Meat texture and antioxidant status are improved when carnosic acid is included in the diet of fattening lambs

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ABSTRACT

Thirty-two Merino lambs fed barley straw and a concentrate alone (CONTROL group) or enriched with carnosic acid [0.6 g kg⁻¹ dry matter (DM), CARN006 group; 1.2 g kg⁻¹ DM, CARN012 group] or vitamin E (0.6 g kg⁻¹ DM, VITE006 group) were used to assess the effect of these antioxidant compounds on meat quality. After being fed the experimental diets for at least 5 weeks, the animals were slaughtered with the 25 kg intended body weight and the different muscles (longissimus lumborum; LL, gluteus medius; GM) were sliced and kept refrigerated under modified atmosphere packaging during 0, 7 and 14 days. The results indicate that carnosic acid seemed to be useful to delay lipid peroxidation in a medium colour-stable muscle such as GM, but this effect was lower than that observed when vitamin E was supplemented to fattening lambs. On the contrary, meat texture and protection against cholesterol oxidation were equally improved with both compounds.

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1. Introduction

The shelf life of meat and meat products is seriously shortened by two main factors: microbial spoilage and lipid peroxidation. Therefore, any finding focused on delaying either of these processes is relevant for the meat industry. The high quality meat demanded by the consumer in developed countries (Boleman et al., 1997) must be tender and free of chemically synthesised additives, so the inclusion of natural compounds (plant origin) in animal feedstuff, thus avoiding any further manipulation of the meat, is suitable to improve all these quality attributes.

In this context, special attention has been paid to the effects promoted by rosemary (Rosmarinus officinalis L.), an herb commonly used as a flavouring agent. The bioactive properties of this herb have been attributed to the phenolic compounds in rosemary plants (Hernández-Hernández, Ponce-Alquicira, Jaramillo-Flores, & Legarreta, 2009). These compounds have demonstrated antimicrobial and antioxidant activities when added to food as additives (McBride, Hogan, & Kerry, 2007), but they have also shown beneficial effects on eggs, milk and meat products when included in the diet of the animal (Botsoglou, Govaris, Giannenas, Botsoglou, & Papageorgiou, 2007; Galobart, Barroeta, Baucells, Codony, & Ternes, 2001; Jordán, Moñino, Martínez, Lafuente, & Sotomayor, 2010; Nieto, Díaz, Bañón, & Garrido, 2010). The main phenolic compound retained in animal tissues after the consumption of rosemary is carnosic acid (Moñino, Martínez, Sotomayor, Lafuente, & Jordán, 2008), so it can be hypothesised that the antioxidant properties on meat quality are mainly due to the increased concentration of this phenolic compound in the muscle. However, the amount of carnosic acid which must be fed to the animals to have beneficial effects on meat quality have not been quantified. It must also be considered that the concentration of phenolic compounds in the plants varies with maturity stage or climatic conditions, mainly drought (Munné-Bosch, Mueller, Schwarz, & Alegre, 2001). This is why feeding rosemary extracts with a known richness of carnosic acid, instead of intact plants will allow recommendations to be established about the amount of rosemary which should be fed to animals according to its level of carnosic acid.

The aim of the present study was to investigate the texture and antioxidant properties of meat when two different doses of rosemary extract (48% carnosic acid) were included in the diet of lambs. Likewise, vitamin E (one of the most frequently used antioxidants in animal nutrition) was included in another group as a positive control.

2. Material and methods

2.1. Animals and diets

Before the commencement of the trial, 32 male merino lambs, aged a month and a half were treated with ivermectin (Ivomec, Merial Labs, Barcelona, Spain) and vaccinated against enterotoxaemia (Milmoxan, Merial Labs, Barcelona, Spain).
After stratification on the basis of body weight (average BW, 15.2 ± 0.749 kg), the lambs were allocated randomly to four different groups (8 lambs per treatment): a control group (CONTROL), a second group fed a single dose (0.6 g carnosic acid kg⁻¹ of concentrate, CARN006) of carnosic acid (Shaaxni Scipher Biotechnology Co., Ltd, Xi’an, China; carnosic acid 470 g/kg dry matter (DM), FAD 12.65 g/kg DM, ash 16.13 g/kg DM), a third group fed a double dose of carnosic acid (1.2 g carnosic acid kg⁻¹ of concentrate, CARN002), and a fourth group fed vitamin E (α-tocopherol acetate 50%, Industrias de Alimentacion Animal, Spain) at a rate of 0.6 g kg⁻¹ of concentrate (VITE006, equivalent to 900 IU of vitamin E kg⁻¹ of concentrate). Animals were then penned individually. All handling practices followed the recommendations of the European Council Directive 86/609/EEC for the protection of animals used for experimental and other scientific purposes (EEC, 1986), and all of the animals were able to see and hear other lambs.

After 7 days of adaptation to the basal diet comprised of concentrate (50% barley, 20% soybean meal, 15% maize, 8% wheat, 4% molasses and 3% mineral premix; DM 888 g kg⁻¹, CP 178 g kg⁻¹ DM, NDF 134 g kg⁻¹ DM, and ash 56 g kg⁻¹ DM) and barley straw (DM 913 g kg⁻¹, CP 35 g kg⁻¹ DM, NDF 757 g kg⁻¹ DM, ash 55 g kg⁻¹ DM), all the lambs were fed barley straw and the corresponding concentrate feed alone (CONTROL group) or supplemented either with vitamin E or carnosic acid 48% (the carnosic acid richness of the extract was measured by HPLC according to Mollito et al., 2008) during 7 weeks. Concentrate (35 g kg⁻¹ BW day⁻¹) and forage (200 g day⁻¹) were weighed and supplied in separate feeding troughs at 9:00 a.m. every day, and fresh drinking water was always available. The orts were also weighed daily, and samples were collected for subsequent analyses.

2.2. Slaughter procedure, packaging, storage and sampling

The animals were slaughtered on four different days, two lambs per group each day. The lambs were selected each day according to their weight (25 kg intended body weight) and slaughtered by stunning and exsanguination from the jugular vein; they were then eviscerated and skinned. The hot carcass of each lamb was weighed, chilled at 4 °C for 24 h and weighed again. The longissimus lumborum (LL) and glutus medius (GM) muscles were removed from the right and left carcass sides.

The LL and GM muscles were cut into slices 2.5 cm thick, placed on impermeable polypropylene trays (a tray per opening day containing both LL and GM slices from only one animal) and wrapped with impermeable polypropylene trays (a tray per opening day containing and left carcass sides. (MAP) using a tabletop Multivac A300 packaging machine (Multivac, Germany). After 7 days of adaptation and exsanguination from the jugular vein; they were then eviscerated and skinned. The hot carcass of each lamb was weighed, chilled at 4 °C for 24 h and weighed again. The longissimus lumborum (LL) and glutus medius (GM) muscles were removed from the right and left carcass sides.

The LL and GM muscles were cut into slices 2.5 cm thick, placed on impermeable polypropylene trays (a tray per opening day containing both LL and GM slices from only one animal) and wrapped with ML40-G bags (Krehalal; Proveedora Hispanola Holandesia S.A., Barcelona, Spain), which were immediately modified-atmosphere packaged (MAP) using a tabletop Multivac A300 packaging machine (Multivac Verpackungsmaschinen, Wolfertschwenden, Germany). The air in the bags was replaced by a commercial gas blend intended for red and poultry meats consisting of 35% CO₂, 35% O₂ and 30% N₂, with a gas: meat volume ratio of about 2:5:1. The ML40-G bags had O₂ and CO₂ transmission rates of 20 and 100 ml m⁻² 24 h⁻¹, respectively, at 23 °C and 80% relative humidity. All packages were stored under simulated retail display conditions (12 h daily illumination and 3 ± 1 °C) and the air temperature was monitored using a Testo175-T2 data logger (Instrumentos Testo S.A., Cabriols, Barcelona, Spain). These trays were opened after 0, 7 and 14 days. On each sampling day, the concentrations of CO₂ and O₂ inside each tray were determined using a CheckMate 9900 (PBI Dansensor, Denmark). LL and GM raw meat samples were frozen for thiobarbituric acid reactive substances (TBARS) analysis and protein oxidation.

2.3. TBARS analysis

Thiobarbituric acid reactive substances (TBARS) were determined on pre-thawed, raw LL and GM samples aged for 0, 7 and 14 days under MAP according to the following procedure. The acid hydrolysis of TEP (1,1,3,3-tetraethoxypropane) was performed, thus yielding malondialdehyde (main product of lipid peroxidation) to construct the standard curve. Meat samples were thawed and a piece of 2.5 g was cut and homogenised for 30 s at 13,000 rpm with 20 ml of distilled water using a T25 digital Ultraturrax provided with a 18 G dispersing tool (IKA®-Werke GmbH & Co. KG, Germany). Thereafter, 5 ml of 25% trichloroacetic acid (TCA) were added, and then centrifuged at 12,100 × g during 15 min at 4 °C, filtered and 3.5 ml transferred to a 10 ml screw cap tube with 1.5 ml of thiobarbituric acid (0.6%). Samples were heated at 70 °C for 30 min and, after being cooled in ice for 10 min absorbance was measured at 532 nm (Schimadzu 160-UV). The results were expressed as µg MDA g⁻¹ meat.

2.4. Protein and cholesterol oxidation

Protein oxidation (P-OX) was performed on raw LL samples aged for 7 days under MAP. This procedure was carried out by the dinitrophenylhydrazine (DNPH) method using the protein carbonyl enzyme immunoassay kit provided by Deltacon S.L. (Spain).

From each opened tray another slice of GM was cooked in a grill at 180 °C, a temperature probe was inserted in the core of the samples, and the sample then cooked to an internal temperature of 70 °C. Finally, GM samples were cooled at 4 °C for 30 min, and then freeze-dried for oxysterols analysis. Cholesterol oxidation products (COPs), also called oxysterols, were determined according to Grau, Codony, Grampa, Baucells, and Guardiola (2001) on cooked GM samples aged for 7 days under MAP. Briefly, lipids were extracted from 1 g of cooked and freeze-dried GM samples using a mixture chloroform/methanol (2:1, v/v) (Folch, Lees, & Stonellane, 1957). 19-hydroxycholesterol (19-HC) was used as an internal standard. Ten ml of 1.5 methanolic KOH was then added and the mixture was kept in an orbital shaker for 20 h at room temperature under N₂ atmosphere and darkness to complete the cold saponification. The unsaponifiable material was extracted three times with diethyl ether in a separating funnel, and then purified by solid-phase extraction (SPE) according to Guardiola, Codony, Rafecas, and Boetella (1995). COPs were derivatised to trimethylsilyl (TMS) ethers prior to gas chromatographic (GC) analysis on an Agilent 7890 Series gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) provided with a FID detector by splitless injection into a VF-5ms CP8947 capillary column (50 m x 25 μm x 0.25 μm, Varian, Palo Alto, CA, USA). Chromatographic conditions were as follows: injection volume 1.5 μl; initial oven temperature 75 °C to 250 °C at 30 °C/min, to 290 °C at 8 °C/min, and to 292 °C at 0.05 °C/min; injector and detector temperatures were 250 and 280 °C, respectively. Helium was used as a carrier gas at a flow rate of 1 ml/min. The oxysterols 7α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol (7β-HC), 5,6α-euphorboxycholesterol (α-CE), 5,6β-euphorboxycholesterol (β-CE), cholestanetriol (CT), 25-hydroxycholesterol (25-HC) and 7- ketocholesterol (7-KC) were identified by comparing their retention times with those of authentic standards (Steraloids, Inc., UK) and quantified with the internal standard.

2.5. Texture (Slice Shear Force, SSF)

From each opened tray another slice of LL was cooked in a grill at 180 °C; a temperature probe was inserted in the core of the samples, and the sample then cooked to an internal temperature of 70 °C. Finally, LL samples were cooled at 4 °C for 30 min, and then frozen at −30 °C until texture analysis. LL cooked slices aged during 0, 7 and 14 days under MAP were used to measure the texture values. A slice 1-cm thick, 5-cm long parallel to the muscle fibres was cut and placed in the single-beam Lloyd Texture Analysers (Lloyd Instruments, UK) so that the blade shears perpendicular to the muscle fibres along the 5-cm dimension of the slice. Maximum shear force was interpolated by plotting the force-time curve and measuring the peak shear force. The peak force was the Slice Shear Force (SSF) for the sample
2.6. Statistical analysis

All the data were subjected to one-way analysis of variance with diet as the only source of variation using the GLM procedure of SAS package (SAS, 1999).

3. Results

3.1. TBARS analysis

Fig. 1 summarises the results of LL and GM muscles when lipid oxidation (TBARS) was studied after packaging in a controlled gas mixture for 0, 7 or 14 days.

The group being fed vitamin E (positive control) showed significantly lower TBARS values when compared to the CONTROL group in both LL and GM muscles at all storage periods (0, 7, and 14 days of refrigerated storage under a high oxygen MAP). As far as carnosic acid is concerned, a significant decrease (P<0.05) only in the LL of the CARN012 group was observed after 14 days of refrigerated storage when compared to the CONTROL group, whereas no significant effect (P>0.05) on TBARS values in LL muscle was detected at any storage time in the CARN006 group. In the GM muscle TBARS values were lower in both groups fed carnosic acid after 7 and 14 days when compared to the CONTROL group.

3.2. Protein and cholesterol oxidation

Table 1 summarises the P-OX results when the protein carbonyl content was measured by the dinitrophenylhydrazine (DNPH) method. As can be observed, the diet had no significant effect on this parameter for raw LL samples aged for 7 days under MAP.

Regarding cholesterol oxidation products (COPs, Table 1), significantly higher values (P<0.05) were observed for 7α-CE, 7β-CE, α-CE, 7-KC and total COPs in the CONTROL samples when compared to the antioxidant groups. On the contrary, β-CE and CT showed no significant differences between groups, whereas 25-HC was not detected. The low levels of the most atherogenic compounds (CT and 25-HC) in all of the cooked meat samples, even in the CONTROL group, are remarkable.

3.3. Texture

Fig. 2 shows the texture values in samples aged for 0, 7 and 14 days under MAP.

Significantly lower values (P<0.0001) for SSF were detected when antioxidant groups (CARN006, CARN012 and VITE006) were compared to the CONTROL at all sampling times (0, 7 and 14 days), with no significant differences between CARN006, CARN012 or VITE006 samples.

4. Discussion

A previous study (López-Bote, Daza, Soares, & Berges, 2001) has described a significant positive effect on lipid oxidation in meat at low supplementation levels of vitamin E (270 mg kg diet−1); however, the protective effects at different storage times were optimised at a dietary inclusion level of 550–625 mg α-tocopheryl acetate kg−1 diet. Consequently, in the present study vitamin E was supplied at 0.6 g kg−1 of concentrated feed (VITE006 group). A similar dose of carnosic acid was supplied to another group (CARN006) to compare the effectiveness of both components. The last group was fed a double dose of carnosic acid (CARN012) in order to clarify if this substance showed a dose dependent effect at meat level.

4.1. TBARS analysis

Faustman and Cassens (1990) reported a strong relationship between lipid oxidation and myoglobin oxidation. This is the reason why TBARS analysis was performed in two muscles with different

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>CARN006</th>
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<td>0.145</td>
<td>0.0443</td>
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**Note:** Different superscripts in the same day indicate statistical differences (P<0.05) between treatments.
colour stability: a high (LL) and medium (GM) colour-stable muscle (McKenna et al., 2005). The increments in lipid oxidation as a consequence of display time could be reduced by the highest dose of carnosic acid (CARN012) in a medium colour stable muscle, GM (days 7 and 14, Fig. 1b), whereas this effect was only significant after 14 days in a high colour stable muscle, LL (day 14, Fig. 1a). No explanation can be found for the high levels in TBARS values observed from the beginning of refrigerated storage in the LL muscle of the CARN012 group (day 0, Fig. 2).

4.2. Protein and cholesterol oxidation

Existing literature suggests that the oxidation of proteins does not follow the same pattern as lipid oxidation. Thus, some phenolic-rich plant (rosemary) and fruit (apple) extracts have been shown to inhibit protein carbonylation in meat, but the antioxidant effect was in general lower than that displayed against lipid oxidation (Estévez, 2011). Additionally, some polyphenols have been reported to promote protein carbonylation (Estévez, 2011). However, the present results differ from those observed by other authors when vitamin E was supplemented to cows (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004). These authors reported a significant correlation between total protein carbonyls and the instrumental texture of refrigerated storage (Table 1), since endogenous proteases were thereafter, they also might have been oxidised.

Regarding oxysterol content in meat samples, these compounds can be absorbed through the intestinal tract into the blood stream, thus increasing the susceptibility of the consumer to coronary heart disease. Since the main source of oxysterols in meats is heat processing, these substances were determined in cooked meat samples (cooked gluteus medius samples after 7 days of refrigerated storage under MAP). Vitamin E supplemented either to pigs (Eder, Müller, Kluge, Hirche, & Brandsch, 2005) or chickens (Grau et al., 2001) has been demonstrated to be effective in reducing oxysterol content in cooked meat samples. This is in agreement with the results observed in the present study, where both vitamin E and carnosic acid seemed to protect cholesterol against oxidation. Broncano, Petrón, Parra, and Timón (2009) reported similar levels of oxysterol content in pork; however they are not fully comparable to the results of the present study since they were obtained in cooked meat with lower lipid oxidation (these meat samples were not kept in refrigerated storage for several days). It must be noted, however, that oxysterol levels were much lower in the present study when compared to those reported for pigs fed unsaturated fats whose meat was cooked after being stored at 4°C for 9 days (Rey et al., 2001). All the samples (even those of the CONTROL group) showed very low levels of CT and 25-HC (Table 1).

It has been suggested that hydroperoxides of polyunsaturated fatty acids formed during lipid oxidation might be necessary to initiate cholesterol oxidation, so oxysterol content might be synergistically increased by unsaturated fat (Smith, 1987). In this sense, biohydrogenation in the rumen undergone by the unsaturated fatty acids consumed by the lambs might have protected their meat against cholesterol oxidation during refrigerated storage and later on during cooking. This is particularly important for the consumer concerned with healthier meat products, since many COPs, and especially CT and 25-HC, have been described as atherogenic oxysterols, responsible for acute injury to the endothelium (Peng, Taylor, Hill, & Morin, 1985, Taylor, Peng, Werthessen, Tham, & Lee, 1979).

4.3. Texture

The lower values (P < 0.0001) for SSF in all the antioxidant groups when compared to the CONTROL (Fig. 2) might have been due to the protection exerted by both vitamin E and carnosic acid against the oxidation of endogenous proteases during the aging process. In this sense, the proteolysis of key cytoskeletal proteins (such as desmin) can reduce the amount of water forced from the intra- and extramyofibrillar spaces of the cell as rigour progresses (Huff-Lonergan & Lonergan, 2005). These cytoskeletal and other myofibrillar proteins are known μ-calpain substrates, an enzyme highly susceptible to oxidation due to histidine and SH-containing cysteine residues at its active site (similar to m-calpain). Therefore the higher values of SSF for CONTROL samples when compared to the antioxidant groups might have been partially due to a reduced functionality of μ-calpain and m-calpain as a consequence of the oxidation undergone by the post-mortem muscle during the aging process, which might have reduced the juiciness and tenderness of the meat (Huff-Lonergan & Lonergan, 2005; Xiong, 2000). These results agree with the higher water holding capacity reported for the meat of the antioxidants groups (Morán et al., 2012). At the same time, these results (Fig. 2) do not disagree with the lack of significant differences in P-OX after 7 days of refrigerated storage (Table 1), since endogenous proteases were expected to catalyse reactions during the first days of the aging process; thereafter, they also might have been oxidised.

A second mechanism to explain the toughening of meat is related to the oxidation of myofibrillar proteins, which promotes aggregation and cross-linking (Estévez, 2011; Huff Lonergan, Zhang, & Lonergan, 2010). Consequently, carnosic acid accumulated in the muscle during the feeding of the lambs might have delayed this oxidation process. However, it must be noted that this has not been confirmed.

5. Conclusion

At the doses used in the present study, it can be concluded that carnosic acid supplemented to fattening lambs seems to protect meat from lipid peroxidation after a long period of time under MAP at refrigerated storage, particularly in medium colour stable muscles such as gluteus medius. It must be noted, however, that this effect is lower than that observed when vitamin E is supplemented. Texture and protection against cholesterol oxidation seem to be equally improved by both compounds.
Acknowledgements

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