Dietary supplementation of suckling lambs with anthocyanins: effects on growth, carcass, oxidative and meat quality traits

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Abstract

The aim of this research was to assess the effect of the dietary inclusion in suckling lambs of a red orange and lemon extract rich in anthocyanins on growth performance, antioxidant status, carcass characteristics and of both dietary antioxidant addition and aging on meat fatty acids, colorimetric and antioxidant profile, and meat texture. The experiment was carried out using 44 Merino male lambs randomly assigned to two groups: one group received the red orange and lemon extract (RLE) (RLE; n = 22) and the control group (CON; n=22) did not receive the anthocyanins. The RLE extract was orally administered (90 mg/kg of live weight) to each lamb every day from birth until slaughter (40 ± 1 days). *Longissimus lumborum* muscle was sampled and aged for 7 days. Rheological, colorimetric and oxidative parameters were affected by aging time and anthocyanins administration. The meat from RLE supplemented lambs had lower cooking loss and Warner-Blatzer Shear Force (WBSF) values. Moreover, thiobarbituric acid reactive substances (TBARS) and hydroperoxides were lower ($P < 0.01$) in RLE meat along the entire aging period, although in both groups they increased during aging ($P < 0.01$). The RLE addition affect yellowness values during aging, showing lower values in CON group at 7 d ($P < 0.01$). The RLE feed addition in able to positively affect oxidative animal status, and consequently animal welfare, enhancing meat oxidative stability and reducing colour deterioration during aging.

Key words: lamb meat, antioxidant additives, oxidative status, fatty acids

Abbreviations: ADF, acid detergent fibre; ADL, acid detergent lignin; AI, atherogenic index; ANOVA, analysis of variance; CAT, catalase; CF, crude fibre; CON, Control group; CP, crude protein; CW, cold carcass weight; DDW, deionized distilled water; DM, dry matter; DNPH, 2,4-dinitrophenyl hydrazine; DW, dry weight; EE, ether extract; FAME, Fatty acid Methyl esters; GHG, greenhouse gas; GLM, general linear model; GPx, glutathione peroxidase; GSH, reduced glutathione;

IMF, intramuscular fat; MDA, malondialdehyde; MUFA, monounsaturated fatty acuds;NADPH, nicotinamide adenine dinucleotide phosphate reductase; NDF, neutral detergent fibre; ORAC, oxygen radical absorbance capacity; PUFA, polyunsaturated fatty acids; RCD, reactive carbonyl derivative; RLE, Red orange and lemon extract group; SEM, standard error of the mean; SFA, saturated fatty acids; SW, slaughter weight; TBARS, thiobarbituric acid reactive substances; TCA, thricloracetic acid; TE, trolox equivalents; TEAC, trolox equivalent antioxidant capacity; TI, thrombogenic index; TMR, total mix ration; UFA: unsaturated fatty acids; WBSF, Warner-Blatzer Shear Force; Wf, final weight; WHC, water holding capacity; Wi, initial weight; WW, warm carcass weight.

1. Introduction

In the last two decades, increasing interest has been focused on the development of feeding strategies useful to induce variation in the meat quality of both ruminants and monogastric animals (Bennato et al., 2020). Small ruminant farming can potentially adopt low-input strategies by using agro-industrial by products as alternative feeding sources (Jabalbarezi Hukerdi et al., 2019). Many studies have been conducted focussing on the dietary effects in small ruminants on their meat of using different natural feeding sources such as olive leaves (Jabalbarezi Hukerdi et al., 2019), cardoon meal, ramie (Wei et al., 2019), spineless cactus (de Abreu et al., 2019), and sea buckthorn pomace (Qin et al., 2020). All these sources are positively perceived by consumers because they are recognised as natural and not synthetic chemical-additive compounds and their antioxidant activity can improve different aspects linked to meat quality, animal welfare and human health (Huang, 2018; Maggiolino et al., 2020). Dietary supplementation with natural antioxidant-rich feeds is also considered a good strategy to modulate the meat fatty acid composition in response to consumer demands (Cimmino et al., 2018), in a market context that requests more and more meat products with healthy and functional claims and characteristics. Several plants or extracts rich in bioactive compounds have demonstrated ability to affect the fatty acid composition of ruminant fat, and some of these compounds may also have

antioxidant activity and other activities (eg. Antimicrobial) (Görgüç et al., 2020). The main reason behind this lies in the fact that animal fats, except for fish, are generally characterized by high saturated fatty acids (SFA) concentration, which are notoriously associated with several human cardiovascular diseases (Wood et al., 2008). Moreover, in addition to reducing fat saturation, these substances may enhance oxidative stability (Vasta and Luciano, 2011). For example dietary inclusion of bioactive substances characterized by antioxidant activity can improve some antioxidant enzyme activity, such as glutathione peroxidase, superoxide dismutase and catalase (Han et al., 2007), can protect cell tissues from lipoperoxidative damage induced by free radicals and prevent lipid oxidation and restore animal oxidative balance improving animal health (Dong et al., 2015). Anthocyanins were just known for their colouring properties, but, in the last few years, they raised importance and scientific interest for their possible use as dietary antioxidants and health benefits (Damiano et al., 2019). It was observed their capacity to protect protein and lipids from direct DNA damages at cellular level (Acquaviva et al., 2003), and it was also hypothesized their capacity to activate some specific enzymes (glutathione reductase, glutathione peroxidase, and glutathione S-transferase) reducing oxidative stress (Shih et al., 2007).

The present study aimed to assess the effect of dietary inclusion of a red orange and lemon extract rich in anthocyanins, obtained from red orange and lemon processing waste, on suckling lamb growth performance, antioxidant status, carcass characteristics, and also of 7 days of aging on meat fatty acids, colorimetric and antioxidant profile, and meat texture analysis.

2. Materials and Methods

2.1. Animal management and feeding

The experiment was authorized by the Animal Welfare organization of the University of Naples Federico II (PG / 2019/0028161 of 03/19/2019).

93 The experimental procedures were carried out at the experimental farm of the Council for Agricultural
94 Research and Economics, Research Centre of Animal Production and Aquaculture (CREA, Bella
95 Muro, Potenza, Italy). Forty-four Merino male lambs, all born as singles, were involved in the trial.
96 All lambs were natural suckled and, from 25 days of life until slaughtering (40 ± 1 days) , *ad libitum*
97 alfalfa hay (188 g/kg crude protein DM, 322 g/kg crude fibre DM) and starter (205g/kg crude protein
98 DM, 18 g/kg fat DM, 250 g/kg crude fibre DM) was offered. Fatty acid composition of the
99 administered feeds is reported in Table 1. After colostrum administration from the mothers within 2
100 h from birth, the animals were randomly assigned to two groups: one group received the red orange
101 and lemon extract (RLE) rich in anthocyanin (n = 22), and the control group (CON; n = 22) did not
102 receive the anthocyanins. After slaughtering, meat samples of each lamb were randomly assigned to
103 three aging times (1, 4 and 7 days), for a completely randomized design.

104 Separation of anthocyanins content in the RLE extract was quantified using an ultra-Fast HPLC
105 system coupled with a photodiode array (PDA) detector and Finnigan LXQ equipped with an
106 electrospray ionization interface (ESI) in series configuration (Thermo Electron, San Jose, CA, USA).
107 The anthocyanins were quantified by UHPLC according to a method described by Fabroni et al.
108 (2016). Five millilitres sample of centrifuged juice were loaded onto C18 Sep-Pak cartridges (Waters,
109 Milford, MA, USA) that were previously conditioned with 5 mL of methanol and 5 mL of pure water.
110 The anthocyanins and other polyphenols were adsorbed by these columns; sugars, acids and other
111 soluble compounds were removed by washing the cartridges with water. Anthocyanins were eluted
112 with methanol containing 10 µL/mL formic acid. The acidified methanol solutions were evaporated
113 to dryness, and the dried fractions were dissolved in 70 µL/mL aqueous formic acid. Then, the
114 samples were filtered through a 0.45 µm membrane filter (Albet, Barcelona, Spain) and injected into
115 the UHPLC-MSn chromatographic system to identify the individual anthocyanins. Anthocyanin
116 separation was conducted on a Chromolith Perfor-mance RP-18 end-capped column (100 × 3.0 mm
117 inner diameter, monolithic particle size; Merck KGaA, Darmstadt, Germany) using an Ultra-Fast

HPLC system coupled to a PDA detector and a Finnigan LXQ ion trap equipped with an ESI interface in series con-figuration (Thermo Electron, San Jose, CA, USA). A binary gradient composed of water containing 70 µL/mL formic acid and methanol was used. The flow rate was 300 μLmin−1, the column temperature was 30°C and the injection volume was 20 μL. The range of wavelengths was set between 210 and 700 nm, and the chromatograms were recorded at 520 nm. The relative composition (g/100g) of individual anthocyanins were measured at 520 nm with a UV–Vis spectrophotometer (Varian Cary 100 Scan, Palo Alto, California, USA) and calculated from peak areas at 520 nm, using Xcalibur v.2.0.7 software. Moreover, flavanonic glycosides, expressed as hesperidin equivalents (g/100 g of powder extract), were determined by HPLC using the HPLC-PDAESI/ MSn apparatus described by an adapted HPLC method (Rouseff et al., 1987) and shown in Table 2.

During the entire suckling period, until slaughter, the two treatment groups were housed in two different pens with their dams and had access to the same feed. The dietary intake was calculated as of 65 and 68 g/day of starter and 18 and 20 g/day of hay respectively for RLE and CON groups, on average during the experiment. Feed intake was determined daily from unconsumed feed before the next feeding. The amount of RLE extract administrated was 90 mg/kg of live weight. The supplement was orally administered to each lamb in the RLE group every day. It was mixed with water to obtain a cream (Maggiolino et al., 2019a), which was then administered directly in the mouth using a large syringe. The lambs in the RLE group were weighed every 2 days at 7:00 am to adjust the daily amount of RLE.

Blood samples were aseptically collected at birth (d 1), and d 20 and 40 via jugular vein puncture using disposable needles (23G) as described by De Palo et al. (2018a), with a negative pressure system for plasma (4 mL tubes with 15 USP U/mL of heparin) (Becton, Dickinson Canada Inc, Vacutainer 1, Oakville, Canada). Heparinized tubes were stored on ice and centrifuged (1500 × g for 10 min) within 1 h. All plasma samples were stored at -20°C until processing.

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144 *2.2. Slaughter procedures and carcass measurements*

145 All lambs were weighed (SW, slaughter weight) after fasting for 12 h with free access to water. The
146 lambs were transported approximately 15 km to the abattoir and the journey time was less than 30
147 min, and they were slaughtered on the same day at a European Community-approved abattoir in
148 compliance with European Community laws on Animal Welfare in transport (1/2005EC) and the
149 European Community regulation on Animal Welfare for slaughter of commercial animals
150 (1099/2009EC).

151 Warm carcass weight of each lamb was recorded after the removal of non-carcass components (head,
152 skin, feet, lungs, trachea, heart, liver, spleen, gastro-intestinal tracts and testicles). Warm dressing
153 percentage was calculated as the formula WW/SW (WW = warm carcass weight; SW = slaughter
154 weight). After, all carcasses were chilled at 4°C for 24 h the cold dressing percentage was calculated
155 using the formula CW/SW (CW = cold carcass weight). Carcass measurements were recorded and
156 some carcass indexes calculated as described by Yakan et al. (2016): carcass length (from the caudal
157 edge of the last sacral vertebra to the dorso-cranial edge of the atlas), internal carcass length (length
158 from the cranial edge of symphysis pubis to the cranial edge of the first rib), leg length (length from
159 the symphysis pubis to the tarsal-metatarsal joint), chest circumference (circumference measurement
160 of chest at the widest rib area), chest width (widest chest measurement between left and right side at
161 the rib area), leg compactness (leg weight/leg length; kg/m) and carcass compactness (cold carcass
162 weight/carcass length; kg/m).

163 The intramuscular pH was recorded at slaughter, 1 and 24 h *post-mortem* with a portable pH meter
164 with glass electrode shaped to easily penetrate meat (Carlo Erba pH 710, Carlo Erba Reagents, Milan,
165 Italy). Before each measurement, the pH meter was automatically calibrated for muscle temperature
166 and using standard solutions with 4 and 7 pH values (Crison, Lainate, Italy).

167

168 *2.3. Meat, kidney, liver and intestine sampling and analysis*

169 The *Longissimus thoracis et lumborum* muscle was sampled (from the 1st thoracic to the 5th lumbar
170 vertebra) on the slaughter day, after chilling 4 h. It was cut in three parts and each part was randomly
171 assigned to one of the three experimental storage days: 1, 3 or 7. All sections were placed extruded
172 polystyrene trays (AERpack PCM0330 produced by Coopbox Italia) and wrapped in film (Cryovac
173 LID2050, Passirana di Rho, Milano, Italy) and stored until the preassigned storage day at a
174 temperature of 4°C. Chemical composition of the muscle was analysed only on day 1 and these
175 samples were ; stored at -20°C until analysis. The pH, texture profile, colorimetric and oxidative
176 profile analysis were performed on samples held for 1, 3 and 7 days of storage. The kidney cortex,
177 the right posterior section of the liver and part of the small intestine were collected for lipid oxidation
178 determination and the measurement of nitric oxide (NO) production and superoxide dismutase (SOD),
179 catalase (CAT) and glutathione peroxidase (GPx) activities.

180

181 *2.3.1. Chemical composition*

182 The muscle epimysium was removed and then the sample was triturated in a domestic blender until
183 a homogeneous mass was obtained and chemical composition (moisture, protein content,
184 intramuscular fat and ash) was determined as described by Maggiolino et al. (2019b).

185 *2.3.2. Water holding capacity, cooking loss and thawing loss*

186 Water-holding capacity (WHC) was calculated using the centrifugation method as described by De
187 Palo et al. (2018b). The cooking loss was determined as described by De Palo et al. (2015) and
188 post-thawing losses were calculated as described by De Palo et al. (2014).

189

190 *2.3.3. Meat Texture profile analysis*

191 The Warner–Bratzler shear force (WBSF) was analysed as described by De Palo et al. (2014). Three
192 pieces of meat of 1 × 1 × 2 cm (height × width × length) were removed parallel to the muscle-fibre
193 direction after a single sample was cooked in a plastic bag up to an internal temperature of 70°C for
194 3 min in a water bath at 85°C (measured with a copper constant in fine wire thermocouple, Model
195 5SC-TT-T-30–36, Omega Engineering Inc., fixed in the geometrical center of the sample). All
196 samples were cut perpendicular to the muscle fiber direction using an Instron 1140 apparatus (Instron,
197 High Wycombe, UK) provided with a computer, using a crosshead speed of 50 mm/min and a load
198 cell of 50 N. Each sample was sheared 3 times and these 3 values measured were used to obtain the
199 mean value for each sample. Maximum shear force, shown by the highest peak of the force–time
200 curve, represents the maximum resistance of the sample to the cut, and was expressed as N.
201 The texture profile analysis was performed on raw meat at room temperature using a TA-XT2 texture
202 analyzer (Stable Micro Systems, Godalming, UK). One cylinder with a 1.5 cm height and 2 cm
203 diameter was prepared from every sample. A double compression cycle test was performed up to 50%
204 compression of the original portion height with an aluminum cylinder probe of 2cm diameter. Five
205 seconds elapsed between the two compression cycles. Force–time deformation curves were obtained
206 with a 25 kg load cell applied at a cross head speed of 2 mm/s. The following parameters were
207 quantified: hardness (maximum force of the first compression cycle required to compress the sample,
208 N), adhesiveness (negative area under the abscissa after the first compression, N/s), springiness
209 (ability of the sample to recover its original form after the deforming force was removed, cm),
210 cohesiveness (extent to which the sample could be deformed prior to rupture, dimensionless),
211 chewiness (work required to masticate a solid food before swallowing, J).
212

213 *2.3.4. Colorimetric analysis*

214 At unpackaging the surface meat colour of samples 2cm thick was determined according to the CIE
215 L*, a*, b* (CIE, 1976) colour system using a Minolta CR-300 colorimeter (light source D65; Minolta

Camera Co. Ltd., Osaka, Japan). Reflectance measurements were collected from a 0° viewing angle with A-pulsed xenon arc lamp with a reading surface of 8 mm diameter. For each day, three measurements were performed on three different points (De Palo et al., 2012). Three measurements were obtained at each point, performed by rotating the detector system by 90° compared to the previous one, giving a total of nine measurements per sample. The colorimeter was calibrated on the Hunter-lab colour space system using a white title (L* = 99.2, a*= 1.0, b*= 1.9). The a* and b* values were used to determine chroma (C*) = $(a^2 + b^2)^{1/2}$ and hue (radians, H) = $\tan^{-1}$ (b/a) according to Maggiolino et al. (2020).

2.3.5. Meat thiobarbituric acid reactive substances (TBARS), protein carbonyls and hydroperoxides analyses

Minced muscle samples (5g) were placed in a 50-mL test tube and homogenized with 15 mL deionized distilled water (DDW). An aliquot of homogenate (1 mL) was transferred to a glass tube for TBARS determination and 0.05 mL of butylated hydroxytoluene (72 µL/mL in ethanol) was added along with 1,950 mL of TBA/trichloracetic acid (TCA)/HCl (3.75 µL/mL TBA, 150 µL/mL TCA and 0.25 N HCl). The sample solution was shaken and then incubated at 90°C for 15 min in a thermostatic bath. After this period, samples were cooled to room temperature (15-30°C) and then centrifuged at 2,000 × g for 15 min. Supernatant absorbance at 531 nm was measured against a blank containing 2 mL of TBA/TCA/HCl solution in 1 mL of distilled water. The TBARS were calculated comparing with a standard curve constructed with 1,1,3,3-tetramethoxypropane, and the concentration of lipid oxidation was expressed as milligrams of malondialdehyde (MDA) per kg of meat (Buege and Aust, 1978).

Four mL of CH_3OH and 2 mL of $CHCl_3$ were added to 2 mL of homogenate (previously prepared for TBARS determination). The samples were vortexed for 30s and then 2 mL of $CHCl_3$ and 1.6 mL of 9 µL/mL NaCl was added. The samples were shaken for 1 min and then centrifuged at 3,500 × g for

241 10 min at 4°C. Two mL of lipid extract were sampled from the lower chloroform phase and processed
242 with 1 mL of $CH_3COOH/CHCl_3$ and 50 µL of KI (1.2 g/L mL distilled water). Samples were stored
243 for 5 min in a dark room and added with 3 mL of 5 µL/mL of CH_3COOCd and then vortexed and
244 centrifuged at 4,500 × g for 10 min at 40°C. Absorbance at 353 nm was measured against a blank
245 tube in which the meat homogenate was replaced by 2 mL of distilled water (De Palo et al., 2013).
246 Results were expressed in millimoles per gram of meat according to Buege and Aust (1978).
247 Meat samples (2 g) were homogenized in 20 mL of 0.15 M KCl for 2 min. Two aliquots of
248 homogenate (50 µL each) were added with 1 mL 100 µL/mL TCA and then centrifuged at 1,200 × g
249 for 3 min at 4°C to measure protein oxidation. The first aliquot was used as a standard and added with
250 1 mL of 2 M HCl solution. The second aliquot was added with 1 mL of 2 M HCl containing 10 mM
251 2,4-dinitrophenyl hydrazine (DNPH). Samples were incubated for 1 h at room temperature (15 to
252 30°C) and shaken every 20 min, and then 1 mL of 100 µL/mL TCA was added. The samples were
253 vortexed for 30 s and centrifuged 3 times at 1,200 × g for 3 min at 4°C and the supernatant removed.
254 Care was taken not to disrupt the pellet. The pellet was washed with 1 mL of ethanol:ethyl acetate
255 (1:1), shaken, and centrifuged 3 times at 1,200 × g for 3 min at 4°C and the supernatant removed. The
256 pellet was then dissolved in 1 mL 20 mM sodium phosphate 6 M guanidine hydrochloride buffer.
257 Samples were then shaken and centrifuged at 1,200 × g for 3 min at 4°C. Carbonyl concentration was
258 calculated on the DNPH treated sample at 360 nm with a Beckman Coulter DU800 (Beckman
259 Instruments Inc., Brea, CA, USA) and expressed as nanomoles carbonyl per milligram protein.
260 Protein concentration was calculated according to the Biuret assay (Tokur and Korkmaz, 2007; De
261 Palo et al., 2013a).

262

263 *2.3.6. Plasma TBARS, protein carbonyls and hydroperoxides analyses*

264 The TBARS were measured fluorometrically according to Gondim et al. (2009) by adding 100 mL
265 of plasma to a 3.7 µL/mL thiobarbituric acid solution. Plasma reactive carbonyl derivative (RCD)

levels were measured according to Faure and Lafond (1995). The RCD levels were determined using the carbonyl reagent DNPH. Plasma (200 mL) was mixed with 1 mL water and 2 mL 200 µL/mL trichloroacetic acid and centrifuged at 1000 × g for 10 min. The pellet was resuspended in 1 mL of 10 mmol/L DNPH and incubated for 60 min at 37.8°C. For control, 1 mL of 1 mol/L hydrochloric acid was used instead of DNPH. Subsequently, 1 mL of 200 µL/mL trichloroacetic acid was added, and the sample was centrifuged at 1000 × g for 10 min. The pellet was washed with 1:1 ethanolethyl acetate solution and centrifuged at 1000 × g for 10 min. The pellet was mixed with 1 mL of 6 mol/L guanidine (diluted in 20 mmol/L dihydrogenphosphate at pH 2.3). Finally, the sample was incubated for 40 min at 37.8°C. The absorbance was measured at 380 nm.

Hydroperoxides were analysed according Södergren et al. (1998). Aliquots (90 mL) of plasma were transferred into eight microcentrifuge vials (1.5 mL). Ten microliters of 10 mM TPP in methanol were added to four of the vials to reduce ROOHs, thereby generating a quadruplicate of blanks. Methanol (10 mL) was added to the remaining four vials to produce a quadruplicate of test samples. All vials were then vortexed and incubated at room temperature for 30 min prior to the addition of 900 mL of FOX2 reagent. After mixing, the samples were incubated at room temperature for 30 min. The vials were centrifuged at 2400 × g for 10 min with a swing-out rotor (Hettich Rotenta / RP centrifuge, Hettich-Zentrifugen, Tuttlingen, Germany). Absorbance of the supernatant was measured at 560 nm using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The ROOH concentration in the plasma samples was calculated using the mean absorbance difference between quadruplicates of test samples and blank samples.

2.3.7. Meat and plasma antioxidant activity evaluation

About 400 mg of meat were homogenized in a tissue homogenizer in 4 mL of saline at 4°C. The homogenate was centrifuged at 4°C for 20 min at 7000 × g and the supernatant was collected to determine the antioxidant enzyme activities. Plasma was analyzed as it was. The SOD (SOD, EC

1.15.1.1), CAT (CAT, EC 1.11.1.6) and GPx (GPx, EC1.11.1.9.) activity were measured as described by Tateo et al. (2020).

2.3.8. Fatty acid Methyl esters (FAME) analysis

The FAME were prepared by transesterification of the lipid extract, as described by De Palo et al. (2013b) both for meat and feed, using methanol in the presence of 3 µL/mL hydrochloric acid in methanol (vol/vol). Then, FA were determined with a Trace GC Thermo Quest Gas Chromatograph (Thermo Electron, Rodano, Milan, Italy) equipped with a flame ionisation detector. The derivatives were separated on a capillary column (Supelco SP-2380 fused-silica column, 60m length, 0.25mm internal diameter and 0.20-mm film thickness; Sigma-Aldrich, St Louis, MO, USA). Injector and detector temperatures were held at 260°C. Column oven program temperatures were as follows: T1 = 80°C, hold 1 min; T2 = 150°C ramp at 15°C/min, hold 2 min; T3 = 220°C ramp at 5°C/min, hold 2 min; and T4 = 250°C ramp at 15°C/min, hold 5 min. The flow rate of the carrier gas (helium) was set at 0.8 mL/min. Identification of FAME was based on the retention times of reference compounds (Sigma-Aldrich, St Louis, MO, USA) and mass spectrometry. The fatty acid composition was expressed as the percentage of total FAME (Supelco TM 37 Component FAME Mix, Catalog Number 47885-U, Sigma-Aldrich). Nutritional implications were assessed by calculating the amount of saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), n-3 and n-6 FA, as well as the SFA:PUFA, SFA:UFA and the n-6:n-3 ratios. Moreover, the atherogenic index (AI) and thrombogenic index (TI) were calculated according to Ulbricht and Southgate (1991).

313 *2.3.9. Malondialdehyde (MDA), nitrite and nitrate assay and markers of oxidative stress in liver,*
314 *kidney and intestine*

315 Lipid peroxidation was determined by assaying the MDA levels according to Ohkawa et al. (1979).
316 It was determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric (532
317 nm) product, proportional to the MDA present. Kidney, liver and intestine were homogenized on ice
318 with MDA Lysis Buffer. To form the MDA-TBA adduct, TBA solution was added to each sample
319 and incubated al 95°C for 60 min. Then, each reaction mixture was placed into a 96 well plate to
320 measure the absorbance at 532 nm. Results were expressed as nanomoles/mg of protein.
321 The production of nitrite (NO_2) and nitrate (NO_3), stable metabolites of NO production, was
322 determined in the supernatant of kidney, liver and intestine by Griess reagent in according to Ciarcia
323 et al. (2010). Nitrate was reduced to nitrite by addition of nitrate reductase (0.4U/mL) in the presence
324 of 10 mM NADPH and 2.5 mM flavin adenin dinucleotide and then assayed as nitrite. The plates
325 were incubated with the Griess reagent at 25°C under reduced light for 20 min. Absorbance was read
326 at 550 nm using a spectrophotometer Glomax Multi Detection System (Promega). The data were
327 expressed as picomoles of nitrite for milligrams of proteins.
328 The activity of SOD, CAT, and GPx was determined by using a spectrophotometer at 450nm, 520nm,
329 and 412nm, respectively, according to previous studies (Sinha, 1972; Akerboom and Sies, 1981; Sun
330 et al., 1988). Samples from all groups were collected at the end of the treatment. One gram of each
331 tissue was homogenized with 9 mL of saline solution using a tissue homogenizer and centrifuged at
332 2000 × g rpm for 15 min at +4°C. The supernatant was collected and used for the measurement of
333 SOD, CAT and GPx activity by a spectrophotometer (Glomax Multi detection system, Promega,
334 Milan, Italy). The antioxidant enzymes activity was expressed as units for milligram of proteins.
335
336 *2.4. Statistical analysis*

The data set was tested for normal distribution and variance homogeneity (Shapiro-Wilk). Each lamb represented an experimental unit. The data of growth performance, carcass traits, indexes and meat chemical composition were subjected to analysis of variance (ANOVA) using the GLM by SAS software (SAS, 2011), according the following model:

$$y_i = \mu + A_i + \varepsilon_{ij,}$$

where y_{ijk} are dependent variables; μ is the overall mean; A was the effect of the i^{th} inclusion of the anthocyanin in the diet (i = 1, 2) and ε_{ij} was the error term.

The colorimetric, texture, oxidative parameters and fatty acid profile were analyzed using the MIXED procedure of SAS (SAS, 2011) with repeated measures, according the following model

$$y_{ijk} = \mu + \alpha_i + A_j + T_k + (A \times T)_{jk} + \varepsilon_{ijkl,}$$

where y_{ijk} are dependent variables; μ is the overall mean; α_i is the constant of the lamb random effect; A was the effect of the j^{th} inclusion of the anthocyanin in the diet (j = 1, 2), T was the effect of the k^{th} ageing (k = 1, …, 3), A × T was the effect of the interaction of the j^{th} anthocyanin inclusion in the diet and k^{th} ageing (1,…,6), and ε_{ijkl} was the error term. When not significant, the binary interaction was dropped from the model. A Tukey test was applied to evaluate the differences according ageing. The significance was set at $P < 0.05$.

3. Results

3.1. Growth performance, cut incidence and meat chemical composition

Anthocyanins inclusion in the lambs' diet during the first 40 days of life did not affect live weight at slaughter (12.2 kg and 12.4 kg for RLE and CON respectively), carcass measurements, dressing percentage (64.2 % and 65.1 % for RLE and CON respectively) or the proportion of cuts in the carcass (Table S1). Moreover, chemical composition of the meat was not different between the RLE and CON groups.

362 *3.2. Rheologic parameters and texture profile analysis*

363 In Table 3 the results of rheologic parameters and texture profile analysis are shown. The pH in both
364 groups was lower (P < 0.01) at 7 days than previous aging times. Similarly, WHC was lower in both
365 groups at 7 days (RLE, *P* < 0.01; CON, *P* < 0.05). Thawing loss was affected by aging, showing
366 higher values on 7th aging day compared to the 1st day in CON (*P* < 0.01) and RLE (*P* < 0.05) groups.
367 Moreover, it was higher (P < 0.01) in CON group. The RLE lamb's meat showed higher (*P* < 0.05)
368 cooking loss values at the 7th day compared to the day 1. The same parameter in CON meat showed
369 higher values at 3 days (*P* < 0.05) and 7 days (*P* < 0.01) compared to day 1. The CON group showed
370 higher (P < 0.01) thawing loss values than RLE. The shear force values were characterized by a drop
371 at 3 days aging (*P* < 0.01) and then remained constant until 7 days aging in both groups (*P* < 0.01),
372 although higher (P < 0.01) in CON group. Juiciness decreased during aging in both experimental
373 groups, with higher (*P* < 0.01) values in RLE animals compared to CON at the 7th day. Chewiness,
374 that was always lower (*P* < 0.01) in RLE meat, decreased (*P* < 0.01) in both groups during aging.

375 *3.3. Meat color, oxidative profile and enzymes*

376 Color and oxidative parameters of the meat are reported in Table 3. Lightness increased (*P* < 0.01)
377 during aging in meat of both experimental groups, with higher (*P* < 0.01) values in RLE meat.
378 Differently, redness and yellowness decreased (*P* < 0.01) during aging time in meat from both the
379 experimental groups. No differences at each ageing time between groups were observed for redness.
380 Moreover, b* values (yellowness) in the CON group at 3 d (*P* < 0.05) and 7 d (*P* < 0.01) were lower
381 compared those from the RLE group. Hue and chroma showed decreasing (*P* < 0.01) values during
382 aging time.

383 The increase of TBARS and hydroperoxides concentration with increasing day aging was greater
384 with control than RLE treatment; adding RLE did not change their concentration at day 1, but
385 decreased (P<0.01) at 3 and 7 d for TBARS and at 7 d for hydroperoxides. Protein carbonyls increased
386 (*P* < 0.01) in both groups, with higher values at 7 d compared to the first day, with no differences

387 between the experimental groups. The superoxide dismutase, catalase and glutathione peroxidase
388 activities in the meat samples decreased ($P < 0.01$) in both the groups during aging, but only
389 superoxide dismutase and glutathione peroxidase showed differences between groups: adding RLE
390 they were higher ($P < 0.01$) during the whole aging period.

391

392 *3.4. Fatty acid profile*

393 Fatty acid results are reported in table 4. Considering SFA, only myristic (C14:0), palmitic (C16:0)
394 and stearic (C18:0) acids showed differences between groups, with lower ($P < 0.01$) values in meat
395 of animals supplemented with RLE. Aging time affected the concentration of the UFA C16:1,
396 C20:5n-3 and C22:5n-3. The concentration of C16:1 after aging for 1 day was lower ($P < 0.05$) than
397 after 3 days in meat from lambs of the RLE group. Both C20:5n-3 of RLE meat and C22:5n-3 of
398 CON group concentration were higher ($P < 0.05$) after 3 and 7 days than after 1 day of aging.
399 Moreover, C20:5n-3 concentration in RLE meat was higher ($P < 0.01$) than CON meat after 1 day of
400 aging, and C22:5n-3 concentration in RLE meat was higher ($P < 0.01$) than CON meat after 3 and 7
401 days of aging. Oleic acid (C18:1) and linoleic acid (C18:2n-6) were found at higher ($P < 0.01$)
402 concentration in the meat from RLE lambs compared with meat from the CON lambs. Moreover, the
403 RLE group was characterized by higher ($P < 0.01$) concentration of C20:5n-3 at day 1 and of C22:5n-
404 3 at days 3 and 7 compared to CON lambs.

405 Results about fatty acids groups and AI and TI are reported in Table 5. Aging affected total n-3 of
406 both groups (Table 5), showing higher ($P < 0.05$) values at day 1 then others in both the CON and
407 REL groups. Moreover, total n-3 were even higher ($P < 0.01$) in the RLE group. By contrast, the n-
408 6/n-3 ratio in the CON group at the beginning of aging was lower than after 3 ($P < 0.05$) and 7 ($P <$
409 0.01) days of aging. The total n-6 percentage in RLE meat was higher ($P < 0.05$) than CON meat at
410 all stages of aging. Meat obtained by RLE lambs was characterized by higher ($P < 0.01$) MUFA,

411 PUFA and UFA concentrations and the lowest ($P < 0.01$) SFA concentrations, SFA/PUFA and
412 SFA/UFA ratio, AI and TI values.

413

414 *3.5. Plasma oxidative profile and antioxidant enzyme quantification*

415 Plasma TBARS (Table 6) did not show differences during the lambs' life in RLE group, instead they
416 showed higher ($P < 0.01$) values at 40 days compared to previous sampling times in the CON group.
417 At this time the CON group was characterized by higher ($P < 0.01$) plasma TBARS values than those
418 in the RLE group. Plasma hydroperoxides increased during the growth of the lambs. The RLE lambs
419 at 40 days of age showed higher ($P < 0.05$) values than at the beginning of the experiment, whereas
420 CON animals exhibited increased ($P < 0.01$) production of plasma hydroperoxides at 20 days of age
421 and this remained constant thereafter. Moreover, at 20 days of age, CON lambs had higher ($P < 0.01$)
422 values than RLE lambs. By contrast, protein carbonyls in plasma didn't show any difference between
423 groups and during the growth of the lambs. Plasma superoxide dismutase and glutathione peroxidase
424 increased ($P < 0.01$) during growth, and at 20 and 40 days of age CON group showed lowest ($P <$
425 0.01) values of both enzymes. The plasma catalase activity showed higher ($P < 0.01$) values at 40
426 days of age compared to previous days in both groups, and at the same age lower ($P < 0.01$) values
427 in CON than RLE group.

428

429 *3.5. Kidney, hepatic and intestinal MDA, NO SOD, CAT and GSPx activity*

430 In Table 7 the lipid peroxidation results in the kidneys, liver and intestine are shown. MDA levels in
431 all tissues did not increase in RLE animals with respect to the CON group. Moreover, there was no
432 difference in NO production, or SOD activity in the kidneys at the end of treatment period between
433 groups. The CAT and GSPx activity of all tissues did not change with RLE administration at the end
434 of the treatment.

435

436 ## 4. Discussion

437 ### *4.1. Growth performance, carcass traits, rheologic parameters and texture profile analysis,*
438 *colorimetric parameters and oxidative profile*

439 Fed addition with plant extract, particularly from agricultural waste, on both animal in vivo
440 performance and meat characteristics is a debated topic, with inconsistent results. The absent dietary
441 treatment effect of RLE on growth performance and carcass characteristics, carcass conformation,
442 carcass indexes and ultimate pH we observed are consistent with other findings in small ruminants
443 (Kotsampasi et al., 2017; Jabalbarezi Hukerdi et al., 2019; Salami et al., 2019), although some others
444 report positive effect (Yusuf et al., 2014). Differences are probably due to great variability of multiple
445 factors as the composition of the extracts involved in the trial, the age at slaughter, and also the
446 bioavailability of phenolic compounds in ruminants and monogastric. Although changes in rumen
447 microbial flora can occur after antioxidant substances addition, it is not well known what can occur
448 in lambs that are still functional monogastric. We can suppose that intestinal microbiome can
449 influence absorption of different additive compounds, although we can't exclude in our trial that,
450 during last days, rumen activity didn't partially start. Also meat chemical composition did not vary
451 when lambs were supplemented with RLE. Similar results were observed after the addition of
452 antioxidant substances in diet of lambs (Qin et al., 2020) (Quiñones et al., 2019) and beef (Maggiolino
453 et al., 2020). Meat composition was similar to that reported previously in lambs slaughtered at a
454 similar age (De Palo et al., 2018b).

455 Multiple conditions, both pre and post-mortem, can affect glycogen synthesis and glycogenolytic
456 pathways in the muscle and consequently the meat acidity (Pastsart et al., 2013; De Palo et al., 2016).
457 During aging, there is an expected decline of pH and it can influence meat quality. The pH variation
458 is particularly linked to meat tenderness because it influences glycolysis (Hopkins et al., 2015) and
459 consequently myofibril length (Ferguson and Gerrard, 2014). In the current study there was no

460 indication that pH change was impacted by the supplementation with RLE, although values are
461 slightly higher than what usually reported in lamb meat, so the reason for meat from RLE
462 supplemented lambs to be more tender than that from the CON lambs is not obvious. Changes are
463 relatively low and unlikely to detected by consumers, however future studies should examine the
464 collagen characteristics of muscle from RLE supplemented lambs. Wood et al. (2008) suggested that
465 oleic acid plays an important role in meat tenderness by affecting the melting point. An increase of
466 oleic acid reduces the melting point, improving tenderness and juiciness in meat. Since our results
467 reported that RLE meat is richer in oleic acid, this may explain why the shear force was lower, but
468 this is speculative and requires clarification as does any impact of the lower cooking loss in the meat
469 from RLE supplemented lambs.

470 Color plays a major role as a sensory property as meat freshness and wholesomeness affects
471 consumer's perception , and any deviation can lead to discounting of meat (Maggiolino et al., 2020;
472 Yang et al., 2020). In both dietary treatments lightness values increased, and redness and yellowness
473 decreased. Luciano et al. (2009) observed similar trends in lightness and redness, but different trends
474 for yellowness. Meat chemical composition represents the most important factor influencing lightness
475 of fresh meat, in particular its water content and its intramuscular fat concentration and composition
476 (Mancini and Hunt, 2005). During aging there is a breakdown of muscle fibers' with the passage of
477 water from the intracellular to the extracellular region, consequently increasing lightness values (De
478 Palo et al., 2013b). This can explain differences during aging time. Redness and yellowness variation
479 during the aging period describes the meat color deterioration from red to brown and is linked to
480 myoglobin concentration and redox status (Mancini and Hunt, 2005), but also to the fatty acid
481 composition. Redness values are negatively correlated with the sensory degradation of color, while
482 yellowness values are positively correlated to visual appreciation by consumers (Insausti et al., 2008;
483 Luciano et al., 2009). Although there were the same decreasing trends for both parameters in both
484 groups, yellowness decreased more in the RLE group after 7 days of aging. Similar results were

observed by Maggiolino et al. (2020) in beef meat after administration of natural polyphenols with antioxidant activity. Differently, Luciano et al. (2009) reported an increasing trend in yellowness values during aging, with lower values in meat of animals that assumed natural substances with antioxidant activity. Yellowness is strictly linked to meat oxidative stability, particularly to myoglobin stability and to lipid oxidation processes (Qin et al., 2020).

Meat is a product susceptible to oxidative rancidity, and lamb is more susceptible than other meats such as pork and chicken because of its high content of iron (Qin et al., 2020). Several studies aimed to use natural dietary antioxidants to improve meat quality by decreasing lipid peroxidation and improve antioxidative status in different species and observed inhibition of lipid oxidation through the lower production of its catabolites (Holman et al., 2019; Maggiolino et al., 2020). In this study, although production of lipid and protein oxidation catabolites increased in both groups due to aging time, we observed lower meat TBARS values and hydroperoxide production and lower TBARS plasma levels when RLE was added to diet. Considering a potential protective effect of dietary anthocyanins on lipid oxidation, and their consequent effect on colour stability, TBARS and hydroperoxides variations during aging detected the differing degree of lipid oxidation. Lipid oxidative stability is generally influenced by the degree of unsaturation of fatty acids (Luciano et al., 2009) and the increasing of degree of unsaturation of the muscle reduced its oxidative stability (Morrissey et al., 1998). It was not the case in this study, considering that although an increased total UFA degree, a major colour stability occurred, particularly of yellowness, probably due to the lipid oxidative stability. However, some fatty acids such as the oleic acid (C18:1), which is more present in meat obtained by lambs fed with RLE addition, and the conjugated linoleic acids (CLA -C18:2) may exert a protective effect on muscle oxidation (Du et al., 2000; Joo et al., 2002) not participating in oxidation processes and reduce the formation of fatty acid free radicals, which results in reduced lipid oxidation and major colour stability (Hur et al., 2004). Vasta and Luciano (2011) observed

509 similar enhanced oxidative stability in meat after consumption of plants secondary compounds with
510 antioxidant activity in small ruminants.

511 Antioxidant defence in animals can be either raised *in vivo* (enzymatic) or derived from the diet (non-
512 enzymatic) (Jabalbarezi Hukerdi et al., 2019). Generally, dietary consumption of high amounts of
513 natural antioxidants results in the transfer of these molecules to animal tissues followed by a
514 significant increase in total antioxidant capacity (Descalzo and Sancho, 2008), but also the antioxidant
515 status of ruminants has been speculated to be influenced by dietary consumption of natural
516 antioxidants, with increasing effect (Jiang et al., 2015; Ognik et al., 2015). Enzymes with antioxidant
517 activities were more active in plasma and muscle tissue due to RLE supplementation, although no
518 differences were observed in liver and kidney tissues. Glutathione peroxidase and superoxide
519 dismutase showed the highest concentration in the meat and plasma of lambs fed with RLE. These
520 results agree with previous reports, which showed increases in antioxidant bioactive substances in the
521 feed of lambs and kids resulted in increased enzyme antioxidant activity in meat (Zhao et al., 2018;
522 Jabalbarezi Hukerdi et al., 2019) and plasma (Emami et al., 2015; Ghavipanje et al., 2016). It is well
523 known that meat oxidative stability depends on the balance between antioxidant and pro-oxidative
524 components (Descalzo and Sancho, 2008) and mechanisms able to counteract oxidation, both
525 endogenous and exogenous, can extend shelf life (Rant et al., 2019). Differences in enzymes, and
526 thus in the oxidative status of both animals and meat, can explain differences in oxidative changes
527 during aging between the experimental groups.

528

529 *4.2. Fatty acid profile*

530 Several studies in recent years examined different agro-industrial by-products as additives in animal
531 feeds because of the presence of secondary metabolites, characterized by antioxidant bioactive
532 activity (poliphenols, α-tocopherol, essential oils), so as to improve the fatty acid composition of
533 ruminant meat (Lanza et al., 2015; Yagoubi et al., 2018; Quiñones et al., 2019; Maggiolino et al.,

2020). Lanza et al. (2015), studied lambs slaughtered at an older age than in the present study, and observed similar results, with a lower UFA concentration, a higher MUFA concentration and a lower SFA/PUFA ratio in the intramuscular fat of lambs fed a diet with the addition of natural substances with antioxidant activity. Wei et al. (2019) obtained similar results in kids, with higher oleic acid content and higher PUFA content. Differently, Salami et al. (2019) did not observe any difference in total MUFA, PUFA and SFA of meat from lambs treated with different levels of dietary antioxidant addition; rather they observed a lower content of stearic and *trans* forms of C18:1. The reduction of SFA concentration and the increasing concentration of UFA, particularly for n-3 and n-6, with the addition of RLE in the diet can be linked to a decreased risk of cardio-metabolic disease in humans (McAfee et al., 2010), resulting in lower TI and AI. This observation, in line with reports by other authors for meat from lamb's fed with natural antioxidant substances (Quiñones et al., 2019), is very important to produce healthier meat. TI and AI values in the current study were below the limits recognized for human health (respectively < 1.3 and < 1.0) (Ulbricht and Southgate, 1991; Costa et al., 2009). However, RLE addition was able to increase some UFA such as oleic one and reduce the SFA acids resulting in lower values of AI and TI, indices that are usually correlated with the possibility of onset of cardiovascular diseases in humans. As reported, the n-6/n-3 ratio in the current study was similar to that reported by others in lamb meat (De Palo et al., 2018b; Quiñones et al., 2019), and just above the threshold of 4 recommended for human health (Bas et al., 2007), although no effect of anthocyanin administration was observed.

5. Conclusions

The results indicate that RLE could be used as a feed supplement for improving and promoting health benefits and qualitative traits of lamb meat, also for improving the oxidative status of animals at the plasma level, but not in specific organs, not compromising animal growth. Aging is characterized by oxidative processes that can result in a short meat shelf life. The RLE supplementation enhanced

559 antioxidant activity and oxidative stability during the 7 aging days. reducing colour deterioration,
560 delaying lipid oxidation and consequent rancidity, and improving the fatty acid profile resulting in a
561 more attractive meat for consumers, in a possibly longer shelf life and in a healthier meat for human
562 consumption.

563

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572

573 6. **References**

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751

752 **TABLES**

753 **Table 1.** Fatty acid composition of alfalfa hay and commercial starter (expressed as % of total fatty
754 acid methyl ester)

755

	Alfalfa hay	**Commercial starter**
C 8:0	8.1	-
C 12:0	2.01	0.11
C 14:0	2.45	1.44
C 15:0	-	0.05
C 16:0	22.54	13.52
C 17:0	-	0.05
C 18:0	3.98	3.78
C 20:0	2.1	0.22
C 22:0	0.95	0.07
C 14:1	-	0.06
C 16:1	-	0.64
C 17:1	-	0.09
C 18:1	3.64	22.24
C 18:2 n-6	18.02	54.16
C 18:3 n-6	36.21	-
C 18:3 n-3	-	4.25
SFA	42.13	19.24
MUFA	3.64	23.03
PUFA	54.23	58.41

756 SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids

757

758 Table 2. Composition of orange and lemon extract (RLE) administered to lambs.

759

Compound	$[M]^+$ (m/z)	MS^n (m/z)	Anthocyanin	Relative composition (%)[(a)]
1	611	449/287	cyanidin 3,5-diglucoside	1.29
2	465	303	delphinidin 3-glucoside	2.67
3	611	287	cyanidin 3-sophoroside	0.41
4	449	287	cyanidin 3-glucoside	39.97
5	595	287	cyanidin 3-rutinoside	1.30
6	479	317	petunidin 3-glucoside	1.59
7	551	465/303	delphinidin 3-(6"-malonyl)glucoside	1.43
8	463	301	peonidin 3-glucoside	2.98
9	565	479/317	petunidin 3-(6"-malonyl)glucoside	1.45
10	535	449/287	cyanidin 3-(6"-malonyl)glucoside	21.76
11	593	449/287	cyanidin 3-(6"-malonyl)glucoside	5.70
12	-	271	pelargonidin derivative	1.44
13	549	463/301	peonidin 3-(6"-malonyl)glucoside	13.80
14	-	287	cyanidin derivative	2.39
15	-	301	peonitin derivative	1.82
			Total anthocyanins (g CGE/100g)	**2.66 ± 0.01**

760 $[M]^+$ (m/z): mass peak; MS^n (m/z): MS fragmentation model;

761 [(a)]Relative composition of anthocyanins calculated from peak areas recorded at 520 nm. The total
762 anthocyanin content was expressed as mg of cyanidin 3-glucoside equivalents (CGE) 100 mL^{-1} and
763 mg CGE 100 g^{-1} for the samples.

764

765 Table 3. Effect of including anthocyanins in the diet of lambs and of aging time on meat rheological parameters, texture profile analysis, meat
766 colour, oxidative parameters and enzyme activity

	RLE			CON				*P values*		
	Day 1	**Day 3**	**Day 7**	**Day 1**	**Day 3**	**Day 7**	**SEM**	**Diet**	**Day**	**Diet × Day**
Rheological parameters										
pH	6.91^{A}	6.87^{A}	6.59^{B}	6.92^{A}	6.84^{A}	6.58^{B}	0.021	0.40	< 0.01	0.26
Water holding capacity (%)	85.12^{A}	82.33	80.06^{B}	85.02^{a}	82.96	80.48^{b}	0.982	0.69	< 0.01	0.92
Cooking loss (%)	37.80^{a}	39.31	40.21^{b,}	39.40^{Aa}	42.61^{b,}	45.00^{B}	0.761	< 0.01	< 0.01	0.27
Thawing loss (%)	5.61^{a}	6.17	7.25^{b}	6.42^{A}	7.19	8.30^{B}	0.342	< 0.01	< 0.01	0.93
Shear Force (N)	22.40^{A}	18.89^{B}	17.42^{B}	25.26^{A}	21.98^{B,}	19.99^{B,}	0.501	< 0.01	< 0.01	0.87
Texture profile analysis										
Adhesiveness (-N × s)	0.32	0.33	0.32	0.34	0.33	0.32	0.020	0.95	0.915	0.85
Springiness (cm)	0.85	0.83	0.83	0.81	0.81	0.83	0.032	0.34	0.510	0.55
Cohesiveness (-)	0.44	0.43	0.43	0.43	0.41	0.41	0.022	0.04	0.255	0.72
Juiciness (-)	0.57^{A}	0.49^{B}	0.46^{B}	0.56^{A}	0.47^{B}	0.39^{C}	0.021	< 0.01	< 0.01	0.07
Chewiness (J × 10-2)	4.26^{A}	3.77^{B}	3.38^{B}	4.76^{A}	4.42	3.99^{B,}	0.102	< 0.01	< 0.01	0.69
Meat colour										
Lightness	44.93^{A}	46.97^{A}	52.03^{B}	42.15^{A}	45.60^{B}	50.41^{C}	0.503	< 0.01	< 0.01	0.33
Redness	11.36^{A}	10.36^{A}	8.91^{B}	12.06^{A}	11.04^{AB}	9.98^{B}	0.281	< 0.01	< 0.01	0.73
Yellowness	2.19^{A}	1.65^{B}	0.87^{C}	2.09^{A}	1.37^{B}	0.52^{C}	0.061	< 0.01	< 0.01	0.11
Hue	0.19^{A}	0.16^{B}	0.10^{C}	0.17^{A}	0.12^{B}	0.05^{C}	0.012	< 0.01	< 0.01	0.06
Chroma	67.29^{A}	55.49^{A}	40.75^{B}	75.48^{Aa}	63.02^{b}	51.92^{B}	2.992	< 0.01	< 0.01	0.81
Oxidative profile										
TBARS (mg MDA/kg of meat)	0.17^{A}	0.23$^{A, X}$	0.38$^{B, X}$	0.21^{A}	0.35$^{B, Y}$	0.59$^{C, Y}$	0.031	< 0.01	< 0.01	< 0.01
Hydroperoxides (mmol/g of meat)	0.34^{A}	0.57^{B}	0.71$^{C, X}$	0.35^{A}	0.59^{B}	1.05$^{C, Y}$	0.031	< 0.01	< 0.01	< 0.01
Protein carbonyl (mmol DNPH/mg protein)	3.18^{A}	3.57	3.96^{B}	3.14^{A}	3.60	4.03^{B}	0.102	0.81	< 0.01	0.87
Enzyme activity										
SOD (U/mg of proteins)	23.20$^{A, X}$	20.07$^{B, X}$	15.67$^{C, X}$	13.59$^{A, Y}$	9.18$^{B, Y}$	6.57$^{C, Y}$	0.302	< 0.01	< 0.01	0.009
CAT (U/mg of proteins)	3.57^{A}	3.08^{B}	2.35^{C}	3.51^{A}	2.99^{B}	2.31^{C}	0.041	0.03	< 0.01	0.78
GSPx (nmol NADPH ox/mg) protein	73.75$^{A, X}$	62.12$^{B, X}$	48.58$^{C, X}$	46.14$^{A, Y}$	36.89$^{B, Y}$	30.39$^{C, Y}$	0.393	< 0.01	< 0.01	< 0.01

767 RLE: red orange and lemon extract group; CON: control group; SEM: standard error of the means; TBARS: Thiobarbituric acid reactive substances;
768 SOD: superoxide dismutase; CAT: catalase; GSPx: glutathione peroxidase

A, B, C = $P < 0.01$; a, b = $P < 0.05$ showed statistical differences among aging days within a group.

771 Table 4. Effect of including anthocyanins in the diet of lambs and of aging time on the meat saturated and unsaturated fatty acid profile (expressed
772 as % of total fatty acid methyl ester)

	RLE			CON				*P values*		
	Day 1	**Day 3**	**Day 7**	**Day 1**	**Day 3**	**Day 7**	**SEM**	**Diet**	**Day**	**Diet × Day**
C 8:0	0.44	0.45	0.45	0.48	0.50	0.50	0.032	0.08	0.91	0.99
C 10:0	0.57	0.52	0.56	0.64	0.60	0.63	0.031	< 0.01	0.09	0.98
C 12:0	0.36	0.40	0.36	0.49	0.46	0.43	0.031	< 0.01	0.28	0.36
C 14:0	3.79	3.80	3.93	4.67	4.46	4.41	0.141	< 0.01	0.74	0.33
C 15:0	0.34	0.39	0.40	0.41	0.48	0.46	0.021	< 0.01	0. 11	0.76
C 16:0	20.12	19.86	19.77	21.51	22.08	21.69	0.282	< 0.01	0.67	0.31
C 17:0	1.96	2.08	1.96	2.23	2.05	2.07	0.080	0.10	0.65	0.19
C 18:0	12.07	12.32	12.13	13.38	12.75	13.16	0.201	< 0.01	0.61	0.07
C 20:0	1.38	1.15	1.23	1.46	1.34	1.25	0.051	0.02	< 0.01	0.19
C 22:0	0.06	0.07	0.06	0.07	0.08	0.08	0.012	0.006	0.42	0.84
C 12:1	0.06	0.04	0.04	0.04	0.05	0.06	0.010	0.47	0.41	0.20
C 14:1	0.60	0.53	0.52	0.54	0.51	0.53	0.022	0.19	0.04	0.19
C 16:1	4.61^{a}	5.19^{b}	4.99	5.05	5.26	5.24	0.141	0.03	0.01	0.39
C 17:1	1.69	1.54	1.64	1.52	1.44	1.54	0.051	0.006	0.07	0.73
C 18:1	40.10	40.06	40.23	36.87	37.20	37.15	0.312	< 0.01	0.79	0.84
C 18:2 n-6	8.91	8.94	9.01	7.97	8.28	8.41	0.153	< 0.01	0.22	0.52
C 18:3 n-6	0.09	0.08	0.10	0.08	0.09	0.09	0.012	0.52	0.24	0.009
C 18:3 n-3	0.77	0.73	0.75	0.73	0.70	0.71	0.022	0.04	0.32	0.95
C 20:1	0.05	0.03	0.04	0.03	0.04	0.03	0.011	0.14	0.12	0.23
C 20:2 n-6	0.03	0.01	0.01	0.04	0.04	0.04	0.011	0.65	0.61	0.06
C 20:4 n-6	0.54	0.49	0.49	0.50	0.47	0.48	0.021	0.12	0.08	0.85
C 20:5 n-3	$0.48^{A, X}$	0.41^{B}	0.40^{B}	0.37^{Y}	0.38	0.39	0.021	< 0.01	0.02	0.001
C 22:1	0.04	0.03	0.04	0.04	0.04	0.03	0.012	0.22	0.06	0.10
C 22:5 n-3	0.65	0.62^{X}	0.61^{X}	0.63^{A}	$0.49^{B, Y}$	$0.42^{B, Y}$	0.022	< 0.01	< 0.01	< 0.01
C 22:6 n-3	0.26	0.23	0.23	0.21	0.23	0.23	0.021	0.29	0.88	0.10

773 RLE: red orange and lemon extract group; CON: control group; SEM: standard error of the means.
774 A, B = $P < 0.01$; a, b = $P < 0.05$ showed statistical differences among aging days within a group.
775 X, Y = $P < 0.01$; x, y = $P < 0.05$ showed statistical differences between groups at the same aging day.

Table 5. Effect of including anthocyanins in the diet and of aging time on n-6, n-3, n-6/n-3, total saturated fatty acids, total monounsaturated fatty acids, total polyunsaturated fatty acids, saturated/polyunsaturated ratio, atherogenic index and thrombogenic index of lamb meat.

	RLE			**CON**				***P values***		
	Day 1	**Day 3**	**Day 7**	**Day 1**	**Day 3**	**Day 7**	**SEM**	**Diet**	**Day**	**Diet × Day**
n-6	9.56	9.54	9.64	8.60	8.87	9.00	0.151	< 0.001	0.30	0.69
n-3	2.16^{Aa}	1.99^{b}	1.98^{B}	1.95^{A}	1.79^{B}	1.75^{B}	0.041	< 0.01	0.34	0.96
n-6/n-3	4.47	4.81	4.88	4.44^{Aa}	4.97^{b}	5.18^{B}	0.112	< 0.01	0.86	0.51
SFA	41.13	41.03	40.87	45.36	44.80	44.67	0.312	< 0.01	0.33	0.69
MUFA	47.14	47.43	47.50	44.09	44.53	44.58	0.331	< 0.01	0.29	0.48
PUFA	11.73	11.54	11.62	10.54	10.67	10.75	0.162	< 0.01	< 0.01	0.84
UFA	58.87	58.96	59.12	54.63	55.20	55.33	0.311	0.12	< 0.01	0.34
SFA/PUFA	3.54	3.57	3.53	4.33	4.21	4.17	0.072	< 0.01	0.43	0.50
SFA/UFA	0.70	0.70	0.69	0.83	0.81	0.81	0.011	< 0.01	0.28	0.67
AI	0.61	0.60	0.61	0.75	0.73	0.72	0.021	< 0.01	0.52	0.54
TI	0.92	0.92	0.91	1.08	1.06	1.06	0.022	< 0.01	0.42	0.69

RLE: red orange and lemon extract group; CON: control group; SEM: standard error of the means; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; UFA: unsaturated fatty acids; AI: atherogenic index; TI: thrombogenic index.

A, B = $P < 0.01$; a, b = $P < 0.05$ showed statistical differences among aging days within a group.

Table 6. Effect of including anthocyanins in the diet on plasma oxidative profile and antioxidant enzymes quantification during 40 days of life

	RLE			**CON**				***P values***		
	Day 1	**Day 20**	**Day 40**	**Day 1**	**Day 20**	**Day 40**	**SEM**	**Diet**	**Day**	**Diet × Day**
TBARS (mg MDA/dl)	1.38	1.41	1.42^{X}	1.34^{A}	1.30^{A}	$1.66^{B, Y}$	0.052	0.40	< 0.01	< 0.01
Hydroperoxides	5.55^{a}	6.12^{X}	6.26^{b}	5.64^{A}	$7.00^{B, Y}$	6.55^{B}	0.701	< 0.01	< 0.01	0.04
Protein carbonyl	97.20	95.91	97.89	102.10	101.21	102.35	2.891	< 0.01	0.29	0.64
SOD (U/mg of proteins)	15.09^{A}	$49.93^{B, X}$	$71.22^{C, X}$	15.06^{A}	$15.40^{A, Y}$	$24.11^{B, Y}$	0.222	< 0.01	< 0.01	< 0.01
CAT (U/mg of proteins)	0.52^{A}	0.54^{A}	$0.78^{B, X}$	0.54^{A}	0.56^{A}	$0.60^{B, Y}$	0.010	< 0.01	< 0.01	< 0.01
GSPx (nmol NADPH ox/mg)	6.57^{A}	$8.35^{B, X}$	$11.74^{C, X}$	6.29^{A}	$7.76^{B, Y}$	$6.69^{C, Y}$	0.081	< 0.01	< 0.01	< 0.01

RLE: red orange and lemon extract group; CON: control group; SEM: standard error of the means; TBARS: Thiobarbituric acid reactive substances; SOD: superoxide dismutase; CAT: catalase; GSPx: glutathione peroxidase.

A, B = $P < 0.01$; a, b = $P < 0.05$ showed statistical differences among aging days within a group.

X, Y = $P < 0.01$ showed statistical differences between groups at the same aging day.

787 Table 7. Effects of RLE on lipid peroxidation measured by malondialdehyde (MDA) test, nitric oxide
788 (NO), SOD, CAT and GSPx activities in renal, liver and intestine at the end of treatment.
789

	RLE	**CON**	**SEM[1]**	**P values**
	Kidney			
MDA (nanomoles/mg of protein)	2.26	2.13	0.172	0.41
Nitric Oxide (pmole/mg)	20.43	18.20	0.581	0.36
SOD (U/mg)	156.80	165.89	4.712	0.25
CAT (U/mg)	117.30	116.67	2.393	0.77
GSPx (U/mg)	43.27	43.89	1.931	0.87
	Liver			
MDA (nanomoles/mg of protein)	2.19	2.12	0.192	0.43
Nitric Oxide (pmole/mg)	21.78	22.09	0.292	0.89
SOD (U/mg)	155.25	149.78	5.471	0.36
CAT (U/mg)	140.20	147.33	2.183	0.36
GSPx (U/mg)	48.11	49.78	1.421	0.89
	Intestine			
MDA (nanomoles/mg of protein)	2.41	2.42	0.222	0.28
Nitric Oxide (pmole/mg)	28.54	29.75	0.291	0.73
SOD (U/mg)	140.96	142.89	3.712	0.66
CAT (U/mg)	153.22	157.33	3.313	0.58
GSPx (U/mg)	61.31	61.00	1.712	0.91

790

791 RLE: red orange and lemon extract group; CON: control group; SEM: standard error of the means;
792 SOD: superoxide dismutase; CAT: catalase; GSPx: glutathione peroxidase.
793

794