oxidation of LDL. *Biochim Biophys Acta* 1256:221–232
- Haak L, Raes K, Van Dyck S, De Smet S (2008) Effect of dietary and α -tocopherol acetate on the oxidative stability of raw and cooked pork following oxidized linseed oil administration. *Meat Sci* 78:239–247
- Hernández-López SH, Rodríguez-Carpena JG, Lemus-Flores C, Grageola-Núñez F, Estévez M (2016) Avocado waste for finishing pigs: impact on muscle composition and oxidative stability during chilled storage. *Meat Sci* 116:186–192
- Mapiye C, Aldai N, Turner TD, Aalhus JL, Rolland DC, Kramer JKG et al (2012) The labile lipid fraction of meat: from perceived disease and waste to health and opportunity. *Meat Sci* 92:210–220
- Monahan FJ, Crackel RL, Gray JJ, Buckley DJ, Morrissey PA (1993) Catalysis of lipid oxidation in muscle model systems by haem and inorganic iron. *Meat Sci* 34:95–106
- Paiva-Martins F, Barbosa S, Pinheiro V, Mourão JL, Outor-Monteiro D (2009) The effect of olive leaves supplementation on the feed digestibility, growth performances of pigs and quality of pork meat. *Meat Sci* 82:438–443
- Rodríguez-Carpena JG, Morcuende D, Andrade MJ, Kylli P, Estévez M (2011a) Avocado (*Persea americana* Mill.) phenolics, in vitro antioxidant and antimicrobial activities, and inhibition of lipid and protein oxidation in porcine patties. *J Agric Food Chem* 59:5625–5635
- Rodríguez-Carpena JG, Morcuende D, Estévez M (2011b) Avocado by-products as inhibitors of color deterioration and lipid and protein oxidation in raw porcine patties subjected to chilled storage. *Meat Sci* 89:166–173
- Rodríguez-Carpena JG, Morcuende D, Estévez M (2011c) Partial replacement of pork back-fat by vegetable oils in burger patties: effect on oxidative stability and texture and color changes during cooking and chilled storage. *J Food Sci* 76:C1025–C1030
- Rodríguez-Carpena JG, Morcuende D, Estévez M (2012) Avocado, sunflower and olive oils as replacers of pork back-fat in burger patties: effect on lipid composition, oxidative stability and quality traits. *Meat Sci* 90:106–115
- Rysman T, Jongberg S, Van Royen G, Van Weyenberg S, De Smet S, Lund MN (2014) Protein thiols undergo reversible and irreversible oxidation during chill storage of ground beef as detected by 4,4'-dithiodipyridine. *J Agric Food Chem* 62:12008–12014
- Soladoye OP, Juárez ML, Aalhus JL, Shand P, Estévez M (2015) Protein oxidation in processed meat: mechanisms and potential implications on human health. *Comp Rev Food Sci Food Saf* 14(2):106–122
- SPSS (1999) SPSS for Windows: advanced statistic release. SPSS, Chicago
- Stadtman ER (1990) Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radic Biol Med* 9:315–325
- Toscas PJ, Shaw FD, Beilken SL (1999) Partial least squares (PLS) regression for the analysis of instrument measurements and sensory meat quality data. *Meat Sci* 52:173–178
- Utrera M, Estévez M (2012a) Analysis of tryptophan oxidation by fluorescence spectroscopy: effect of metal-catalyzed oxidation and selected phenolic compounds. *Food Chem* 135:88–93
- Utrera M, Estévez M (2012b) Oxidation of myofibrillar proteins and impaired functionality: underlying mechanisms of the carbonylation pathway. *J Agric Food Chem* 60:8002–8011
- Utrera M, Rodríguez-Carpena JG, Morcuende D, Estévez M (2012) Formation of lysine-derived oxidation products and loss of tryptophan during processing of porcine patties with added avocado byproducts. *J Agric Food Chem* 60:3917–3926
- Utrera M, Parra V, Estévez M (2014) Protein oxidation during frozen storage and subsequent processing of different beef muscles. *Meat Sci* 96:812–820
- Ventanas S, Ventanas J, Tovar J, García C, Estévez M (2007) Extensive feeding versus oleic acid and tocopherol enriched mixed diets for the production of Iberian dry-cured hams: effect on chemical composition, oxidative status and sensory traits. *Meat Sci* 77:246–256
- Villaverde A, Morcuende D, Estévez M (2014) Effect of curing agents on the oxidative and nitrosative damage to meat proteins during processing of fermented sausages. *J Food Sci* 79:1331–1342
- Wood JD, Enser M (1997) Factors influencing fatty acids in meat and the role of antioxidants in improving meat quality. *Brit J Nutr* 78:49–60
- Wu X, Beecher GR, Holden JM, Haytowitz DB, Prior RL (2004) Lipophilic and hydrophilic antioxidant capacity of common foods in the U.S. *J Agric Food Chem* 52:4026–4037