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Dietary pomegranate by-product improves oxidative stability of lamb meat

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Highlights

- The inclusion of 20% whole pomegranate by-product (WPB) in lamb diet was evaluated
- WPB is rich in bioactive compounds (e.g. conjugated FA, vitamin E and phenols)
- WPB-lamb meat contained higher concentration of vitamin E and PUFA
- Lipid oxidation and metmyoglobin formation of meat were reduced by WPB treatment
- Dietary WPB increased the meat antioxidant capacity in the lipophilic fraction

ABSTRACT:

This study investigated the effect of including whole pomegranate by-product in lamb diet on meat oxidative stability. Seventeen lambs were assigned to two experimental treatments and fed a cereal-based concentrate (CON) or the same concentrate where 200 g/kg DM of cereals were replaced by whole pomegranate by-product (WPB). Meat from WPB-fed lambs had a greater concentration of vitamin E (α- and γ-tocopherols), polyunsaturated fatty acids (PUFA), highly peroxidizable PUFA and a higher peroxidability index ($P < 0.05$). Feeding WPB limited the formation of metmyoglobin ($P = 0.05$) and reduced lipid oxidation (TBARS values) after 7 days of storage for raw meat ($P = 0.024$) or 4 days for cooked meat ($P = 0.006$). Feeding WPB increased meat antioxidant capacity (ORAC assay) in the lipophilic fraction ($P = 0.017$), but not in the hydrophilic. These results suggest that vitamin E in the pomegranate by-product contributed to the higher antioxidant capacity of meat from the WPB-fed lambs.

KEYWORDS: pomegranate by-product; lipid oxidation; meat quality; vitamin E; antioxidants; phenolic compounds.

1. Introduction

The agro-industrial by-products have long been used in ruminant feeding as an effective strategy to reduce the cost of the diet. Furthermore, the use of alternative feeds that do not compete with human foods is currently one of the primary objectives for the scientific community and the re-use of agro-industrial wastes is pivotal to mitigate their impact on the environment (Salami et al., 2019). Among the many agro-industrial wastes available, pomegranate (*Punica granatum* L.) by-products are gaining enormous interest due to the global increase in consumption of pomegranate juice or ready-to-eat arils, also linked to the recognition of its potential health properties. Pomegranate fruit contains numerous bioactive compounds, such as peculiar conjugated fatty acids, phenolic compounds and vitamins, which possess antioxidant, antimicrobial, anti-inflammatory, antitumoral and immunomodulatory properties (Viuda-Martos, Fernández-López, & Pérez-Álvarez, 2010; Johanningsmeier & Harris, 2011).

The pomegranate by-products have a high nutritional value as ruminant feeds, and can be effectively used in ruminant diets to replace cereals. In a previous study, dietary administration of fresh pomegranate peels to beef calves increased feed intake and the concentration of α-tocopherol in plasma (Shabtay et al., 2008). Subsequent studies reported evidences of the beneficial effects of dietary pomegranate seed pulp on goat kids' antioxidant status (Emami, Ganjkhanlou, Fathi Nasri, Zali, & Rashidi, 2015) and meat fatty acid composition (Emami, Fathi Nasri, Ganjkhanlou, Rashidi, & Zali, 2015). Recently, we also observed a desirable increment of the polyunsaturated fatty acids (PUFA) in meat (Natalello et al., 2019) and milk (Valenti, Luciano, et al., 2019) when sheep diets were supplemented with a dried whole pomegranate by-product (WPB), which contained both seeds and peels. Nevertheless, although a high concentration of PUFA in meat is considered desirable from a human health perspective, the higher susceptibility of PUFA to peroxidation may drastically reduce meat shelf-life

(Bekhit, Hopkins, Fahri, & Ponnampalam, 2013). Few studies have investigated the shelf-life of meat from ruminants fed with pomegranate by-products. Emami, Fathi Nasri, Ganjkhanlou, Zali, & Rashidi (2015) observed a greater lipid and colour stability of meat when kids were fed with 150 g/ kg (dry matter basis) of pomegranate seed pulp. These authors mainly attributed the reduction in lipid oxidation and colour discoloration to the phenolic compounds present in the by-product. Nevertheless, the concentration of total phenols in pomegranate seed is not high, especially if compared to other portions of the fruit, such as peels (Pande & Akoh, 2009; Orak, Yagar,& Isbilir, 2012; Natalello et al., 2020). Moreover, as stated above, pomegranate fruits and the by-products residual after juice extraction contain other bioactive compounds, such as vitamin E, which could play a major role in determining the stability of meat to oxidative deterioration (Bellés, Campo, Roncalés, & Beltrán, 2019).

To our knowledge, no other studies have tested the dietary inclusion of whole pomegranate by-product on meat oxidative stability. Therefore, the aim of the present study was to investigate the effect of feeding lambs with WPB on the resistance of meat to oxidation. We hypothesized that the diverse bioactive molecules present in the WPB could delay the oxidative deterioration even in meat with a high PUFA content. To test this hypothesis, we used the same animals from the experiment by Natalello et al. (2019) and we evaluated the vitamin E and the antioxidant capacity in lipophilic and hydrophilic fraction in the muscle, as well as the colour and lipid stability in meat preserved in common retail conditions.

2. Materials and methods

2.1. Whole pomegranate by-product

The experimental feeding trial is described in detail by Natalello et al. (2019). Briefly, fresh pomegranate fruits, from Wonderful variety, were processed in a local juice manufacturing company (Catania, Sicily, Italy) by mechanically halving and squeezing the fruit. After

processing, the residual part containing peels, seeds, membranes and portion of arils was collected and dried in a ventilated oven set at 40 °C for approximately 36 hours until constant weight. Chemical composition and antioxidant capacity of the dried residual part are presented in Table 1.

2.2. Animals and experimental treatments

The experimental procedures were approved by the University of Catania (approval: 015CT325). The animals were raised at the university's experimental farm (Catania, Italy; 37°24'35.3''N 15°03'34.9''E) and handled by specialized personnel following the European Union Guidelines (2010/63/ EU Directive). As described by Natalello et al. (2019), the trial involved seventeen Comisana male lambs, born within an interval of 10 days in the same commercial farm. At 60 days of age, animals were transported to the university facilities, weighed (average body weight 14.82 kg ± 2 kg) and allocated indoors in individual pens (1.5 m^2 each). Lambs were randomly assigned to two dietary treatments, balanced for bodyweight, and adapted to the experimental diet over 8 days, during which the pre-experimental concentrate was gradually replaced with the experimental diets. After this adaptation period, lambs were fed *ad libitum* for 36 days with a barley-corn based concentrate diet (CON, 8 lambs) or a concentrate diet containing 200 g/kg dry matter (DM) of whole pomegranate by-product to partially replace barley and corn (WPB, 9 lambs). Ingredients and chemical composition of the experimental diets are reported in Table 1. All the ingredients were ground (5-mm screen), mixed and pelleted (at 40 °C) using a pelleting machine (CMS-IEM - Colognola ai Colli, Verona, Italy) to avoid selection. Lambs had free access to fresh water throughout the experiment. Every day, the amount of offered and refused diet was recorded in order to calculate the dry matter intake (DMI). Lambs were weighed every week from the beginning to the end of the trial to calculate average daily gain (ADG).

2.3. Slaughter procedure and samplings

At the end of the trial, all animals were slaughtered on the same day at a commercial abattoir according to the European Union welfare guidelines (Council Regulation no. 1099/2009). Lambs were firstly stunned by a captive bolt and exsanguinated. Each carcass was immediately weighted and stored at 4° C for 24 h. Then, carcasses were halved and the entire *longissimus thoracis et lumborum* muscle (LTL) was excised from both sides. The right LTL was immediately vacuum-packed and stored at −80 °C until analysis of intramuscular fatty acid composition, antioxidant vitamins and antioxidant capacity. The left LTL was firstly used to measure the muscle pH by a pH-meter (HI-110; Hanna Instruments, Padova, Italy), then was aged vacuum-packaged for 3 days at 4 °C, after which it was used for oxidative stability measurements.

2.4. Feedstuffs analyses

Samples of the experimental diets were collected at the beginning, middle, and end of the trial, vacuum-packed and stored at -30 °C. Feed sample for analysis was obtained by mixing equal amounts the above subsamples collected during the trial. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined according to Van Soest, Robertson, & Lewis (1991). Furthermore, crude protein, crude fat (ether extract) and ash were analysed according to the AOAC methods (1995).

Total phenolic compounds and total tannins in the feeds were extracted and determined as described by Natalello et al. (2019). Briefly, finely ground feeds (200 mg) were extracted sequentially in a sonicating water-bath with acetone 70% (v/v) followed by methanol 80% (v/v). The combined supernatants were evaporated and the residue was dissolved in methanol 70% (v/v). Total phenolic compounds were quantified by reaction of the extract with the Folin-

Ciocalteu reagent (1N) and sodium carbonate 20% (w/v), after which the absorbance at 725 nm was measured using a double-beam spectrophotometer (model UV-1601; Shimadzu Corporation, Milan, Italy). Non-tannin phenolics were determined with the same procedure, after removal of the tannins from the extract with insoluble polyvinylpyrrolidone (PVPP). The concentration of total tannins was calculated as difference between total phenols and total non-tannin phenols. Standard solutions of tannic acid (TA) were used to prepare an external calibration curve in order to quantify phenolic compounds, which were expressed as g TA equivalents/100 g dry matter. Additionally, total condensed tannins were determined by the *in situ* thiolysis assay according to the method described by Gea, Stringano, Brown, and Mueller-Harvey (2011) with slight modifications. In short, 200 mg of ground feedstuffs were weighed into a screw-top glass tube, and a reagent containing 2 mL of MeOH, 1 mL of 3.3% HCl in MeOH, and 100 µL of benzyl mercaptan (BM) was added. The tubes were heated at 40 °C for 2 h under vigorous stirring. Then, 9 mL of 1% formic acid in water was added, and the tubes were subsequently vortex mixed and centrifuged for 5 min. The supernatant was transfer to 2-mL vials and analysed within 48 h by liquid chromatography-mass spectrometry (LC-MS; Agilent 1100 series, Agilent Technologies, Waldbronn, Germany) with taxifolin as an external standard. The concentration of condensed tannins was expressed as g/100 g dry matter.

Tocopherols from feedstuffs were extracted and analysed as described by Valenti et al. (2018). Briefly, feed samples were homogenized with an ethanolic butylated hydroxytoluene (BHT) solution (0.06%, w/v) and saponified with KOH (60%, w/v) at 70 °C for 30 min. Tocopherols were extracted three times using hexane/ethyl acetate (9/1, v/v), dried under N_2 and dissolved with acetonitrile. A 50 µL volume was injected in a HPLC system (pump model Perkin Elmer series 200), equipped with an autosampler (model AS 950-10, Tokyo, Japan) and a Synergy Hydro-RP column (4 µm, 4.6 × 100 mm; Phenomenex, Bologna, Italy). A mobile phase consisting of acetonitrile/methanol/tetrahydrofuran/1% ammonium acetate (68/22/7/3, v/v/v/v)

was used and the flow rate was set at 2 mL/min. The tocopherols were identified using a fluorescence detector (model Jasco, FP-1525) set at excitation and emission wavelengths of 295 nm and 328 nm, respectively and were quantified by using external calibration curves of commercial standard compounds (Sigma Aldrich, Steinheim, Germany).

The feed antioxidant capacity was determined using the oxygen radical absorbance capacity (ORAC). The hydrophilic and lipophilic fractions were extracted from 1 g finely powdered feed using 10 ml of either hexane or phosphate buffer (pH 7.2), for the lipophilic and hydrophilic fractions, respectively. Both fractions were extracted by vortex-mixing the samples for 1 min, followed by centrifugation at 4000 x *g* for 30 min at 25 °C. The supernatant (2 ml) was stored at -80 °C prior to analysis. The ORAC assays were carried out on a FLUOstar OPTIMA microplate fluorescence reader (BMG LABTECH, Offenburg, Germany) following the procedure previously described by Valenti, Luciano, et al. (2019). In short, 2,20-azobis (2-methylpropionamide) dihydrochloride (AAPH; Sigma-Aldrich) was used as peroxyl radical generator, Trolox was used as the reference antioxidant standard and fluorescein was used as a fluorescent probe. A 100 µL volume of diluted sample, blank or Trolox calibration solution (10–80µmol) was mixed with 1 mL of fluorescein (80 nM); then, 200 µL of each mixture was placed in a well of the microplate. The microplate was placed in the reader and pre-incubated for 20 min at 37 °C, after which AAPH (60 µL) was automatically added in each well to initiate the reaction. The fluorescence was recorded every 1.9 min, using excitation and emission wavelengths of 485 nm and 520 nm, respectively. The area under the fluorescence decay curve was measured for each sample and compared to that obtained with the Trolox standard solutions in order to express the data as µmol Trolox equivalents (TE) / g of sample. All the reaction mixtures were prepared in duplicate, and at least three independent assays were performed for each sample.

2.5. Myoglobin concentration, fatty acids, antioxidant vitamins and antioxidant capacity of meat

As described by Krzywicki (1982), myoglobin (Mb) was extracted by homogenization of muscle samples with phosphate buffer, followed by centrifugation at 6800 × *g* at 4 °C and filtration through Whatman 541 paper. The filtered supernatant was scanned in a UV/VIS spectrophotometer (UV-1601; Shimadzu Co., Milan, Italy) and the absorbance at 525 nm was used to calculate Mb concentration, expressed as mg/g of fresh tissue.

Intramuscular fatty acid composition from the same muscles used here was determined by Natalello et al. (2019). In this previous investigation, the detailed fatty acid profile was reported as g/100 g of total fatty acids according to the purpose of that study. In the present study, the main classes of fatty acids, such as saturated, monounsaturated and polyunsaturated fatty acids (SFA, MUFA and PUFA, respectively), n-3 and n-6 PUFA are expressed as mg/g of muscle. In addition, the susceptibility of fatty acids to oxidation was estimated by the amount of the highly peroxidizable polyunsaturated fatty acids (HP-PUFA) with unsaturation degree ≥ 3 and the peroxidability index was calculated according to Valenti, Natalello, et al. (2019).

The concentration of vitamin E in muscle (α- and γ-tocopherols) was analysed as described by Luciano et al. (2017). Briefly, 2 g of sample was homogenized with aqueous BHT (0.06%), saponified with ethanolic KOH (60%) at 70 °C for 30 min and extracted with hexane/ethyl acetate (9/1, v/v). The extracted solution was dried under nitrogen and resuspended with in acetonitrile. The HPLC analysis of tocopherols was performed as described above for feeds.

The muscle antioxidant status was measured on the lipophilic and hydrophilic fraction by the ORAC assay, using the conditions described above for feed samples.

2.6. Meat oxidative stability

Oxidative stability was measured in fresh and cooked meat over aerobic storage, as described by Valenti, Natalello, et al. (2019). Briefly, six slices (2 cm thickness) of each left LTL muscle were cut from the 9 to the 13 ribs using a knife. Three slices were packed under vacuum and cooked for 30 min at 70 °C in a water bath. One of these was used immediately for measurement of lipid oxidation (day 0), whereas the other two slices were placed in polystyrene trays, over-wrapped with 3-layers of domestic cling film and stored in the dark at 4 °C for 2 and 4 days. The other three raw slices were immediately placed in polystyrene trays, covered as cooked meat and stored at 4 °C in dark for 0 (after 2 hours of blooming), 4 and 7 days. At the end of the respective storage time, each slice of raw meat was used for measuring colour stability by a Minolta CM 2022 spectrophotometer (d/8° geometry; Minolta Co. Ltd. Osaka, Japan) set to operate in the specular components excluded (SCE) mode and to measure with the illuminant A and 10° standard observer. Two measurements were taken on the meat surface and the mean value was calculated. The colour descriptors L* (lightness), a* (redness), b* (yellowness), C (saturation) and h_{ab} (hue angle) were measured in the CIE L* a* b* colour space. The reflectance spectra from 400 to 700 nm wavelength were recorded for calculation of metmyoglobin percentage (MMb) formation as described by Krzywicki (1979).

For both raw and cooked slices, lipid oxidation was determined by measuring the 2-thiobarbituric acid reactive substances (TBARS) at the end of each storage time, as described by Valenti, Natalello et al. (2019). Meat samples (2.5 g) were homogenized with 12.5 mL of distilled water using a Heidolph Diax 900 tissue homogenizer (Heidolph ElektroGmbH & Co. KG, Kelheim, Germany) operating at 9500 rpm. During the homogenization, samples were put in a water/ice bath. Subsequently, 12.5 mL of 10% (w/v) trichloroacetic acid (TCA) was added to precipitate proteins, after which samples were filtered through Whatman No. 1 filter paper. The clear filtrate (4 mL) was added to 1 mL of 0.06M aqueous thiobarbituric acid into pyrex-glass tubes. The tubes were incubated in a water bath at 80 °C for 90 min and the absorbance

of each sample was read at 532 nm using a Shimadzu UV/vis spectrophotometer (UV-1601; Shimadzu Corporation, Milan, Italy). The assay was calibrated with solutions of TEP (1,1,3,3,-tetraethoxypropane) in distilled water ranging from 0 to 65 nmoles/4mL. Results were expressed as mg of malonaldehyde (MDA)/kg of meat.

2.7. Statistical Analysis

Data on animal performances and intakes, as well as on fatty acid classes, myoglobin, tocopherols and antioxidant capacity of meat were analysed using the general linear model (GLM) to test the effect of the dietary treatment. Data on oxidative stability measured in raw and cooked meat were analysed using a mixed model to test the effect of the dietary treatment and of the time of storage, as well as of their interaction as the fixed factors, while individual lamb was considered a random effect. Differences between means were assessed using the Tukey's Honest Significant Difference test. Significance was declared at $P \leq 0.05$, while trends toward significance were considered when $0.05 < P \leq 0.10$. Statistical analyses were performed using Minitab, version 16 (Minitab Inc., State College, PA, USA).

3. Results

3.1. Feed composition, animal performances and intakes

As shown in Table 1, the partial replacement of barley and corn with the whole pomegranate by-product produced slight variations in the nutrient composition parameters of the diet mostly related to the fibre fractions, with higher NDF and ADF in the WPB diet compared to CON. The whole pomegranate by-product contained almost 10% DM of total phenolic compounds, mostly represented by tannins (98.21% of total phenols). Consequently, the WPB diet had a greater content of total phenolic compounds and total tannins compared to the CON diet. Regarding vitamin E, α-tocopherol represented the main compound when compared with γ-

tocopherol in the pomegranate by-product. Both compounds were found at a greater concentration in the WPB diet when compared with CON. The hydrophilic fraction accounted for most of the antioxidant capacity (ORAC) in all the experimental feeds analysed and the WPB diet exhibited a greater antioxidant capacity (ORAC) of both hydrophilic and lipophilic fractions compared to CON.

The dietary treatment did not affect ($P > 0.05$) the performance parameters of lambs, measured as final bodyweight, carcass weight, average daily gain, voluntary feed intake and feed conversion ratio (Table 2). The above differences in the concentration of phenolic compounds between the diets, led to a greater intake of total phenolic compounds and tannins by lambs fed the WPB diet ($P < 0.001$). Similarly, feeding the WPB diet increased the daily intake of α- and γ-tocopherols compared to the CON treatment ($P < 0.001$).

3.2. Myoglobin, vitamin E, fatty acids and antioxidant capacity of meat.

As shown in Table 3, the dietary treatment did not affect the ultimate pH of meat, the concentration of myoglobin and the intramuscular fat content ($P > 0.05$). Feeding the WPB diet increased the concentration of vitamin E in meat (α- and γ-tocopherols; $P < 0.001$). Regarding the fatty acid composition of the intramuscular fat, the dietary treatment did not affect the concentration of saturated and monounsaturated fatty acids (SFA and MUFA, respectively; $P > 0.05$), while a greater concentration of polyunsaturated fatty acids (PUFA) was found in meat from the WPB-fed lambs ($P < 0.05$). Particularly, compared to CON, feeding WPB increased the concentration of highly peroxidizable (HP) PUFA with at least three double bonds and the peroxidability index of intramuscular fatty acids ($P < 0.05$). Lastly, as shown in Figure 1, the WPB diet increased the antioxidant capacity (ORAC) of the lipophilic fraction of meat ($P < 0.05$).

3.3. Meat oxidative stability

The effect of the dietary treatment and time of storage on the oxidative stability parameters measured in raw and cooked meat are reported in Table 4. The time of storage affected some of the colour parameters measured in raw meat, with a* values decreasing over the 7 days of storage, while b* and h_{ab} values were increased ($P < 0.001$). Also, the percentages of metmyoglobin (MMb%) increased over time, indicating meat browning ($P < 0.001$). The dietary treatment did not affect any of the colour parameters overall measured in meat during storage, except for L* values which were lower in meat from the CON group ($P < 0.01$). Additionally, feeding the WPB diet reduced the average MMb% measured in meat across the 7-day storage period ($P = 0.05$). In both raw and cooked meat, lipid oxidation (TBARS values) increased over storage duration ($P < 0.001$) and the WPB diet reduced the extent of lipid oxidation overall measured in meat over time ($P < 0.05$). A significant diet × time interaction was found for TBARS values measured in raw meat. Specifically, compared to day 0, while the TBARS values increased already after 4 days in meat from CON-fed animals, lipid oxidation increased in WPB meat after 7 days ($P < 0.05$; Figure 2). Raw meat from lambs in the WPB treatment had lower TBARS values compared to the CON group after 7 days of storage ($P < 0.05$). Statistically comparable results were observed between fresh meat (day 0) from control group and the WPB meat stored over 7 days ($P > 0.05$).

4. Discussion

The resistance of meat to oxidation depends upon the complex balance between pro-oxidant factors, such as readily oxidizable substrates and catalysts, and various antioxidant defences, including both endogenous systems and exogenous antioxidants of dietary origin (Bekhit et al., 2013). Among the pro-oxidant factors, heme iron in myoglobin has been demonstrated to promote the initiation of lipid oxidation (Baron & Andersen, 2002), so that a greater content of

myoglobin in muscle might increase its susceptibility to lipid peroxidation. Nevertheless, in the present study, we did not observe differences in the concentration of myoglobin between meat from CON- and WPB-fed lambs. The fatty acid composition of the intramuscular fat is another factor determining meat oxidative stability. Particularly, polyunsaturated fatty acids (PUFA) are the primary target for lipid oxidation (Bekhit et al., 2013) and their susceptibility to oxidation increases with increasing degree of unsaturation (Johnson & Decker, 2015). Therefore, feeding strategies aimed at increasing the PUFA concentration in muscle may present the drawback of impairing the oxidative stability of meat if not balanced by adequate antioxidant interventions (Bekhit et al., 2013). In the present study, we found that the concentration of PUFA was higher (+38%) in meat from lambs fed WPB compared to the control. In a previous study, Natalello et al. (2019) investigated the effect of the whole pomegranate by-product on the fatty acid metabolism of lambs and reported the detailed fatty acid composition of muscle, liver and ruminal digesta from the same animals used in the present study. As reported in that study, the greater concentration of total PUFA observed in meat from the WPB-fed animals was due to the combined effect of both PUFA and bioactive substances (such as tannins, able to alter the ruminal lipid metabolism) present in the pomegranate by-product. Consequently, compared to CON, meat from the WPB-fed lambs contained specific PUFA derived from the whole pomegranate by-product (i.e., conjugated linolenic acid isomers). Also, other PUFA, such as rumenic acid, were more abundant in meat from animals in the WPB treatment compared to the CON group (Natalello et al., 2019). In the present study, caused by the greater amount of highly unsaturated PUFA, we also observed a greater peroxidability index in meat from the WPB-fed lambs. Therefore, a possible higher susceptibility to lipid oxidation could be expected in meat from the WPB-fed animals. Nevertheless, no reduction of meat shelf-life was observed; on the contrary, the formation of the secondary lipid oxidation products (TBARS) was reduced in raw meat. Furthermore, meat

from the WPB-fed lambs displayed a greater resistance to lipid oxidation even under more stressful oxidative conditions, such as cooking. These results demonstrate that feeding the whole pomegranate by-product improved the antioxidant capacity of meat.

In the present study, we measured the overall antioxidant capacity of meat using the ORAC (oxygen radical absorbance capacity) assay which was adopted because it first offers a high sensitivity compared to other common assays (Cao & Prior, 1998). Moreover, while most of the other tests only measure antioxidants in the hydrophilic fraction, the ORAC assay has been successfully adopted to separately determine the antioxidant capacity of the lipophilic and hydrophilic fractions of the sample (Prior et al., 2003; Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002). In the case of dietary phenolic compounds, this approach can be useful to assess their antioxidant activities in the diet and their possible antioxidant effects in animal tissues, as most of these compounds and their metabolites have hydrophilic nature. Therefore, due to the greater concentration of phenolic compounds in the WPB diet compared to the CON, it is not surprising that the former displayed a much higher ORAC value in the hydrophilic fraction and this finding agree with previous reports on the antioxidant capacity of pomegranate fruits (Elfalleh et al., 2011; Valenti, Luciano et al., 2019).

Nevertheless, although the animals fed WPB ingested a higher quantity of polyphenols than those fed the CON diet control, the antioxidant capacity of the hydrophilic fraction of meat was not affected by the dietary treatment. Even if the bioavailability of phenolic compounds was not tested, this result might lead to suppose that pomegranate phenolic compounds did not contribute to improving meat oxidative stability with a mechanism that involved their intestinal absorption and transfer to the muscle as previously suggested. Kotsampasi et al. (2014) reported that a greater content of phenolic compounds in meat from lambs fed with a silage pomegranate by-product. However, the Folin-Ciocalteu method used by the authors is biased by several interfering substances, some of which present in muscle, with a consequent

erroneous measure of the phenolic compounds (Georgé, Brat, Alter, & Amiot, 2005). Also, among the heterogeneous class of phenolic compounds, tannins are considered to be poorly bioavailable in animals (Vasta & Luciano, 2011; López-Andrés et al., 2013). In this context, it is of note that phenolic compounds in the WPB used in the present study were almost exclusively represented by tannins (approximately 98%), in agreement with previous reports (Seeram, Lee, Hardy, & Heber, 2005; Mphahlele, Fawole, Mokwena, & Opara, 2016; Natalello et al., 2020).

Differently from the hydrophilic fraction, our results demonstrate that feeding the whole pomegranate to lambs increased the antioxidant capacity in the lipophilic fraction of muscle. This result might be explained by the greater concentration of Vitamin E (α- and γ-tocopherols) in muscle from lambs in the WPB treatment, as tocopherols react in the ORAC assay (Huang et al., 2002). The composition of the diets can directly explain the results found on the concentration of vitamin E in meat. Indeed, vitamin E is highly bioavailable as it is largely hydrolysed in the intestine and then absorbed in combination with lipid micelles. For this reason, the concentration of vitamin E in muscle responds to its content in the diet, as extensively demonstrated in different animal species (Bellés et al., 2019; Sales & Koukolová, 2011). Therefore, in the present study, the greater deposition of vitamin E in meat from the WPB-fed lambs could derive from their higher intake of tocopherols, especially α-tocopherol which is the most bioavailable among the vitamin E isoforms (Bellés et al., 2019). Moreover, compared to the CON diet, the WPB diet contained a greater amount of lipids, which can further increase the intestinal absorption of vitamin E (Lodge, Hall, Jeanes, & Proteggente, 2004). Finally, other possible effects of feeding whole pomegranate by-product on the concentration of vitamin E in meat cannot be excluded. For example, it has been suggested that dietary phenolic compounds could exert indirect antioxidant effects. Among these, polyphenols could protect and/or regenerate other antioxidant compounds, such as vitamin E, in the

gastrointestinal tract or in the animal tissues for the most bioavailable compounds (Halliwell, Rafter, & Jenner, 2005; Iglesias, Pazos, Torres, & Medina, 2012). In agreement with this observation, recent studies demonstrated a greater concentration of tocopherols in meat and milk from sheep fed diets supplemented with phenolic compounds (Lobón, Sanz, Blanco, Ripoll, & Joy, 2017; Ortuño, Serrano, & Bañón, 2015; Valenti, Natalello et al., 2019). Therefore, it can be supposed that the polyphenols contained in the pomegranate by-product used in the present study might have contributed to the greater deposition of vitamin E in meat from the WPB-fed animals and future studies would be necessary to investigate this possible effect.

Vitamin E has been extensively shown to be one of the main determinants of meat oxidative stability (Bellés et al., 2019). Indeed, it has been demonstrated that pro-oxidant factors, such as intramuscular polyunsaturated fatty acids and hem iron content, play a less important role when feeding strategies promote an adequate deposition of vitamin E in meat (Ponnampalam, Norn, Burnett, Dunshea, Jacobs, & Hopkins, 2014). Therefore, in the present study, it is plausible that vitamin E exerted a considerable role in reducing the extent of lipid oxidation in raw and cooked meat from lambs fed the WPB diet. This is the first study demonstrating that vitamin E could contribute to the antioxidant capacity of dietary pomegranate by-products as, to our knowledge, no previous studies have determined the vitamin E concentration, or the lipophilic antioxidant capacity, in muscle from animals fed with pomegranate by-products. Therefore, it is not possible to fully compare our results with the existing literature and further studies would be necessary to confirm these results. Although comparisons between studies should always be made with caution due to the different experimental conditions, it is possible that vitamin E contributed to the improvement of meat oxidative stability observed also in previous studies where lambs and kids were fed with pomegranate by-products. For example, Emami, Fathi Nasri, Ganjkhanlou, Zali, et al. (2015) used a by-product mostly composed of

pomegranate seeds and attributed the observed antioxidant effects mainly to the phenolic compounds. Nevertheless, it has been shown that tocopherols occur at higher concentrations in pomegranate seeds than in other parts of the pomegranate fruit, such as peels and pulp (Pande & Akoh, 2009). Furthermore, tocopherols were shown to be the main contributors to the antioxidant capacity of pomegranate seed oil, while phenolic compounds are associated to the antioxidant capacity of other portions of the fruits such as peels (Elfalleh, et al. 2011). Similar considerations may apply to the results provided by other studies in which dietary pomegranate seed pulp or other by-products containing seeds reduced lipid oxidation and improved antioxidant activity in meat (Emami, Fathi Nasri, Ganjkhanlou, Rashidi, et al., 2015; Kotsampasi et al., 2014).

Regarding meat colour, it is not easy to propose a plausible explanation for the higher L* values found, in the present study, in meat from lambs given the WPB diet. Indeed, factors potentially affecting meat lightness, such as ultimate pH, intramuscular fat and the concentration of myoglobin in meat did not differ between treatments. It is possible to suppose an effect of tannins from pomegranate in increasing L* values, as it was reported in studies where lambs were fed with different tannin-containing feeds, such as acacia foliage, fresh sulla or carob pulp (Priolo, Waghorn, Lanza, Biondi, and Pennisi, 2000; Priolo, Ben Salem, Atti, and Nefzaoui, 2002; Priolo et al., 2005). On the other colour parameters, it is known that meat browning, caused by the redox conversion of myoglobin forms, can be evaluated by the instrumental colour measurement, with some descriptors being particularly relevant (Mancini & Hunt, 2005). Specifically, the decrease of a* values and the increase of b* and h_{ab} values over time of storage observed in the present study are consistent with several shelf-life studies on lamb meat stored in comparable conditions (Aouadi et al., 2014; Valenti, Natalello, et al., 2019; Luciano et al., 2019). Although the saturation index (C values) has often been reported to decrease following meat browning, C values did not change over time in the present study. In

agreement with our results, Emani, Fathi Nasri, Ganjkhanlou, Zali, et al. (2015) studied the effects of feeding kids with pomegranate seed pulp on meat oxidative stability and reported that, despite the variation of a*, b* and h_{ab} values, the saturation index was not affected by the storage time. Lastly, it has been reported that strategies to reduce the extent of lipid oxidation often improve the stability of myoglobin to oxidation (Faustman, Sun, Mancini, & Suman, 2010). Therefore, in the present study, the ability of dietary pomegranate by-product to reduce lipid oxidation might explain the reduction of metmyoglobin accumulation observed in meat from WPB-fed animals compared to the CON treatment. However, it should be stressed that the accumulation of metmyoglobin over time was overall numerically small, albeit significant. This could partially explain the absence of differences in the colour descriptors between treatments, as well as their negligible numerical variation over time.

5. Conclusions

The results of this study provided evidence that a high amount of conventional cereal grains in the diet of lambs can be replaced by whole pomegranate by-product without negative effects on animal performances. Furthermore, this dietary strategy led to a reduction of lipid oxidation in fresh and cooked meat during refrigerated storage, despite the greater concentration of polyunsaturated fatty acids, while the formation of metmyoglobin was also slightly reduced. These results could be linked to the higher concentration of vitamin E in muscle from animals fed whole pomegranate by-product, which was associated to the higher antioxidant capacity measured in the lipophilic fraction of muscle. Therefore, these findings suggest for the first time vitamin E from dietary pomegranate by-products as a main factor contributing to improve meat oxidative stability.

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Table 1.
Ingredients, chemical composition and antioxidant capacity of the experimental diets and whole pomegranate by-product.

	Whole pomegranate by-product	Experimental diet[1]	
		CON	WPB
Ingredients, g/100 g dry matter (DM)			
Corn		22.6	11.6
Barley		22.6	11.6
Alfalfa hay		19.8	19.8
Wheat bran		20.0	20.0
Soybean meal		12.0	14.0
Whole pomegranate by-product		-	20.0
Molasses		0.9	0.9
Mineral premix[2]		2.1	2.1
Chemical composition, g/100 g DM			
DM, g/100 as fed	90.0	88.7	89.2
Crude Protein	6.52	17.6	17.8
NDF[3]	28.8	23.3	26.3
ADF[3]	20.7	12.9	15.5
ADL[3]	5.52	2.98	2.70
Ash	3.52	5.87	4.40
Crude Fat	3.99	2.11	2.51
Phenolic compounds, g/100g DM			
Total phenols[4]	9.51	0.30	1.89
Total tannins[4]	9.34	0.14	1.70
Condensed tannins	0.80	0.10	0.20
Tocopherols, mg/kg DM			
γ-Tocopherol	11.1	0.74	2.04
α-Tocopherol	48.3	7.82	16.8
Antioxidant capacity (ORAC), μmol TE/g DM[5]			
Hydrophilic fraction	684	103	342
Lipophilic fraction	27.3	21.1	31.1

[1] CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product.
[2] Containing: 25% calcium carbonate, 25% sodium bicarbonate, 25% bicalcic phosphate and 25% sodium chloride.
[3] NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin (ADL).
[4] Expressed as tannic acid equivalents.
[5] ORAC: oxygen radical absorbance capacity; TE: trolox equivalents.

Table 2.
Effect of the dietary treatment on lamb performances and intakes.

	Dietary treatment[1]		SEM[2]	*P*-value
	CON	WPB		
Performances				
Final body weight, kg	23.6	23.1	0.524	0.637
Carcass weight, kg	11.1	10.2	0.330	0.179
DMI[3], g/day	821	882	19.50	0.125
ADG[3], g/day	234	235	7.200	0.921
FCR[3], g DMI/g ADG	3.56	3.79	0.101	0.278
Intakes of phenolic compounds g/day				
Total phenols	2.49	16.7	1.700	<0.001
Total tannins	1.16	15.0	1.660	<0.001
Condensed tannins	0.08	0.18	0.012	<0.001
Intakes of tocopherols, mg/day				
γ-Tocopherol	0.61	1.80	1.444	<0.001
α-Tocopherol	6.42	14.8	1.300	<0.001

[1] CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product.
[2] SEM: standard error of the mean.
[3] DMI: dry matter intake; ADG: average daily gain; FCR: feed conversion ratio.

Table 3.
Effect of the dietary treatment on the muscle pH, myoglobin, antioxidant vitamins and oxidisable fatty acids in the intramuscular fat.

	Dietary treatment[1]		SEM[2]	*P*-value
	CON	WPB		
pH	5.72	5.78	0.036	0.427
Myoglobin, mg/g of muscle	2.68	2.46	0.093	0.259
Intramuscular fat, g/100 g muscle	1.88	2.01	0.156	0.690
Tocopherols, ng/g of muscle				
γ-Tocopherol	13.0	32.0	2.610	<0.001
α-Tocopherol	162	309	22.00	<0.001
Fatty acids classes and oxidizable fatty acids, mg/g of muscle				
Saturated	6.39	6.27	0.637	0.928
Monounsaturated	6.62	6.15	0.566	0.702
Polyunsaturated (PUFA)	1.30	1.80	0.121	0.041
PUFA *n*-3	0.13	0.16	0.011	0.091
PUFA *n*-6	1.08	1.34	0.086	0.137
HP-PUFA[3]	0.39	0.57	0.041	0.024
Peroxidability index[4]	2.07	2.85	0.191	0.042

[1] CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product.
[2] SEM: standard error of the mean.
[3] Highly peroxidizable-PUFA, calculated as the sum of PUFA with three or more unsaturated bonds.
[4] Calculated as: peroxidability index = (∑dienoic×1) + (∑trienoic×2) + (∑tetraenoic×3) + (∑pentaenoic×4) + (∑hexaenoic×5).

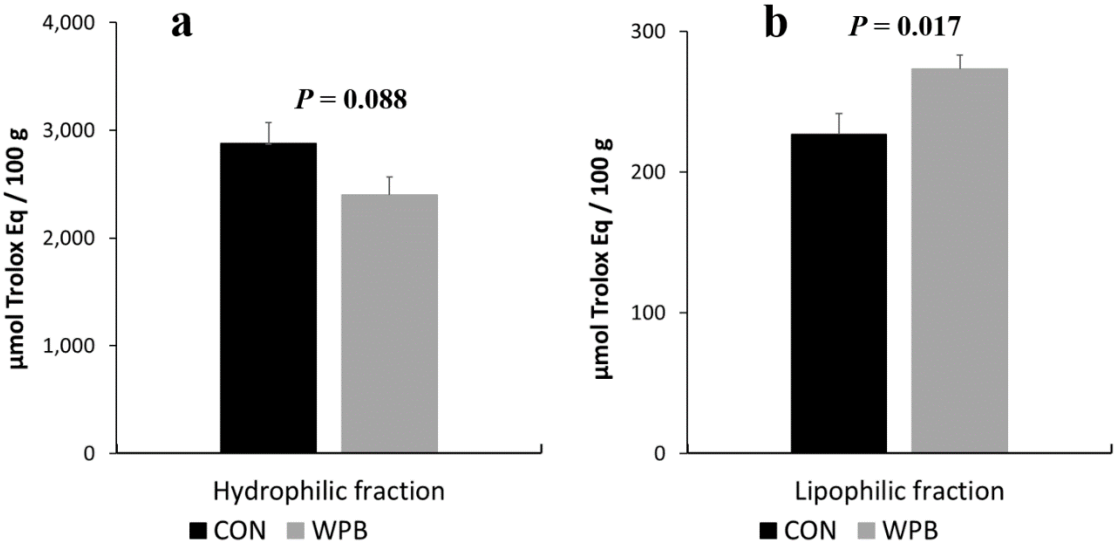
Table 4.
Effect of the dietary treatment and time of storage on the oxidative stability parameters of meat.

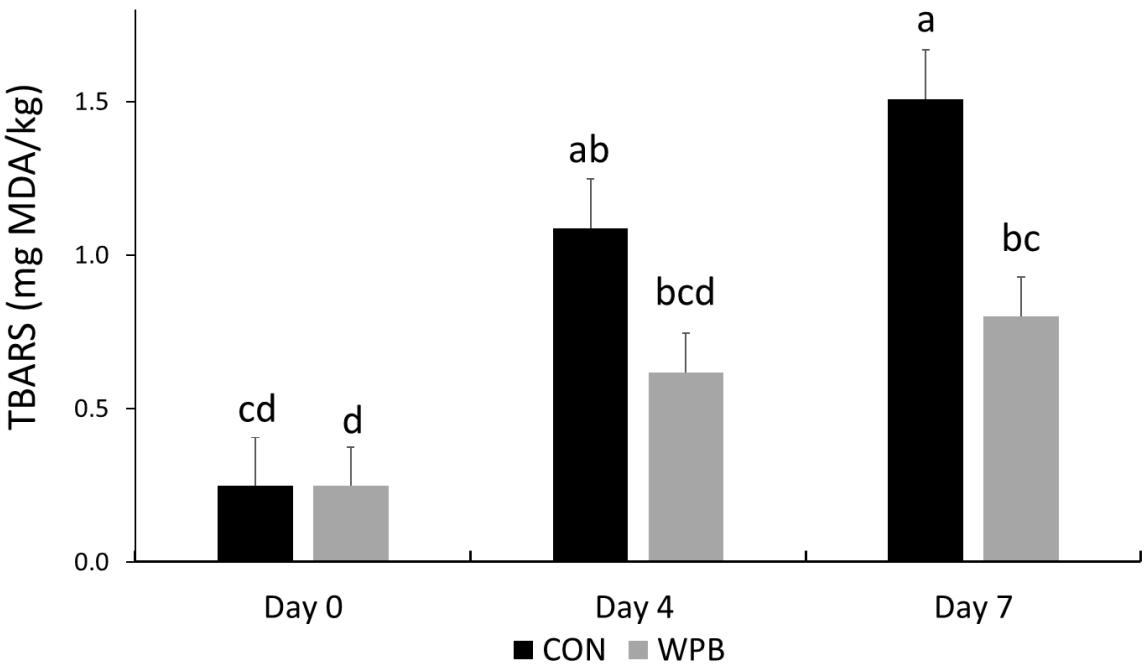
	Dietary treatment (D)[1]		Time of storage (T)[2]			SEM[3]	*P*-values[4]		
	CON	WPB	0	1	2		D	T	D × T
Colour descriptors and metmyoglobin % of raw meat									
L* (lightness)	46.99	49.02	48.57	48.65	47.48	0.296	0.005	0.068	0.533
a* (redness)	11.56	11.60	12.24^{a}	11.58^{ab}	10.93^{b}	0.166	0.929	<0.001	0.181
b* (yellowness)	11.21	11.39	10.15^{b}	11.87^{a}	11.93^{a}	0.194	0.663	<0.001	0.159
C (saturation)	16.14	16.29	15.91	16.59	16.19	0.214	0.789	0.474	0.168
h_{ab} (hue angle)	44.08	44.29	39.55^{c}	45.61^{b}	47.45^{a}	0.494	0.620	<0.001	0.087
MetMb, % of Mb	47.60	46.03	39.52^{c}	49.16^{b}	51.25^{a}	0.762	0.050	<0.001	0.498
Lipid oxidation (TBARS values), mg/kg meat									
Raw meat	0.95	0.56	0.25^{b}	0.80^{a}	1.08^{a}	0.080	0.024	<0.001	0.013
Cooked meat	3.74	3.07	1.69^{c}	3.60^{b}	4.70^{a}	0.192	0.006	<0.001	0.266

[1] CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product.
[2]Time of storage 0, 1, 2 correspond to: days 0, 4, 7 (raw meat); days 0, 2, 4 (cooked meat)
[3]SEM: standard error of the mean.
[4]*P*-values for the effects of the dietary treatment, time of storage and of the Diet × Time interaction
[a, b, c] Within row, different superscript letter indicates differences ($P < 0.05$) between times of storage tested using the Tukey's Honest Significant Difference test.

Fig 1. Effect of the dietary treatment (CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product) on raw meat antioxidant capacity of (a) hydrophilic fraction and (b) lipophilic fraction.
Values presented are the estimated least squares means and standard error bars.

Fig 2. Effect of the dietary treatment (CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product) and time of storage (days 0, 4 and 7) on TBARS (thiobarbituric acid reactive substances) values of raw meat over aerobic storage at 4 °C. Values presented are the estimated least squares means and standard error bars.
MDA: Malondialdehyde
[a,b,c]Indicate differences between mean values (P <0.05) tested using the Tukey's Honest Significant Difference test.


a
P = 0.088
µmol Trolox Eq / 100 g
4,000
3,000
2,000
1,000
0
Hydrophilic fraction
CON
WPB
b
P = 0.017
µmol Trolox Eq / 100 g
300
200
100
0
Lipophilic fraction
CON
WPB


TBARS (mg MDA/kg)
1.5
1.0
0.5
0.0
cd
d
ab
bcd
a
bc
Day 0
Day 4
Day 7
CON
WPB