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XXXII CYCLE

# Pomegranate by-products as ruminant feeds: Effects on meat quality

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## List of publications

1) **Natalello, A**., Luciano, G., Morbidini, L., Valenti, B., Pauselli, M., Frutos, P., Biondi, L., Rufino-Moya, P.J., Lanza, M. and Priolo, A., **2019.** Effect of feeding pomegranate byproduct on fatty acid composition of ruminal digesta, liver, and muscle in lambs. *Journal of Agricultural and Food Chemistry*, *67*(16), 4472-4482.

2) **Natalello, A.**, Hervás, G., Toral, P.G., Luciano, G., Valenti, V., Mendoza, A.G., Pauselli, M., Priolo, A. and Frutos, P., **2020.** Bioactive compounds from pomegranate by-products increase the *in vitro* ruminal accumulation of potentially health promoting fatty acids. *Animal Feed Science and Technology*, *259*, 114355

3) **Natalello, A.**, Priolo, A., Valenti, B., Codini, M., Mattioli, S., Pauselli, M., Lanza, M., Stergiadis, S. and Luciano, G., **2020.** Dietary pomegranate by-product improves oxidative stability of lamb meat. Submitted to *Meat Science*

4) Luciano, G., Roscini, V., Mattioli, S., Ruggeri, S., Gravador, R.S., **Natalello, A**., Lanza, M., De Angelis, A. and Priolo, A., **2017.** Vitamin E is the major contributor to the antioxidant capacity in lambs fed whole dried citrus pulp. *Animal, 11*(3), 411-417.

5) Valenti, B., Luciano, G., Pauselli, M., Mattioli, S., Biondi, L., Priolo, A., **Natalello, A**., Morbidini, L. and Lanza, M., **2018.** Dried tomato pomace supplementation to reduce lamb concentrate intake: Effects on growth performance and meat quality. *Meat Science*, *145*, 63-70.

6) Valenti, B., **Natalello, A**., Vasta, V., Campidonico, L., Roscini, V., Mattioli, S., Pauselli, M., Priolo, A., Lanza, M. and Luciano, G., **2019.** Effect of different dietary tannin extracts on lamb growth performances and meat oxidative stability: comparison between mimosa, chestnut and tara. *Animal*, *13*(2), 435-443.

7) Valenti, B., Luciano, G., Morbidini, L., Rossetti, U., Codini, M., Avondo, M., Priolo, A., Bella, M., **Natalello, A**. and Pauselli, M., **2019.** Dietary pomegranate pulp: effect on ewe milk quality during late lactation. *Animals*, *9*(5), 283.

8) Luciano, G., **Natalello, A**., Mattioli, S., Pauselli, M., Sebastiani, B., Niderkorn, V., Copani, G., Benhissi, H., Amanpour, A. and Valenti, B., **2019.** Feeding lambs with silage mixtures of grass, sainfoin and red clover improves meat oxidative stability under high oxidative challenge. *Meat Science*, *156*, 59-67.

9) Biondi, L., Randazzo, C.L., Russo, N., Pino, A., Natalello, A., Hoorde, K.V. and Caggia, C., **2019.** Dietary Supplementation of Tannin-Extracts to Lambs: Effects on Meat Fatty Acids Composition and Stability and on Microbial Characteristics. *Foods, 8*(10), 469.

10) Biondi, L., Luciano, G., Cutello, D., Natalello, A., Mattioli, S., Priolo, A., Lanza, M., Morbidini, L., Gallo, A. and Valenti, B., **2020.** Meat quality from pigs fed tomato processing waste. *Meat Science*, 159, 107940.

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# List of abbreviations

| | |
|---|---|
| ACC | Acetyl-CoA carboxylase |
| ADF | Acid detergent fibre |
| ADG | Average daily gain |
| ADL | Acid detergent lignin |
| BCFA | Branched chain fatty acids |
| BH | Biohydrogenation |
| BW | Body weight |
| CA | Catalpic acid |
| CLA | Conjugated linoleic acid |
| CLnA | Conjugated linolenic acid |
| CON | Control |
| CP | Crude protein |
| DM | Dry matter |
| DMD | Dry matter disappearance |
| DMI | Dry matter intake |
| FA | Fatty acids |
| FAME | Fatty acid methyl esters |
| FAS | fatty acid synthase |
| LTL | *Longissimus thoracis et lumborum* |
| MDA | Malondialdehyde |
| ME | Metabolizable energy |
| MMb | Metmyoglobin |
| MUFA | Monounsaturated fatty acids |
| n-3 | Omega-3 fatty acids |
| n-6 | Omega-6 fatty acids |
| NDF | Neutral detergent fibre |
| NEFA | Non-esterified fatty acids |
| OBCFA | Odd- and branched-chain fatty acids |
| OCFA | Odd- and linear-chain fatty acids |
| P$^{\bullet}$ | Protein radical |
| PA | Punicic acid |
| PE | Phenolic extract |
| PEG | Polyethylene glycol |
| PO | Pomegranate oil treatment |
| POO$^{\bullet}$ | Peroxidic radical |
| POOH | Alkyl peroxide |

| | |
|---|---|
| PPP | Pomegranate peels and pulp |
| PS | Pomegranate seeds |
| PSO | Pomegranate seed oil |
| PT | Pomegranate tannin treatment |
| PTO | Pomegranate phenolic and oil treatment |
| PUFA | Polyunsaturated fatty acids |
| $R^{\bullet}$ | Alkyl radical |
| RA | Rumenic acid |
| RNS | Reactive nitrogen species |
| $ROO^{\bullet}$ | Peroxyl radical |
| ROOH | Hydroperoxides |
| ROS | Reactive oxygen species |
| SEM | Standard error of means |
| SFA | Saturated fatty acids |
| TBA | Thiobarbituric acid |
| TBARS | Thiobarbituric reactive substances |
| TCA | Tricholoacetic acid |
| TMR | Total mixed ration |
| VA | Vaccenic acid |
| VFA | Volatile fatty acid |
| VLDL | Very low-density lipoproteins |
| WPB | Whole pomegranate by-product |
| α-ESO | α-eleosearic acid |
| α-LnA | α-linolenic acid |
| β-ESO | β- eleosearic acid |

# List of units and symbols

| | |
|---|---|
| < | Lower than |
| % | Percentage |
| > | Higher than |
| ± | Standard deviation |
| Σ | Sum |
| µg | Microgram |
| cm | Centimetre |
| g | Gram |
| h | Hour |
| kg | Kilogram |
| L | Litre |
| mg | Miligram |
| mL | Mililitre |
| mmol | Milimol |
| ºC | Degree Celsius |
| vol | Volume |
| wt | Weight |
| α | Alpha |
| β | Beta |
| Δ | Delta |
| *x g* | Times gravity |
| a* | Redness descriptor measured in the CIELab colour space |
| b* | Yellowness descriptor measured in the CIELab colour space |
| C | Chroma measured in the CIELab colour space |
| $h_{ab}$ | Hue angle measured in the CIELab colour space |
| L* | Lightness descriptor measured in the CIELab colour space |

# ABSTRACT

Increasing demand and production of pomegranate has led to a large amount of by-products that might be used in ruminant feeding. In this PhD thesis, we have investigated the effect of dietary whole pomegranate by-product (WBP) on performance and meat quality of lambs. The WBP was collected from a processing company after squeezing to obtain juice, and it included peels, seeds, membranes and small residual parts of pulp. These parts of pomegranate are particularly rich in bioactive compounds: seeds contain a high amount of lipids, which are mainly characterised by conjugated linolenic acids (CLnA) and among which, the punicic acid (C18:3 *c*9 *t*11 *c*13) is the predominant. Whereas, pomegranate peels are a valuable source of phenolic compounds, and in particular tannins.

In the first study (Chapter 5), 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 barley and corn were replaced by whole pomegranate by-product (WPB). After 36 days of trial the lambs were slaughtered and samples of rumen digesta, liver and muscle were collected. The dietary treatment did not affect all the animal performance parameters. While, total polyunsaturated FA (PUFA), linolenic, rumenic (RA), and vaccenic (VA) acid were increased in liver and muscle of WPB lambs ($P < 0.05$). Punicic acid and three isomers of conjugated linolenic acid were detected exclusively in the rumen and tissues of WPB-lambs. The C18:1 *t*10/*t*11 ratio in rumen digesta or in tissues was reduced by feeding WPB ($P < 0.001$), suggesting that the WPB prevented the t10-shift rumen biohydrogenation pathway.

The second study (Chapter 6) was designed to fill in the knowledge gaps found in the first study. Indeed, it remains unclear whether this effect on FA metabolism was due to the action of the bioactive CLnA or of the tannins or perhaps to their interaction. Therefore, two *in vitro* experiments were conducted: the first one tested the effects of pomegranate oil and tannins, alone or in combination, on the biohydrogenation process, and the second one compared the ruminal responses to by-products rich in CLnA (pomegranate seeds, PS), in tannins (pomegranate peels and pulp, PPP) or in both bioactive components (i.e., the whole pomegranate by-product; WPB). Three cannulated ewes were used as donors of inocula for batch cultures of rumen microorganisms. Incubations lasted for 12 and 24 h and were repeated on 3 different days. The consistency in the results from both trials would confirm that pomegranate tannins and CLnA played different roles in modulating ruminal FA composition. Specifically, tannins would favour the accumulation of potentially health-promoting FA present in dietary lipids (e.g., 18:2n-6 or 18:3n-3) and cis-9 trans-11 conjugated linoleic acid (CLA), whereas the observed increase in trans-11 18:1 would mainly derive from the biohydrogenation of CLnA isomers. Changes in ruminal fermentation parameters showed that pomegranate tannins protected dietary protein from degradation. Nevertheless, a negative impact on *in vitro* ruminal fermentation was observed when 20% of by-products were included in the diet. Finally, there seem to be no evident synergistic but additive effects between pomegranate bioactive compounds (i.e., tannins and CLnA) on ruminal biohydrogenation or fermentation.

The third study (Chapter 7) investigated the effect of including whole pomegranate by-product in lamb diet on meat oxidative stability, using the meat samples from the first experiment. 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 fresh 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.

# SECTION I – General introduction

# CHAPTER 1 - Pomegranate by-products

Pomegranate (*Punica granatum* L.) is typically a shrub or a small tree that belongs to the Punicaceae family. Its leaves are lance shaped, deciduous and their colour changes from red as soon as formed to glossy green as complete development. The flowers are hermaphroditic, red, white, or variegated and have a tubular calyx from which the fruit originates. The fruit is a berry and can be up to 12 cm wide with a deep red and leathery skin. The fruit contains many seeds (arils) separated by a white, membranous pericarp, and each is surrounded by small amounts of tart, red juice (Jurenka, 2008).

Pomegranate fruit is known and consumed since ancient times. Also, this fruit was lauded in the Egyptian, Greek and Roman cultures as a sacred fruit conferring fertility, abundance and good luck (Jurenka, 2008). Pomegranate has been used as medicinal food in the Middle East for thousands of years (Johanningsmeier and Harris, 2011).

**Figure 1.** Pomegranate fruit (left) and its section (Right) from Viuda-Martos *et al.* 2010.

The pomegranate is native from the Near East (Melgarejo *et al.,* 2012), but it has been widespread over the centuries in the entire Mediterranean region, Southeast Asia and tropical Africa (Jurenka, 2008). Nowadays, it is also grown in the United States (California and Arizona), Japan and Russia (Fadavi *et al.,* 2006).

Although in recent years there has been a sudden increase in production and cultivation of pomegranate, there are no reliable data on the global production of pomegranate fruit, probably because it is still considered a minor crop. Melgarejo *et al.* (2012) have estimated the world pomegranate production, associating data provided by several reports and communications, and calculated approximately 3,086,000 tons of pomegranate fruits produced in the world around 2011. They also reported an area of 300,000 ha dedicated to pomegranate production. India and Iran are the largest producers, followed by China, Turkey and USA (Melgarejo *et al.,* 2012).

Although, as mentioned above, this fruit has been known since ancient times, its consumption and interest has exploded in recent years. This trend can mainly be attributed to the recognition of the health properties of this fruit, also thanks to the research findings. In fact, the interest of the scientific community has been focused on the potential beneficial effects of pomegranate on human health. This is easily observable from the trend of articles published in peer-reviewed journal in the last decades by searching “pomegranate health” on PubMed website (Figure 2).

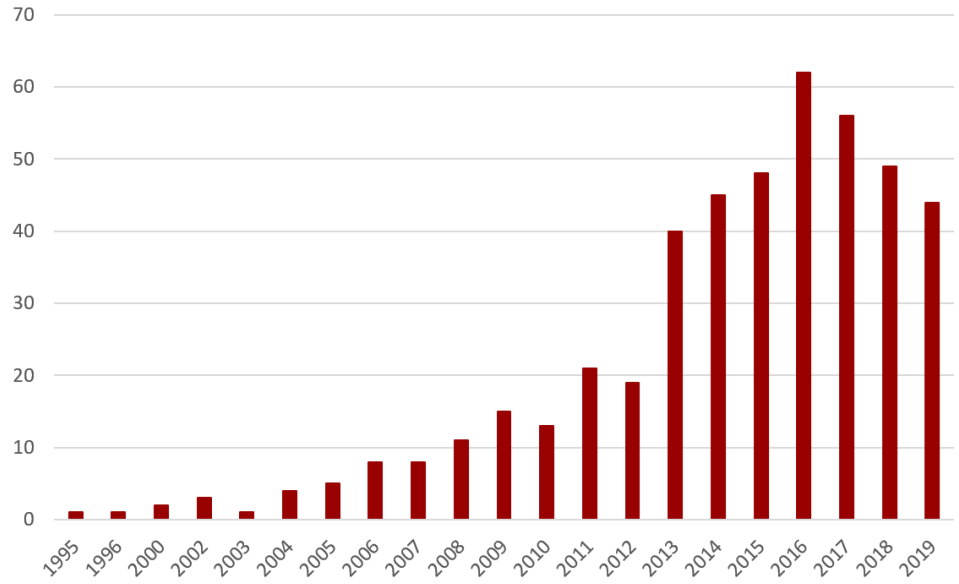


**Figure 2.** Scientific papers relating to health of pomegranate published between 1995 and 2019 (PubMed – July 2019).

From *in vitro* and *in vivo* studies, it would seem that the pomegranate has multiple functional and medicinal effects such as antioxidant, antidiabetic, antimicrobial, anti-inflammatory, antiviral, and anticarcinogenic properties (Viuda-Martos *et al.,* 2010).

These beneficial properties are undoubtedly linked to the occurrence of many bioactive compounds present in all the parts of pomegranate fruit. Specifically, pericarp (peel) contains large amounts of phenolic compounds, among which hydrolysable tannins and anthocyanins are the predominant (Lansky and Newman, 2007; Gumienna *et al.,* 2016). In turn, pomegranate hydrolysable tannins are mainly characterised by punicalin and punicalagin (Figure 3), which release ellagic acid when hydrolysed (Johanningsmeier and Harris, 2011). It has been

widely demonstrated that pomegranate phenolic compounds possess strong antioxidant properties (Gil *et al.,* 2000; Tzulker *et al.,* 2007) as measured by different assays (e.g., FRAP, DPPH, TEAC, AEAC).

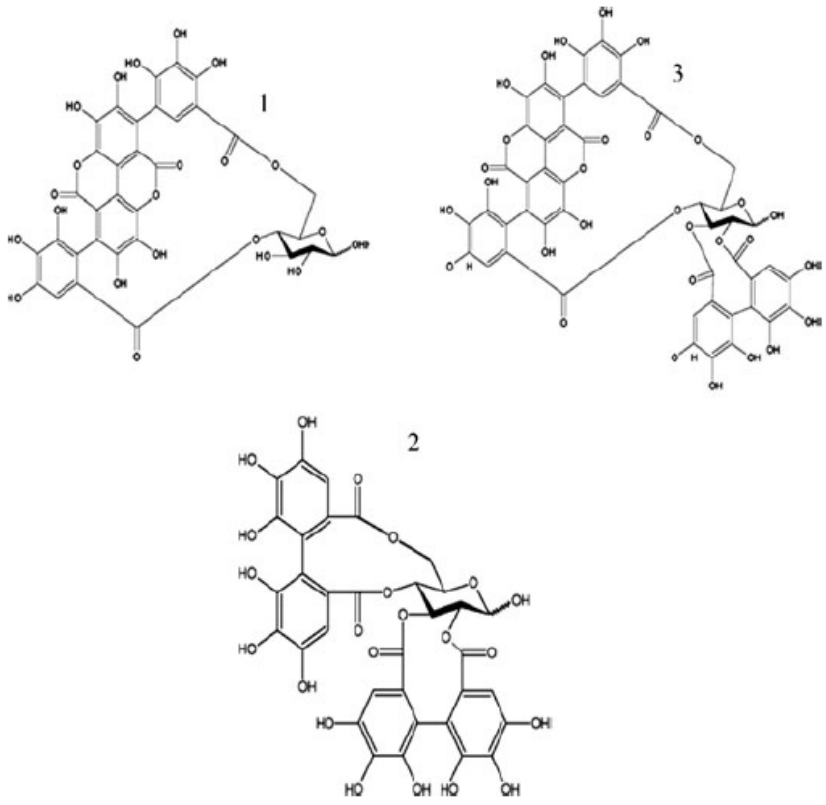


**Figure 3.** Principal ellagitannins present in pomegranate peel. 1: punicalin; 2: pedunculagin; 3: punicalagin. From Viuda-Martos *et al.* 2010.

Pomegranate seeds contain remarkable amounts of lipids, ranging from 6 - 24 % of the seed total weight (Aruna *et al.,* 2016). The pomegranate seed lipids are mainly represented by fatty acids (FA; over 95%), which are almost all in the form

of triglycerides (Lansky and Newman, 2007). The fatty acid profile of pomegranate oil is very particular, if not unique. In fact, it is mostly composed of peculiar fatty acids with 18-carbon molecules and 3 alternating double bonds (also known as conjugated linolenic acids (CLnA) and trienoic acids). Among these, punicic acid (C18 *c*9 *t*11 *c*13) is the predominant compound, reaching up to 85% of the total FA (Aruna *et al.,* 2016), while other CLnA isomers, such as α-eleostearic acid (C18:3 *c*9 *t*11 *t*13; α-ESA), catalpic acid (C18:3 *t*9 *t*11 *c*13; CA), and β-eleostearic acid (C18:3 *t*9 *t*11 *t*13; β-ESA), occur at minor concentrations in pomegranate seed oil (Sassano *et al.,* 2009; Figure 4).

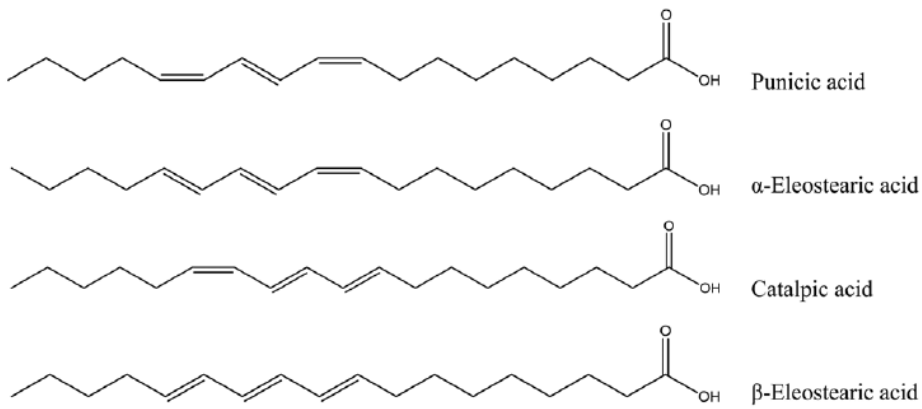


**Figure 4.** Chemical structure of pomegranate conjugated linolenic acids (Yuan *et al.,* 2014).

These CLnA isomers have been shown to have beneficial biological functions, even with stronger effects than CLA (Igarashi and Miyazawa, 2000; Tsuzuki *et al.,* 2004), which are considered unequivocally beneficial compounds for human health. Furthermore, it has been well demonstrated both *in vivo* (Tsuzuki *et*

*al.,* 2004; Yuan *et al.,* 2009) and *in vitro* (Schneider *et al.,* 2013) that CLnA are effectively absorbed in the organism and converted into CLA, probably via a Δ13 saturation reaction (de Melo *et al.,* 2014).

In addition to recognizing the health properties of the pomegranate, the development of advanced industrial technologies that provide products more appealing to the consumer, such as fresh fruit and ready-to-eat arils, has certainly contributed to the increase in global consumption.

However, the development of agri-food industries has led to a substantial increase in waste biomass. For instance, huge quantities of peels and seeds are produced after processing the pomegranate to obtain the juice. These by-products could have a negative environmental impact, if not properly disposed. Consequently, their disposal constitutes a substantial cost for the industries.

However, the chemical composition of pomegranate by-products makes these wastes interesting for ruminant feeding and promising results have already been achieved in the last decades. For instance, Shabtay *et al.* (2008) investigated the pomegranate peels in the forms of fresh or stored (ensiled and dried), analysing the chemical composition to assess their nutritional value for ruminants. Additionally, they evaluated *in vivo* the fresh pomegranate peels offered to bull calves *ad libitum* as cafeteria. These authors reported that dietary pomegranate peels increased the dry matter intake (DMI) and α-tocopherol concentration in blood plasma, with a trend toward increased average daily gain (ADG) of the calves. Subsequently, Modaresi *et al.* (2011) and Razzaghi *et al.* (2015) did not observe effects on DMI and milk production when pomegranate seed pulp (PSP) was

included up to 12 % in diets of dairy goats. Interestingly, these authors noted improvements in the fatty acid composition of milk, with a greater content of potentially health-promoting fatty acids. Similarly, the inclusion of pomegranate seed pulp at levels up to 150 g/kg DM in kid diets did not affect the animal performance parameters, such as DMI, ADG and carcass weight (Emami *et al.,* 2015a), but improved meat FA composition (Emami *et al.,* 2015b) and meat oxidative stability (Emami *et al.,* 2015c), as well as the overall antioxidant status of kids (Emami *et al.,* 2015a). Also, Safari *et al.* (2018) observed improvements of the overall antioxidant capacity, without effect on DMI, when transition dairy cows were fed with pomegranate seeds or pomegranate seeds plus peels.

Similar results were achieved using an ensiled pomegranate by-product that included both seeds and peels. Indeed, Kotsampasi *et al.* (2014) supplemented this by-product up to the level of 240 g/kg in the diet of growing lambs and no differences were found in animal performances, while the fatty acid composition of meat was enhanced of desired FA. The same authors also evaluated the inclusion (up to 150 g/kg DM) of pomegranate pulp silage into total mixed rations for lactating dairy cows and observed no negative effects on animal productive performance and on milk chemical composition, whereas the milk fatty acids profile and animal antioxidant status were improved (Kotsampasi *et al.,* 2017). Recently, the replacement of 120 g/kg DM of corn silage with pomegranate by-product silage led to an increase in milk production, without affecting the DMI, in *postpartum* Holstein cows under heat stress condition (Khorsandi *et al.,* 2019). Conversely, Shaani *et al.*

(2016) concluded that the level of 8% pomegranate peel silage impaired DMI, milk production and digestibility of DM, CP, and NDF in lactating cows.

Likewise, Hatami *et al.* (2018) reported a reduction of DMI and ADG in growing lambs fed with 160 g of a dried pomegranate by-product /kg DM diet, while no adverse effect on performance was observed at the level of 80 g/kg DM.

To conclude, generally speaking, it is necessary to stresse that the chemical characteristics of the various pomegranate by-products is variable in relation to many factors such as production place, cultivar, phenological state of the fruit and/or plant, type of processing and conservation, etc. Moreover, these differences are greatly accentuated when considering the bioactive compounds of a by-product. Therefore, the presence of partially contrasting results on the use of pomegranate by-products in ruminant feeding can certainly be explained by the possible chemical variations of this waste. Moreover, other factors such as the species and physiological conditions of the animal, interaction with the basal diet, etc. can further complicate the interpretation of the results. Notwithstanding all this, most of the studies in the literature have shown promising results on the use of pomegranate by-products as alternative feeds in ruminant feeding, also suggesting high levels of inclusion of these by-products (up to 24%) in replacement of conventional ingredients, such as cereals. Further studies are needed to confirm the effective and practical use of pomegranate by-products, but also to assess their effect on the quality parameters of ruminant products such as milk and meat. Indeed, as also investigated in some studies, the numerous bioactive molecules contained in this

fruit could influence not only the animal health status or their productive performance, but also the nutritional characteristics of milk and meat.

# CHAPTER 2 - Lipid metabolism in ruminants

Ruminant products such as milk and meat are often blamed for favouring the onset of cardiovascular diseases, mainly due to their high content of saturated fatty acids (SFA). In fact, despite these animals consume mainly feeds rich in polyunsaturated fatty acids (PUFA), during the processes that take place in the rumen most of the unsaturated fatty acids are saturated. The rumen transformations of fatty acids can be attributed to two main processes: lipolysis and biohydrogenation (BH) (Vasta and Bessa, 2012).

**Lipolysis**

The lipids present in the diet of ruminants are mainly in the form of (i) triglycerides or (ii) sulfo-, galacto- and phospholipids respectively for cereals and forages. Once in the rumen, dietary lipids are rapidly transformed into non-esterified free fatty acids (Vasta and Bessa, 2012). This process, named lipolysis, involves the splitting of triglycerides allowing the release of glycerol and free fatty acids as well as small amounts of mono- and diglycerides (Buccioni *et al.,* 2012). The presence of a free carboxylic group at the Δ-end of the free fatty acid allows subsequent biohydrogenation processes. Lipase is the enzyme involved in this process and has plant and microbial origin (Lourenço *et al.* 2010). However, the hydrolysis of the esterified fatty acids in the rumen is mainly performed by the microbial lipase (Lourenço *et al.* 2010) and, although the microorganisms capable of hydrolysing are few, their activity is highly specific (Buccioni *et al.,* 2012). In normal conditions,

the lipolysis process occurs very rapidly and more than 85% of esterified dietary lipids are hydrolysed (Shingfield *et al.,* 2013). Nevertheless, several factors such as the presence of low pH, accumulation of PUFA or bioactive substances could inhibit this process (Jenkins *et al.,* 2008; Lourenço *et al.,* 2010; Vasta and Bessa, 2012).

**Biohydrogenation**

Biohydrogenation process consists of a series of chemical reactions, which involve isomerization, hydratation, or hydrogenation of unsaturated dietary FA after their lipolysis by rumen microbial populations. The reason why the microorganisms hydrogenate extensively the unsaturated FA is still not entirely clear (Vasta and Bessa, 2012); however, among the various theories, the hypothesis that microorganisms conduct the BH process to reduce the toxicity of PUFA would seem to be the most plausible.

Dietary fatty acids are mainly represented by molecules with 18 carbon atoms, and in particular linoleic (C18:2 *c*9 *c*12) and linoleic (C18:3 *c*9 *c*12 *c*15) acids. From the biohydrogenation of these two FA, many intermediates are generated which vary in relation to the stage and the pathways of the BH process. Indeed, different rumen BH pathways (mainly of linoleic and linolenic acids) have been described in many excellent publications (Shingfield *et al.,* 2010; Alves and Bessa, 2014). Although alternative pathways occur when animal ingest diets rich in starch and/or PUFA, due to the change in rumen pH and rumen bacteria community (Ferlay *et al.,* 2017), the major BH pathway of linoleic and linolenic acid seems to be clear (Figures 5 and 6, respectively). Indeed, in normal conditions, the rumen

biohydrogenation of these FA starts from the isomerization of the *c*12 to *t*11 double bond. Next, the *c*9 double bond is hydrogenated to produce C18:1 *t*11 or C18:2 *t*11 *c*15. The process continues with further isomerizations and saturations until the completion of the process with the formation of stearic acid (C18:0).

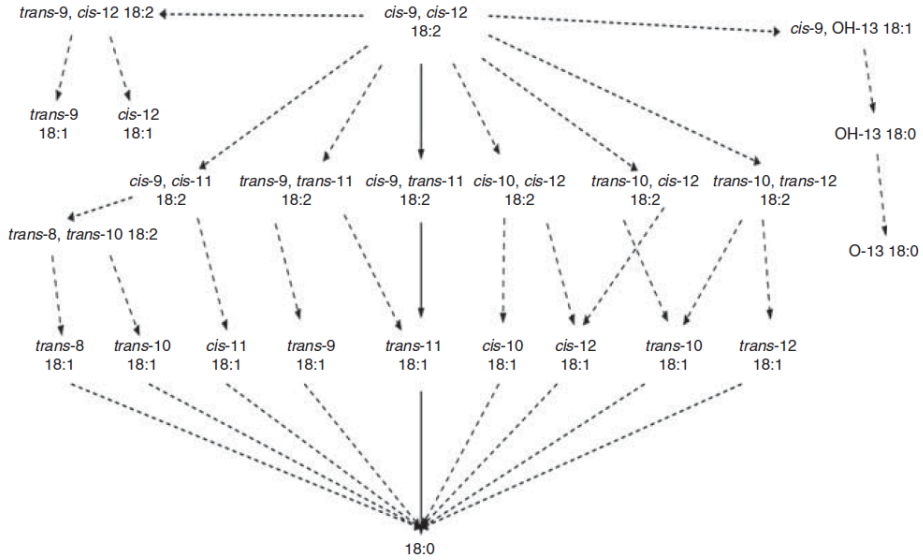


**Figure 5.** Putative pathways describing linoleic acid metabolism in the rumen (Shingfield *et al.,* 2010)

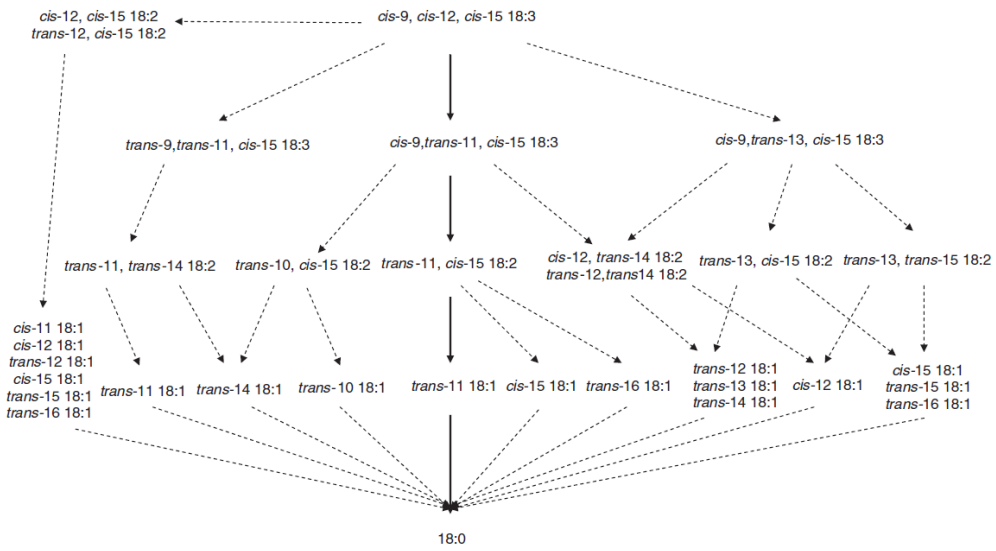


**Figure 6.** Putative pathways describing linolenic acid metabolism in the rumen (Shingfield *et al.,* 2010)

Among the various intermediates of the biohydrogenation process, several conjugated fatty acids are formed, and in particular the conjugated linoleic acids (CLA). These particular fatty acids have received enormous interest from the scientific community in the last few decades for their antitumoral properties (Pariza *et al.,* 2001). Although many CLA isomers have been identified in the rumen, the main isomer formed by the biohydrogenation of linoleic acid is the C18:2 *c*9 *t*11 (Lourenço *et al.,* 2010), also known as rumenic acid (RA). In particular this CLA would also seem to have a more potent beneficial effect on human health than other CLA isomers (McGuire and McGuire, 2000). While, from the biohydrogenation of linolenic acid (C18:3 *c*9 *c*12 *c*15) several conjugated triene isomers are usually formed, also called conjugated linolenic acids (CLnA), such as C18:3 *c*9 *t*11 *c*15,

C18:3 *t*9 *t*11 *c*15 and C18:3 *c*9 *t*13 *c*15 (Shingfield *et al.,* 2010). Also in this case, it has been demonstrated that CLnA have beneficial effects on human health (Yuan *et al.,* 2014; Białek *et al.,* 2017).

Vaccenic acid (C18:1 *t*11) is the predominant *trans* monounsaturated FA (MUFA) originated during the biohydrogenation process (Wolff, 1995). It is also a desired fatty acid because in animal tissues it can be converted into rumenic acid (as detailed in the next paragraph). However, when certain conditions occur, the rumen biohydrogenation pathway cannot follow the main route, and another *trans* isomer (i.e., C18:1 *t*10) may become the most abundant *trans* MUFA at the expense of C18:1 *t*11. This change in the rumen pathway is defined as "*t*-10 shift" and occurs mainly when the ruminant diet is richer in concentrates than in forages (Bessa *et al.,* 2015). Although the effect of C18:1 *t*10 on human health is an issue still under debate (Salter, 2019), this FA is often accused to increase the risk of cardiovascular diseases (Hodgson *et al.,* 1996).

In the rumen content there are also considerable quantities of odd- and branched- chain fatty acids (OBCFA). They are mainly synthesized *de novo* by rumen microorganisms, as only small traces are present in plants and are ingested with the diet (Vlaeminck *et al.,* 2006). The OBCFA are generally included in the membranes of microorganisms to maintain fluidity, thanks the low melting points relative to chain length (Vlaeminck *et al.,* 2006). Odd linear chain FA (C15:0, C17:0), even *iso* acids (C14:0 *iso*, C16:0 *iso*), odd *iso* acids (C15:0 *iso*, C17:0 *iso*) and odd *anteiso* acids (C15:0 *anteiso*, C17:0 *anteiso*) are usually detected in the rumen content.

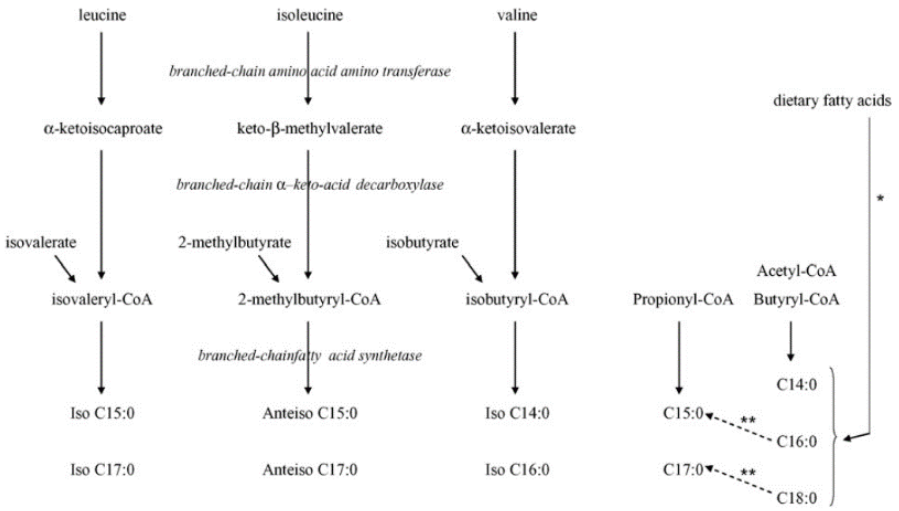


**Figure 7.** Synthesis of odd and branched chain fatty acids (from Vlaeminck *et al.,* 2006).

The OBCFA are also found in meat and milk of ruminants and their appearance is directly derived from ruminal bacteria activity (Vlaeminck *et al.,* 2006). Therefore, Fievez *et al.* (2012) proposed the use of OBCFA as biomarkers of rumen functions to predict the rumen volatile fatty acids (VFA), microbial protein, methane production and the early detection of ruminal acidosis.

**Fatty acid metabolism: post-rumen fate**

After the processes that take place in the rumen, the fatty acids that arrive in the small intestine are mainly in the form of non-esterified FA (NEFA).

Subsequently, these fatty acids are absorbed by epithelial intestinal cells, and processed depending on the length of the FA chain. Indeed, the short and medium chain FA (<C12) are directly secreted as NEFA in the blood system.

Otherwise the long chain FA are esterified into triacylglycerols, phospholipids and cholesterol esters, which are incorporated into chylomicrons and very low-density lipoproteins (VLDL). The latter two are later secreted into intestinal lymph and then in the blood plasma through the thoracic duct (Hocquette and Bauchart, 1999; Wang *et al.,* 2013; Shingfield *et al.,* 2013).

In this way the fatty acids carried throughout the organism can enter the various cells. In muscle cells, fatty acids are converted to fatty acyl-CoA thioesters, which can then be sequestered through binding to acyl-CoA binding proteins. Alternatively, fatty acyl-CoA thioesters can be oxidized or incorporated into triglycerides and phospholipids with or without further modification such as elongation/desaturation (Sampath and Ntambi, 2005).

Fatty acids in the muscle can be also synthesized *de novo* through the activity of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) enzymes, which can yield fatty acids up to 16 carbons as the final end products. *De novo* fatty acids are substrate for further elongations and desaturations (Shingfield *et al.,* 2013).

Saturated and monounsaturated FA can be modified by elongases 1, 3 and 6, while PUFA are extended by elongases 2, 4 and 5 (Cherfaoui *et al.,* 2012 – Shingfield 2013). Therefore, long-chain fatty acids (e.g., C20:5 n-3, C22:5 n-3, C22:6 n-3, etc.) are commonly found in meat and milk, thanks to the action of these enzymes, although the ruminant diet does not contain very long-chain fatty acids (> C20) unless supplemented with particular oils such marine or fish oils.

Another important process that occurs with fatty acids in mammalian tissues is the desaturation, which is performed by the activity of Δ-5, Δ-6 or Δ-9 desaturases

(Shingfield *et al.,* 2013). By the action of these enzymes, SFA are converted into MUFA, and part of MUFA into PUFA. Among these enzymes, the Δ-9 desaturase (also known as stearoyl-CoA desaturase) has received particular attention from the scientific community for its effectiveness in improving the FA composition of milk and meat from a healthy point of view. Indeed, Δ-9 desaturase is able to introduce a *cis* double bond at carbon 9 position of the fatty acid chain, counting from the Δ-end. Although the C16:0 and C18:0 are the preferential substrates of Δ-9 desaturase, this enzyme is able to insert the *cis* double bond in a large spectrum of methylene-interrupted fatty acyl-CoA (Ntambi, 1999). In the light of this, considering that stearic acid is among the most abundant FA of the rumen outflow, due to the extensive biohydrogenation process of dietary FA, the oleic acid (C18:1 *c*9) is largely produced in muscle and mammary gland. As a consequence, meat and milk from ruminants contain a high concentration of oleic acid. Moreover, the Δ-9 desaturase is able to convert the vaccenic acid (C18:1 *t*11) formed in the rumen into rumenic acid (C18:1 *c*9 *t*11), which is well known to exert favourable effects on human health. The efficiency of Δ-9 desaturase in the conversion of the vaccenic acid into rumenic acid is very high and many studies have shown that up to 90% of the rumenic acid content in the milk and meat is originated by this enzyme (Vasta and Bessa, 2012).

# CHAPTER 3 - Meat oxidative stability

Oxidation of meat is an irreversible and inevitable process that arises under post-mortem conditions. This process leads to a series of changes over time at the expense of the main muscle components, such as proteins and lipids, with the consequent development of off-flavour, formation of toxic compounds and deterioration of colour (Bekhit *et al.,* 2013). Therefore, the oxidative processes are among the major responsibles for the qualitative deterioration of the meat (Falowo *et al.,* 2014). Indeed, the quality from a nutritional point of view is worsened by oxidation due to the loss of polyunsaturated fatty acids, essential amino acids and vitamins, while food safety can be compromised by the production of some harmful compounds (Estévez, 2017).

Oxidative stress is termed as the imbalance between oxidants and/or oxidisable substrates and antioxidants in favour of the former, causing potential damage (Sies, 1997). During normal metabolic processes, a series of aerobic reactions generate free radicals. Among these, reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a crucial role in different homeostatic processes by interacting with nucleic acids, proteins and fatty acids (Falowo *et al.,* 2014). Free radicals are mainly formed in the mitochondria (Cadenas and Davis, 2000) and in normal conditions they are present in minimal concentrations in the organism (El-Aal, 2012). Usually the animal antioxidant defences are able to limit the actions of free radicals *in vivo*, however, when in excess and the activity of antioxidant defence is low (which also occurs obviously under post-mortem

conditions), free radicals could cause damage to cellular components (Morrissey *et al.,* 1998; Falowo *et al.,* 2014), inducing harmful autoimmune responses and causing oxidative damage such as mutagenesis, carcinogenesis and membrane damage.

**Lipid oxidation**

Lipid oxidation is a complex process that involves the formation and propagation of lipid radicals, an absorption of oxygen, a reorganization of double bonds of unsaturated lipids and the possible destruction of membrane lipids, with the subsequent production of several products of decomposition (Repetto *et al.,* 2012). Polyunsaturated fatty acids are the primary target for the lipid oxidation, due to the higher susceptibility of double bonds. As a result, although the phospholipids are in minority compared to triglycerides in muscle, they are much more susceptible to oxidation due to their high content of unsaturated fatty acids (Labuza and Dugan, 1971). Since phospholipids are found mainly in cell membranes, the action of free radicals in these sites can cause alterations of adhesion, fluidity and permeability as well as metabolic functions.

The process of lipid oxidation is generally classified into three stages: initiation, propagation, and termination (Morrissey *et al.,* 1998). The initiation step of lipid oxidation is the abstraction of a hydrogen atom from a methylene carbon in the lipid substrate, resulting in the formation of a highly reactive alkyl radical ($R^{\bullet}$). The latter quickly reacts with the oxygen to form peroxyl radical ($ROO^{\bullet}$) in the propagation phase. This peroxyl radical, in turn, subtracts a hydrogen atom from the

following fatty acid generating hydroperoxides (ROOH), which are the primary oxidation products. Further interactions between hydroperoxides and radicals lead to the formation of secondary products such as aldehydes, ketones, alcohols, hydrocarbons, esters, furans and lactones (Rivas-Cañedo *et al.,* 2013). Alternatively, antioxidant molecules such as α-tocopherol can donate hydrogen to peroxyl radicals, generating in turn a hydroperoxide and an inert α-tocopherol radical (Morrissey *et al.,* 1998). The propagation phase is followed by the termination phase in which the radicals react with themselves to yield in non-radical products (Repetto *et al.,* 2012).

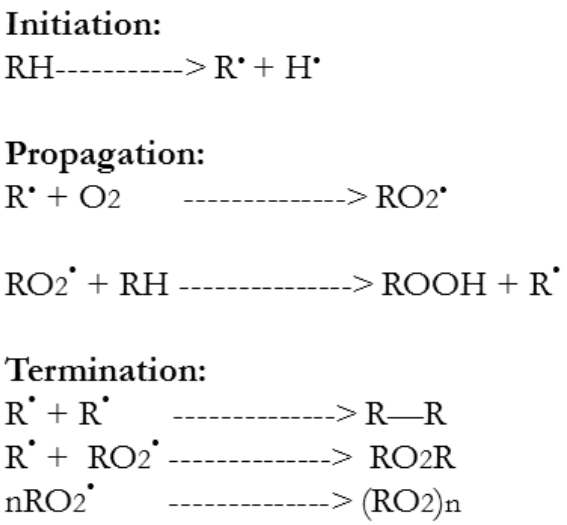


**Figure 8.** Basic steps in the autoxidation of fatty acid (From Rufielyn Gravador PhD Thesis, 2014).

**Protein oxidation**

Although the proteins are also targets of ROS, their oxidation has been much less studied compared to lipids (Lund *et al.,* 2011). However, this process is very important, as it is associated with many negative effects on the quality of meat. Indeed, protein oxidation can lead to the development of off-flavour, discolouration, undesirable textural changes, loss of essential amino acids, decreased water-holding capacity, decreased emulsification capacity and decreased tenderness (Faustman *et al.,* 2010a).

Similarly to lipid oxidation, protein oxidation proceeds through a free radical chain reaction. A protein radical ($P^{\bullet}$) is generated by the removal of the hydrogen atom. In the presence of oxygen, this radical is then converted into a peroxidic radical ($POO^{\bullet}$) and to an alkyl peroxide (POOH) by removing hydrogen atoms from another susceptible molecule. Subsequent reactions with $HO_2^{\bullet}$ cause the formation of alkoxy radicals ($PO^{\bullet}$) and its hydroxyl derivative (POH) (Lund *et al.,* 2011).

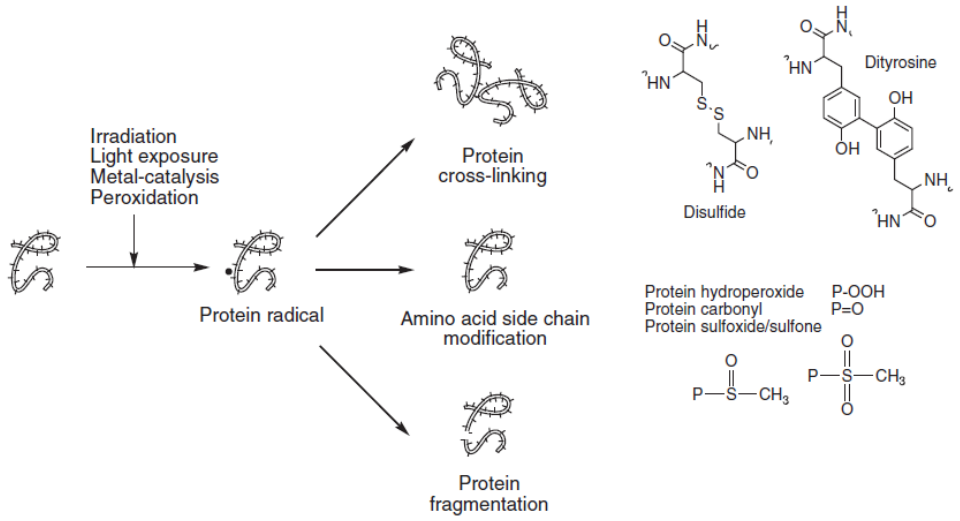


**Figure 9.** The most common consequences of oxidation of proteins (From Lund *et al.,* 2011).

Myoglobin is an essential haem protein in the muscle. This protein is the main pigment of meat, thus influencing its colour. Myoglobin is composed of 153 amino acids and contains a haem group, which holds an iron atom in the centre (Faustman *et al.,* 2010a). This protein can be present in the fresh meat in three different forms: deoxymyoglobin, oxymyoglobin and metmyoglobin (Mancini and Hunt, 2005). After cutting the meat, the myoglobin of the meat surface gets in contact with oxygen, and quickly passes from the deoxymyoglobin to the oxymyoglobin form. As a result, the meat colour changes from a purplish-red to a bright red, which is the most appreciated by the consumer. Subsequently, the oxymyoglobin is slowly oxidized in metmyoglobin, and the meat colour loses its

vividness and attractive appearance, causing a browning of the meat (Mancini and Hunt, 2005; Figure 10).

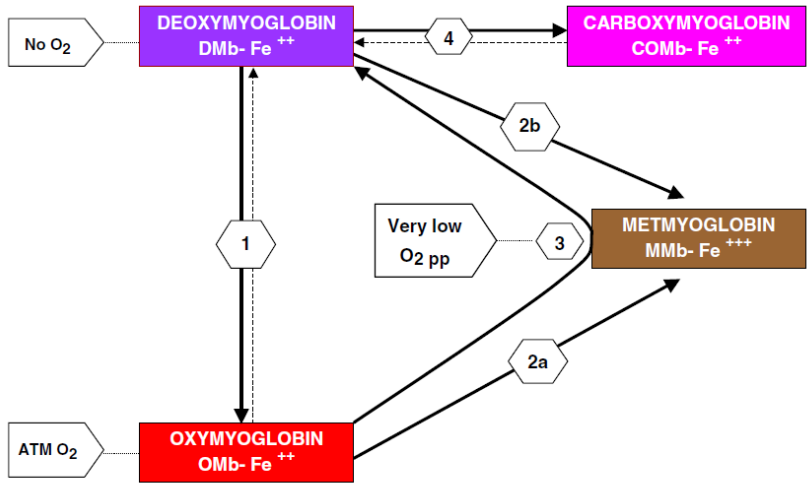


**Figure 10.** Visible myoglobin redox interconversions on the surface of meat (From Mancini and Hunt, 2005).

**Biological defence systems against oxidation.**

The animal organism has a series of defences against the action of free radicals to try to keep the oxidative balance stable and avoid oxidative stress (Sies, 1993). The most relevant groups of antioxidants in muscle can be divided into enzymatic and non-enzymatic compounds. Antioxidant enzymes include superoxide dismutase, glutathione peroxidase and catalase, which continue to perform their function also post-mortem. Superoxide dismutase (SOD) is

responsible for the conversion of superoxide radicals ($O_2$-$^{\bullet}$) in non-reactive species. Glutathione peroxidase converts hydrogen peroxide into two molecules of water, inactivating the oxidative process. Catalase is located mainly in the peroxisomes and mitochondria and it is able to catalyse the decomposition of $H_2O_2$ to $H_2O$ (Yu, 1994).

Non-enzymatic antioxidants are a heterogeneous group of molecules capable of capturing free radicals causing less harmful chemical species or non-reactive radicals. Thus, the non-enzymatic antioxidant system includes a long series of low molecular weight compounds, the most important being glutathione and vitamins E and C. In addition, there are other compounds such as phenolic compounds, β-carotene, lycopene and ubiquinols (Sies, 1993). These all of those compounds are able to donates hydrogen to a peroxyl radical, thus interrupting the propagation of lipid oxidation. These molecules are often of dietary origin and carry out their antioxidant action animal tissue at very low concentrations. For instance, the vitamin E reacts about 104 quicker than the propagation reaction of lipid oxidation (Morrissey *et al.,* 1998), which means that vitamin E is able to inactivate the peroxyl radicals much faster than the latter can react with other fatty acids.

In post-mortem conditions, several biochemical processes occur in the muscle, converting the latter into meat. However, during these processes many of the enzymatic antioxidant defences are altered, causing conditions in which oxidative stress is not as adequately balanced as in a living organism (Buckley *et al.,* 1995). In this context, an adequate concentration of non-enzymatic exogenous

antioxidants, that could be increased with the diet, plays a predominant role in protecting meat against oxidation.

# CHAPTER 4 - Feeding strategies on lipid metabolism and meat oxidative stability

The nutritional composition of meat is strongly connected with the animal diet and different feeding strategies have been proposed with the aim of improving the nutritional value of meat and milk, as well as their shelf-life. The ruminant diets play an important role in the modulation of the lipid metabolism (Toral *et al.,* 2018) and the regulation of the balance between pro-oxidants and antioxidants, which then affect the oxidative stability (Bekhit *et al.,* 2013).

**Modulation of FA metabolism**

In recent years the scientific community has been very focused on improving the FA profile of ruminant products. The reduction of SFA, the increase in PUFA and in particular the *n*-3 PUFA at the expense of *n*-6 PUFA, the increase of vaccenic and rumenic acids are among the goals most pursued by animal nutritionists.

Many of these objectives were successfully achieved through the use of PUFA-rich lipid feeds such as vegetable oils, forage, marine and fish oils and lipid-rich seeds (Scollan *et al.,* 2014; Shingfield *et al.,* 2013; Toral *et al.,* 2018). Although some of these oils also have influences on the rumen bacterial community, these strategies are mainly based on the supplementation of high levels of PUFA, which subsequently ends up enriching the ruminant-derived products. However, unlike monogastrics, this approach is not always successful in ruminants due to the

extensive biohydrogenation processes occurring in the rumen (Vahmani *et al.,* 2015).

Instead, a different approach is to alter the ruminal biohydrogenation process, trying to inhibit the action of microorganisms on the whole BH process or in some particular phases. In this regard, a wide variety of plant secondary compounds, such as tannins, saponins, polyphenol oxidase, essential oils and oxygenated FA, have been studied both *in vitro* and *in vivo* with the purpose of modulating biohydrogenation (Toral *et al.,* 2018). Polyphenol oxidase seems to impair the lipolysis phase due to the formation of protein-phenol complexes, which incorporate the lipids and making them unavailable for lipolysis and subsequent BH (Vasta and Bessa, 2012).

Among the phenolic compounds, the tannins have been very studied in the last 10 years for their antibacterial properties, which could modulate the rumen BH. Although their ability to influence the rumen microorganism population is well known, their mechanism of action and the affected BH phase is still controversial (Toral *et al.,* 2018). For instance, pioneer studies (Vasta *et al.,* 2009; Khiaosa-ard *et al.,* 2009) reported an inhibiting action on the last step of BH, where the vaccenic acid is converted into stearic acid. As a result, the formation of stearic acid was reduced while the vaccenic acid was accumulated in the rumen content. On the other hand, subsequent studies have observed an inhibition of the early stages or a general depression of the BH process, reducing the biohydrogenation of the dietary PUFA (Carreño *et al.,* 2015; Campidonico *et al.,* 2016; Costa *et al.,* 2017).

These contradictory results can certainly be due to the different chemical characteristics of these compounds (e.g., condensed or hydrolysable tannins), but also to the interaction with the basal diet (Vasta *et al.,* 2009; Alves *et al.,* 2017), the dosage used (Carreño *et al.,* 2015) or the type of experimental approach (i.e., *in vitro* vs *in vivo*; Toral *et al.,* 2018).

**Feeding on meat oxidative stability**

The oxidative stability of the meat is also influenced by the diet and several dietary strategies have been proposed to increase the resistance of meat to oxidative processes (Vasta and Luciano, 2011; Scollan *et al.,* 2014). However, feeding strategies designed to increase PUFA (as mentioned above) often have a negative impact on the shelf life of meat. Indeed, polyunsaturated fatty acids are the primary substrate of lipid oxidation in meat. Therefore, if diets are not adequately balanced with antioxidants, these strategies aimed at improving the healthiness of FA lead to a faster deterioration of meat (Scollan *et al.,* 2014).

Tocopherols and tocotrienols (i.e., vitamin E) are the main fat-soluble antioxidant vitamins, which act both in live animals and in post-mortem conditions (Bellés *et al.,* 2019). It was well established that the dietary administration of vitamin E promotes the deposition of tocopherol in the muscle, increasing the resistance of meat to oxidative deterioration (Bellés *et al.,* 2019). In particular, among the eight isomers of tocopherols and tocotrienols, the α-tocopherol possesses the highest biological activity (Descalzo and Sancho, 2008).

In addition to vitamin E, other natural antioxidants have been investigated in animal feeding looking at the effects on oxidative stability (Vasta and Luciano, 2011). Phenolic compounds are certainly part of this class given their pronounced antioxidant properties. They are one of the most ubiquitous group of plant secondary compounds, which range from simple molecules (e.g., phenolic acids and flavonoids) to the highly polymerized compounds, such as tannins (Bravo, 1998). Promising results have been found with administration of quebracho (*Schinopsis lorentii* L.) tannin extract. For instance, Luciano *et al.* (2009; 2011) observed an improvement of colour stability of fresh meat from lambs fed a concentrate supplemented with 8.96% DM of tannin extract. While, Morán *et al.* (2012) enriched a lamb concentrate with carnosic acid (phenols present in rosemary plant) and noted a delay in lipid oxidation of meat. Conversely, in a recent study, no effects on colour and lipid stability of meat were observed when three different tannin extracts were included in a lamb diet (Valenti *et al.,* 2019b). In this study lambs were not challenged with any oxidative stressors (e.g., heat stress, PUFA-rich diet. Etc.) and this could partially explain the lack of appreciable antioxidant effect of dietary tannins. Indeed, Liu *et al.* (2016) reported that dietary chestnut tannin extract improved oxidative stability of meat only when lambs were subjected to heat stress. Similarly, no antioxidant effects were observed in late stage lactation ewes after dietary supplementation of quebracho or chestnut extracts (Buccioni *et al.,* 2017).

However, when talking about phenolic compounds in ruminant feeding, the results must always be taken with caution. In fact, the great chemical heterogeneity of these compounds can certainly influence the observable effects. In addition, the

chemical characteristics and even more the size of the molecules greatly affect the *in vivo* bioavailability of these compounds. As reviewed by Vasta and Luciano (2011), it seems that the bioavailability is limited by the size of phenols molecules, as the small molecules are easily absorbed via the intestine, while the bigger polymers are not metabolised.

Furthermore, the interaction with the basal diet, the animal species and the dose are certainly several factors that must be considered to reach clearer conclusions on the effects of phenolic compounds in ruminant feeding.

# SECTION II – Experimental part

# Objectives

On the basis of the general section (above), the present thesis was designed to provide insight on the use of the pomegranate by-product in ruminant feeding, with particular attention to the nutritional and commercial (shelf-life) quality of meat, considering the growing availability of these by-products and the growing attention of the consumer to the health aspects.

To achieve this general purpose, we planned an experimental trial with lambs used as a ruminant model, although the comparison with other ruminant species must always be done with caution.

Considering the notable bioactive compounds of pomegranate waste and supported by the scientific achievements that these substances could have beneficial effects on the animal organism, we hypothesized that the inclusion of whole pomegranate by-product in a lamb diet would modulate the rumen biohydrogenation due to the simultaneous administration of a high level of PUFA (i.e., pomegranate CLnA) and a considerable amount of phenolic compounds (mainly tannins). Furthermore, we also hypothesized that the remarkable concentration of antioxidant molecules (i.e., vitamin, phenols, etc.) present in this by-product may affect the balance that regulates oxidative stability of meat stored under retail conditions.

To our knowledge, there are no studies in literature that have evaluated the effects of the whole pomegranate by-product, which includes all the fruit components residual from the production of juice (i.e., seeds and peels), on lipid metabolism and meat oxidative stability.

In addition, three other aspects must be mentioned: (i) the pomegranate by-product (like all by-products in general) is a cheaper resource to partially replace the conventional feedstuffs used in ruminant rations, therefore reducing the feeding cost. (ii) The conventional feed used in animal diets are often edible for humans. Thus, decreasing them in the animal diet reduces the competition between feed and food, which is becoming increasingly important in view of population growth. (iii) Agro-industrial by-products represent a cost for the industries and, if not properly disposed, they could have a negative impact on the environment.

In this thesis, the experimental part includes three chapters, which had the following specific objectives:

**Chapter 5 –** Feeding lambs with a concentrate diet supplemented with whole pomegranate by-product at 200 g/kg DM, in order to investigate the fatty acid metabolism, analysing the three main districts involved in lipid modifications (i.e., rumen, liver and muscle). The specific objective was to evaluate whether the bioactive compounds of the pomegranate influenced lipid metabolism, with consequent improvements of the profile of fatty acids in meat.

**Chapter 6 -** After the promising results obtained in the first experiment (Chapter 5), we wanted to understand which bioactive substance had most contributed to our results or whether there was a synergistic or additive effect. Therefore, this study was conducted to better understand the mechanisms and

contribution of each of these bioactive compounds. To this aim, two *in vitro* experiments were carried out in parallel with the main objectives of assessing the effects of pomegranate oil and tannins on the rumen biohydrogenation of dietary FA (Experiment 1), and evaluating the consequences of different pomegranate by-products on the processes of ruminal fermentation and biohydrogenation (Experiment 2).

**Chapter 7 -** This experiment was designed to evaluate the effects of the dietary whole pomegranate by-product on the oxidative stability of meat. To this purpose, lipid oxidation, colour stability and myoglobin formation were analysed in meat stored in retail conditions, using the meat samples obtained from the *in vivo* experiment (Chapter 5). In addition, to investigate the main factors involved in meat oxidative processes, vitamin E and the antioxidant capacity of the lipophilic and hydrophilic fractions of the muscle were assessed.

# CHAPTER 5 - Effect of feeding pomegranate by-product on fatty acid composition of ruminal digesta, liver and muscle in lambs

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**Abstract**

This work investigated the effects of feeding whole pomegranate by-product (WPB) to lambs on ruminal, liver and intramuscular fatty acids (FA). Seventeen lambs, divided into two groups, were fed for 36 days with a cereal-based concentrate diet (CON) or with a concentrate diet containing 200 g/kg DM of WPB to partially replace barley and corn (WPB).

The dietary treatment did not affect the final body and carcass weight, the dry matter intake or the average daily gain. However, total polyunsaturated FA (PUFA), linolenic, rumenic (RA) and vaccenic (VA) acid were increased in liver (+15%, +32%, +344% and +118%, respectively) and muscle (+46%, +38%, +169% and +89%, respectively) of WPB lambs ($P < 0.05$). Punicic acid and three isomers of conjugated linolenic acid were detected exclusively in the rumen and tissues of WPB-lambs. The C18:1 *t*10/*t*11 ratio in rumen digesta or in tissues was reduced by feeding WPB (-791%, -690% and -456%, respectively in rumen, liver and muscle; $P < 0.001$), suggesting that the WPB prevented the *t*10-shift rumen biohydrogenation pathway. In conclusion, the inclusion of WPB into a concentrate-based diet can be a strategy to improve the FA composition of meat, without effects on the animal performances.

***Key words:*** *pomegranate, rumen, liver, meat quality, fatty acid metabolism, conjugated linoleic and linolenic acids*

## 1. Introduction

In the last decade there has been an enormous increase in the demand of pomegranate fruits (*Punica granatum* L.) (Shaani *et al.,* 2016), especially destined to juice production, due to the recognition of its beneficial effects on human health (Lasky and Newman, 2007). The current increasing trend in the industrial production of pomegranate juice has led to great amounts of residual biomasses. The primary by-product is the whole pomegranate by-product (WPB), which contains peels, membranes, seeds and residual arils pulp. The WPB is produced in massive amounts in many parts of the world and, if not destined to other uses, its disposal as a waste represents a cost for the processing industries. However, WPB is a natural source of bioactive compounds. Specifically, pomegranate peel is rich in phenolic compounds, such as flavonoids, anthocyanidins and tannins, with the latter being mostly represented by ellagitannins (Lasky and Newman, 2007). These compounds have been shown to possess antimicrobial, antioxidant, anti-inflammatory, antitumoral and immunomodulatory properties both *in vivo* and *in vitro* (Viuda-Martos *et al.,* 2010). Also, pomegranate seeds contain variable amounts of oil ranging from 6 to 24%, and approximately 80% of the fatty acids (FA) in pomegranate oil are conjugated linolenic acids (CLnA), characterized by the occurrence of three double bonds conjugated together. Punicic acid (C18:3 *c*9 *t*11 *c*13; PA), in particular, is the predominant CLnA in the pomegranate seed oil, where it can represent more than 70% of total fatty acids (Aruna *et al.,* 2016). Other CLnA isomers, such as α-eleostearic acid (C18:3 *c*9 *t*11 *t*13; α-ESA), catalpic acid (C18:3 *t*9 *t*11 *c*13; CA) and β-eleostearic acid (C18:3 *t*9 *t*11 *t*13; β-ESA), occur at

lower concentrations in pomegranate seed oil (Kýralan *et al.,* 2009; Sassano *et al.,* 2009). All these fatty acids are among the few CLnA found in nature. *In vivo* and *in vitro* studies showed that CLnA isomers can exert several health benefits in humans (Yuan *et al.,* 2014). Tsuzuki *et al.* (2004) reported that CLnA would have a stronger antitumor propriety than conjugated linoleic acid (CLA). Furthermore, studies have demonstrated that CLnA isomers can be converted to *c*9 *t*11 CLA (rumenic acid; RA) in rat tissues (Tsuzuki *et al.,* 2004; Yuan *et al.,* 2009) and in *in vitro* cell cultures (Schneider *et al.,* 2013).

The fatty acid composition of ruminant meat and milk is mostly determined by complex interactions between dietary factors and rumen metabolism (Harfoot and Hazlewood, 1997). Several studies have focused on possible feeding strategies to increase the content of polyunsaturated fatty acids (PUFA) and CLA in meat and milk, via the manipulation of the ruminal biohydrogenation (BH). Diets rich in PUFA are certainly among the most effective feeding strategies to achieve this objective. Indeed, it has been well documented that diets enriched of unsaturated FA increase the amount of PUFA and other BH intermediates escaping the complete saturation in the rumen (Scollan *et al.,* 2006). Also, the inclusion of phenolic compounds in the ruminant diets is being investigated, as it has been demonstrated that dietary phenolic compounds affect the ruminal microbial community (Vasta and Luciano, 2011) and lead to alterations of the ruminal BH with the consequent accumulation of unsaturated FA.

In the light of all above, WPB could be usefully included in animal diet to increase the deposition of desirable fatty acids in meat. Indeed, some studies have

demonstrated that feeding ruminants with pomegranate by-products, such as seed pulp, increased the amount of PUFA and RA in meat (Emami *et al.,* 2015) and milk (Modaresi *et al.,* 2011; Razzaghi *et al.,* 2015). To our knowledge, only ensiled WPB was tested in milk (Kotsampasi *et al.,* 2017) and meat production (Kotsampasi *et al.,* 2014), with controversial results on fat composition, and no studies have investigated in depth the effects of pomegranate by-products on ruminant lipid metabolism. As WPB contains both CLnA and tannins, different mechanisms might explain its effect in increasing the deposition of PUFA and RA in meat. Such mechanisms, alone or in combination, may involve the reduction of the ruminal BH extent, as well as the direct conversion of punicic acid into RA both in the rumen and in muscle. For these reasons, it would be of interest to get a deeper insight on the effects of pomegranate by-products on the lipid metabolism in ruminants. Therefore, here we have investigated, for the first time, the effect of dietary dried whole pomegranate by-product on the composition of fatty acids in lamb ruminal digesta, liver and muscle, which represent the main districts involved in lipid metabolism.

## 2. Materials and methods

The trial was conducted between October and December 2016 at the experimental farm of the University of Catania (37°24’35.3’’N 15°03’34.9’’E). All experimental procedures were accomplished following the European Union Guidelines (2010/63/ EU Directive) and according to the protocol approved by the Universities of Catania and Perugia.

*2.1. Whole pomegranate by-product*

Fresh WPB was obtained from a local juice manufacturing company (Catania, Sicily, Italy). At the factory, the pomegranate fruits (Wonderful variety) were halved and squeezed mechanically. The residual part containing peel, 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. Approximately 300 kg of dried biomass was obtained for the use in the feeding trial. This product is referred to as "whole pomegranate by-product" (WPB) throughout the article.

*2.2. Experimental Design*

Seventeen Comisana male lambs, born within 10, were selected. At 60 days of age, lambs were weighed (initial body weight 14.82 ± SD 2 kg) and individually penned indoor. Animals were randomly assigned to two dietary treatments balanced for the body weight. After 8 days of adaptation period, where pre-experimental concentrate was gradually replaced with the experimental diets, lambs were fed for 36 days with barley-corn based concentrate diet (CON, 8 lambs) or a concentrate diet containing 200 g/kg DM of WPB to partially replace barley and corn (WPB, 9 lambs). The table 1.1 reports the ingredients and the chemical composition of the experimental diets, which were planned to provide similar levels of energy and nitrogen. 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. During the experimental period all the lambs were fed *ad libitum* with their respective diets (10% minimum amount of residual allowed). For each

lamb, offered and refused feed was recorded every day to calculate dry matter intake (DMI). Water was continuously available. From the beginning of the trial, the body weight was measured weekly in order to calculate average daily gain (ADG).

*2.3. Slaughter Procedure and Samplings*

At the end of the trial, all the lambs were slaughtered on the same day at a commercial slaughterhouse according to the European Union welfare guidelines. The experimental diet and water were available to lambs until around 3 h before slaughter. Lambs were firstly stunned by a captive bolt then exsanguinated. Individual ruminal content was sampled within 15 min from butchery. After cutting the ruminal wall with a scalpel, the whole rumen content was placed into a 4-L-plastic beaker and homogenized with a ladle. Ruminal pH measurement was performed by a pH meter (HI-110; Hanna Instruments, Padova, Italy). An aliquot of approximately 120 mL of rumen content was immediately placed in dry ice prior to storage at -80 °C. Liver samples were collected immediately after slaughter, vacuum-packed and stored at -80 °C. Each carcass was immediately weighted and stored at 4° C for 24 h. Thereafter the entire *longissimus thoracis et lumborum* muscle (LTL) was removed from the right side of each halved carcass, packed under vacuum and stored at -80 °C.

**Table 1.1.** Ingredients and chemical composition of the experimental diets.

| | Experimental diet[a] | |
|---|---|---|
| | CON | WPB |
| Ingredient, g/kg of dry matter | | |
| Corn | 226 | 116 |
| Barley | 226 | 116 |
| Alfalfa hay | 198 | 198 |
| Wheat bran | 200 | 200 |
| Soybean meal | 120 | 140 |
| Whole pomegranate by-product | 0 | 200 |
| Molasses | 9 | 9 |
| Mineral mix[b] | 21 | 21 |
| Chemical composition, g/kg DM | | |
| Dry matter (DM), g/kg as fed | 887 | 892 |
| Crude Protein | 176 | 178 |
| NDF | 233 | 263 |
| ADF | 129 | 155 |
| ADL | 29.8 | 27.0 |
| Ash | 58.7 | 44.0 |
| Crude Fat | 21.1 | 25.1 |
| Metabolizable Energy[c] | 10.6 | 10.3 |
| Total phenols[d] | 3.04 | 18.9 |
| Total tannins[d] | 1.41 | 17.0 |
| Vitamin E (α-tocopherol) | 7.82 | 16.8 |
| Total fatty acids (FA) | 13.1 | 15.5 |
| Individual FA, g/100 g total FA | | |
| C14:0 | 0.28 | 0.17 |
| C16:0 | 17.3 | 14.0 |
| C18:0 | 2.36 | 2.11 |
| C18:1 *c*9 | 17.6 | 12.5 |
| C18:2 *c*9 *c*12 | 42.1 | 32.5 |
| C18:3 *c*9 *c*12 *c*15 (α-LnA[e]) | 5.11 | 5.09 |
| C18:3 *c*9 *t*11 *c*13 (PA[e]) | - | 11.1 |
| C18:3 *c*9 *t*11 *t*13 (α-ESO[e]) | - | 1.03 |
| C18:3 *t*9 *t*11 *c*13 (CA[e]) | - | 1.38 |
| C18:3 *t*9 *t*11 *t*13 (β-ESO[e]) | - | 0.62 |

[a] CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product.

[b] Containing: 25% Calcium carbonate, 25% Sodium bicarbonate, 25% Bicalcic phosphate and 25% Sodium chloride
[c] Mj/kg DM Estimated using Feed Tag (University of California, Davis, CA, USA).
[d] Expressed as g tannic acid equivalents/kg DM
[e] α-LnA, α-linolenic acid; PA, punicic acid; α-ESO, α-eleostearic acid; CA, catalpic acid; β-ESO, β-eleostearic acid.

*2.4. Feedstuffs analyses*

Samples of the feedstuffs were collected at the beginning, middle, and end of the experimental period, vacuum-packed and stored at -30 °C. Feed sample for analysis was obtained by mixing equal amounts the collected subsamples during the trial. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined according to the method described by Van Soest *et al.* (1991). Furthermore, crude protein, crude fat (ether extract) and ash were analyzed according to the AOAC methods (1995).

Total phenolic compounds and total tannins in the feeds were analyzed following the procedure originally described by Makkar *et al.* (1993), with modifications as follows. Briefly, finely ground feeds (200 mg) were first extracted with acetone 70% (v/v) in a sonicating water-bath at 4 °C for 15 min. Following centrifugation, the supernatant was collected, and the residual solid pellet was re-extracted using methanol 80% (v/v) in a sonicating water-bath at 4 °C for 15 min. The supernatants were combined, solvents were evaporated using a rotary evaporator system (Rotavapor R-114, Büchi, Flawil, Switzerland) and the residue was dissolved in methanol 70% (v/v). As described by Makkar *et al.* (1993), total phenolic compounds were determined using Folin-Ciocalteu reagent (1N) and sodium carbonate 20% (w/v) via a 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). Tannin concentration 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 curves in order to quantify phenolic compounds, which were expressed as g TA equivalents / kg dry matter.

Vitamin E (α-tocopherol) was extracted and quantified as reported by Valenti *et al.* (2018). Briefly, α-tocopherol was extracted 3 times using hexane/ethyl acetate (9/1, v/v), after saponification with KOH (60%) at 70 °C for 30 min. The extracted solution was dried under nitrogen, dissolved with acetonitrile and 50 µL injected via an autosampler (Jasco, model AS 950–10, Tokyo, Japan) into a HPLC system (pump model Perkin Elmer series 200, Norwalk, CT, USA). A Synergy Hydro-RP column (4 µm, 4.6×100 mm; Phenomenex, Bologna, Italy) was fitted to the HPLC. The sample was run in isocratic condition with a flow rate of 2 ml/min and a mobile phase prepared with: acetonitrile/methanol/tetrahydrofuran/ 1% ammonium acetate (68/22/7/3, v/v/v/v). α-tocopherol was identified using a fluorescence detector (Jasco, model FP-1525, Tokyo, Japan) set at excitation and emission wavelengths of 295 nm and 328 nm, respectively. Quantification was achieved through an external calibration curves with increasing amounts of pure standard compounds (Sigma-Aldrich, Bornem, Belgium).

*2.5. Fatty Acid Analyses*

Fatty acids were extracted from 200 mg of freeze-dried sample of the experimental diets and converted to fatty acid methyl esters (FAME) with a 1-step procedure using chloroform and 2% (v/v) sulfuric acid in methanol (Shingfield *et al.,* 2003) and nonadecanoic acid (Sigma-Aldrich, St. Louis, MO, USA) as an internal standard.

Rumen digesta FA were directly converted to fatty acid methyl esters (FAME) by combining basic and acid methylation as described by Alves *et al.* (2013), with some modifications to prevent the isomerization of CLnA. Briefly, rumen digesta samples were freeze-dried (Christ, alpha 2–4 LD plus, Osterode am Harz, Germany) and 500 mg were incubated at 50 °C for 10 min with 4 mL of sodium methoxide in methanol (0.5 M). After cooling to room temperature, 6 mL of 3 N HCl solution in methanol was added and the solution was allowed to react for 15 min at 50 °C. Once cooled, 8 mL of 6% aqueous potassium carbonate were added to prevent excessive effervescence, followed by addition of 4 mL of hexane. Following centrifugation, the organic phase was collected, and the extraction step was repeated twice. The final solution was dried over anhydrous sodium sulfate, evaporated under nitrogen at room temperature and resuspended in 1 mL of hexane. The internal standard used was methyl nonadecanoate (1 mg/mL).

Liver fat was extracted from 10 g of tissue with a mixture of chloroform and methanol (2:1, v/v) and 50 mg of lipids were converted to FAME by base catalyzed transesterification, using 1 mL of sodium methoxide in methanol 0.5 M and 2 mL of hexane containing C19:0 as an internal standard (Valenti *et al.,* 2018).

LTL muscle, at the level of the 13th thoracic rib, was deprived of any visible external fat and 10 g were used to obtain the FAME as described above for the liver.

Fatty acid methyl esters, obtained from feeds, rumen digesta, liver and muscle samples, were separated and quantified using a gas chromatograph (ThermoQuest, Milan, Italy) equipped with a flame ionization detector (FID) and a 100-m high-polar fused silica capillary column (25 mm i.d., 0.25-μm film thickness; SP. 24056; Supelco Inc., Bellefonte, PA). One μL of sample was injected, split at a ratio of 1:80 and carried by a constant flow (1 mL/min) of helium. The oven temperature was set at 50 °C and held for 4 min, then increased to 120 °C at 10 °C/min, held for 1 min, then increased up to 180 °C at 5 °C/min, held for 18 min, then increased up to 200 °C at 2 °C/min, held for 15 min, and then increased up to 230 °C at 2 °C/min, held for 19 min. The temperature of injector and detector was set at 270 °C and 300 °C, respectively. FAME was identified by comparison with the retention times of a commercial mixture of standard FAME (GLC-674, Nu-Chek Prep Inc., Elysian, MN, USA), individual standard FAMEs (21-1615; 21-1614; 21-1413; 21-1412; 20-1823; Larodan Fine Chemicals, Malmo, Sweden) and with the chromatograms published by Alves and Bessa (2007) and Kramer *et al.* (2008). Punicic acid was identified by comparing retention time with the pure standard compound (10-1875; Larodan Fine Chemicals, Malmo, Sweden). The identification of the other CLnA isomers was based on the comparison between published chromatograms of pomegranate seed oil (Sassano *et al.,* 2009) and the chromatograms of a commercial pomegranate seed oil analyzed in our laboratory both under the same conditions reported by Sassano *et al.* (2009) and the GC

conditions described above for the present study. Fatty acids were expressed as g/100g of total fatty acids. The biohydrogenation indexes (%) for C18:1 *c*9, C18:2 *c*9 *c*12, α-LnA and PA, and the biohydrogenation completeness (%) in rumen digesta were estimated as reported by Alves *et al.* (2017).

*2.6. Statistical Analysis*

All data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC), with the dietary treatment as the main effect. Data were reported as least-squared means and standard error of means (SEM). Significance was declared at $P \leq 0.05$, while trends toward significance were considered when $0.05 < P \leq 0.10$.

**3. Results**

*3.1. Animals Performance and Intakes*

As shown in Table 1.2, no effect of the dietary treatment was found on the final body and carcass weight, dry matter intake (DMI) and average daily gain (ADG). The intake of NDF was greater for lambs in the WPB group, compared to the control group ($P < 0.001$). Moreover, WPB-fed lambs ingested a greater quantity of total phenols, total tannins and Vitamin E as compared to CON lambs ($P < 0.001$). The daily intake of total FA and stearic acid (C18:0) was higher ($P < 0.001$ and $P = 0.019$, respectively) in WPB group than CON group. The intake of oleic acid (C18:1 *c*9) was greater ($P = 0.024$) in the control group, whereas a comparable intake of linoleic acid (LA; C18:2 *c*9 *c*12) was observed between groups ($P = 0.547$). The intake of α-linolenic acid (α-LnA; C18:3 *c*9 *c*12 *c*15) resulted greater ($P < 0.001$)

for animals in the WPB group compared to those in the CON group. Moreover, a remarkable amount of punicic acid (PA; C18:3 *c*9 *t*11 *c*13) was ingested only by lambs fed the WPB diet (1.52 g/day).

**Table 1.2.** Effect of the dietary treatment on lamb performance and intakes.

| | Dietary treatment[a] | | SEM[b] | *P*-value |
|---|---|---|---|---|
| | CON | WPB | | |
| No. of animals | 8 | 9 | | |
| Final body weight, kg | 23.6 | 23.1 | 0.524 | 0.637 |
| Carcass weight, kg | 11.1 | 10.2 | 0.330 | 0.179 |
| DMI[c], g/day | 821 | 882 | 19.500 | 0.125 |
| ADG[c], g/day | 234 | 235 | 7.200 | 0.921 |
| NDF intake, g/day | 191 | 232 | 6.650 | <0.001 |
| Total phenols intake, g/day | 2.49 | 16.7 | 1.700 | <0.001 |
| Total tannins intake, g/day | 1.16 | 15.0 | 1.660 | <0.001 |
| Vitamin E[d] intake, g/day | 6.42 | 14.8 | 1.300 | <0.001 |
| Total FA[c] intake, g/day | 10.8 | 13.6 | 0.424 | <0.001 |
| C18:0 intake, g/day | 0.26 | 0.29 | 0.007 | 0.019 |
| C18:1 *c*9 intake, g/day | 1.90 | 1.71 | 0.044 | 0.024 |
| C18:2 *c*9 *c*12 intake, g/day | 4.56 | 4.43 | 0.095 | 0.547 |
| C18:3 *c*9 *c*12 *c*15 intake, g/day | 0.55 | 0.69 | 0.022 | <0.001 |
| C18:3 *c*9 *t*11 *c*13 intake, g/day | 0 | 1.52 | 0.181 | - |

[a] CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product.
[b] SEM, standard error of the mean.
[c] DMI, dry matter intake; ADG, average daily gain; FA, Fatty Acids.
[d] α-tocopherol

*3.2. Fatty Acid Composition of Rumen Digesta*

The FA composition of the rumen digesta and the BH indexes are presented in Table 1.3. Feeding the WPB diet tended to increase the sum of saturated fatty

acids (SFA; $P = 0.085$) and to decrease total (MUFA; $P = 0.059$). Among the SFA, the proportion of C14:0 tended to be greater in the rumen content from CON group ($P = 0.093$), while stearic acid (C18:0) tended to be greater in the WBP treatment compared to CON group ($P = 0.073$). The proportion of C24:0 was greater in the CON treatment ($P = 0.007$). Within the MUFA, C16:1 *c*9, C18:1 *c*11, C18:1 *t*6 + *t*7 + *t*8 and C22:1 *c*13 were found at greater proportion in the rumen digesta from the CON lambs compared to the WPB group ($P < 0.05$). Vaccenic acid (VA; C18:1 *t*11) tended to be greater in the rumen digesta of WPB lambs as compared to CON ($P = 0.093$), whereas C18:1 *t*10 was reduced ($P < 0.001$) by feeding lambs the WPB diet. The dietary treatment did not affect the total PUFA in the rumen content ($P > 0.05$), while the inclusion of WPB in the diet increased the RA (C18:2 *c9 t*11) by three times in the rumen of WPB in comparison with the control group ($P = 0.050$). The pomegranate CLnA (PA, α-ESO, CA and β-ESO) were detected only in the rumen of lambs receiving the WPB diet. In relation to the odd- and branched-chain fatty acids (OBCFA), the inclusion of WPB in the diet decreased their total concentration in the rumen digesta ($P < 0.001$). In particular, C15:0, C15:0 *iso*, C15:0 *anteiso*, C17:0 and C17:0 *anteiso* were significantly higher in the rumen content of CON group compared to WPB group ($P < 0.05$). No significant difference was found for the index of BH completeness or for the BH index of individual unsaturated C18:1 fatty acids, with the only exception of α-LnA, which tended to be greater in the WPG group ($P = 0.072$).

**Table 1.3.** Effect of the dietary treatment on pH and fatty acid composition of rumen digesta (g/100 g of total FA)

| | Dietary treatment[a] | | SEM[b] | *P*-value |
|---|---|---|---|---|
| | CON | WPB | | |
| pH | 5.85 | 6.02 | 0.093 | 0.375 |
| Fatty acids | | | | |
| C12:0 | 0.34 | 0.29 | 0.023 | 0.255 |
| C13:0 | 0.10 | 0.08 | 0.009 | 0.186 |
| C13:0 *iso* | 0.03 | 0.03 | 0.002 | 0.199 |
| C14:0 | 0.95 | 0.68 | 0.078 | 0.093 |
| C14:0 *iso* | 0.21 | 0.28 | 0.026 | 0.237 |
| C15:0 | 1.02 | 0.60 | 0.059 | <0.001 |
| C15:0 *iso* | 0.39 | 0.29 | 0.024 | 0.041 |
| C15:0 *anteiso* | 1.98 | 1.00 | 0.170 | 0.002 |
| C16:0 | 16.24 | 15.94 | 0.732 | 0.851 |
| C16:0 *iso* | 0.45 | 0.32 | 0.048 | 0.202 |
| C16:1 *c*9 | 0.18 | 0.10 | 0.016 | 0.007 |
| C17:0 | 0.54 | 0.33 | 0.028 | <0.001 |
| C17:0 *iso* | 0.16 | 0.09 | 0.019 | 0.086 |
| C17:0 *anteiso* | 0.60 | 0.19 | 0.068 | 0.001 |
| C18:0 (SA) | 25.35 | 34.17 | 2.410 | 0.073 |
| C18:1 *c*9 | 9.37 | 7.80 | 0.470 | 0.105 |
| C18:1 *c*11 | 1.21 | 0.60 | 0.099 | 0.001 |
| C18:1 *c*12 | 1.03 | 0.70 | 0.171 | 0.369 |
| C18:1 *c*13 | 0.06 | 0.07 | 0.005 | 0.145 |
| C18:1 *t*5 | 0.08 | 0.05 | 0.007 | 0.090 |
| C18:1 *t*6 + *t*7 + *t*8 | 1.35 | 0.50 | 0.163 | 0.006 |
| C18:1 *t*9 | 0.60 | 0.37 | 0.079 | 0.167 |
| C18:1 *t*10 | 5.47 | 0.83 | 0.745 | <0.001 |
| C18:1 *t*11 (VA) | 4.35 | 6.51 | 0.626 | 0.093 |
| C18:2 *c*9 *c*12 | 12.30 | 9.68 | 0.781 | 0.103 |
| C18:2 *c*9 *t*11 (RA) | 0.60 | 2.07 | 0.376 | 0.050 |
| C18:2 *t*9 *c*12 | 0.28 | 0.29 | 0.017 | 0.889 |
| C18:3 *c*9 *c*12 *c*15 (α-LnA) | 1.35 | 1.18 | 0.082 | 0.334 |

(*continued*)

**Table 1.3.** (*continued*)

| | Dietary treatment[a] | | SEM[b] | *P*-value |
|---|---|---|---|---|
| | CON | WPB | | |
| C18:3 *c*9 *t*11 *c*13 (PA) | n.d. | 1.20 | 0.165 | - |
| C18:3 *c*9 *t*11 *t*13 (α-ESO) | n.d. | 0.51 | 0.069 | - |
| C18:3 *t*9 *t*11 *c*13 (CA) | n.d. | 0.99 | 0.131 | - |
| C18:3 *t*9 *t*11 *t*13 (β-ESO) | n.d. | 0.91 | 0.107 | - |
| C20:0 | 0.58 | 0.63 | 0.024 | 0.368 |
| C20:1 *c*11 | 0.32 | 0.24 | 0.020 | 0.057 |
| C20:4 *n*-6 | 0.07 | 0.06 | 0.002 | 0.276 |
| C21:0 | 0.11 | 0.11 | 0.010 | 0.813 |
| C22:0 | 0.39 | 0.38 | 0.009 | 0.544 |
| C22:1 *c*13 | 0.16 | 0.07 | 0.013 | <0.001 |
| C23:0 | 0.14 | 0.13 | 0.004 | 0.062 |
| C24:0 | 0.42 | 0.35 | 0.013 | 0.007 |
| SFA | 44.27 | 52.44 | 2.310 | 0.085 |
| MUFA | 27.27 | 20.34 | 1.810 | 0.059 |
| OBCFA | 5.82 | 3.58 | 0.301 | <0.001 |
| PUFA | 15.21 | 17.19 | 0.777 | 0.225 |
| Biohydrogenation indexes (%) | | | | |
| C18:1 *c*9 | 44.98 | 41.35 | 3.033 | 0.575 |
| C18:2 *c*9 *c*12 | 70.00 | 72.41 | 1.874 | 0.547 |
| C18:3 *c*9 *c*12 *c*15 (α-LnA) | 72.86 | 78.49 | 1.531 | 0.072 |
| C18:3 *c*9 *t*11 *c*13 (PA) | n.d. | 90.02 | 10.78 | - |
| Completeness (%) | 64.72 | 69.09 | 1.784 | 0.242 |
| SA/(SA+VA) | 0.81 | 0.84 | 0.024 | 0.489 |

[a] CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product.
[b] SEM, standard error of the mean.

### *3.3. Fatty Acid Composition of Liver*

Table 1.4 reports the effect of dietary treatment on the fatty acid composition of liver. Total hepatic fat was not affected by the dietary treatment (*P*

= 0.173). The sum of SFA was comparable between the treatments ($P = 0.384$) while the proportion of C14:0 and C16:0 was greater in the liver of lambs fed the control diet compared to the WPB lambs ($P = 0.025$ and $P = 0.015$, respectively) and the stearic acid (C18:0) was detected at greater concentration in liver from the animals fed the WPB diet compared with animals in the CON group ($P = 0.033$). Dietary WPB decreased the concentration of total MUFA in liver compared to the CON treatment ($P = 0.002$). Regarding the individual MUFA, most were found in a greater proportion in the liver from the CON-fed lambs, except for C18:1 *c*6, C18:1 *c*14, C18:1 *t*9 and VA, which were increased by feeding the WPB diet ($P < 0.05$).

The sum of PUFA was greater when lambs received the WPB diet compared to the control diet ($P < 0.001$). Within the PUFA, the WPB increased the accumulation of RA, LA, C18:2 *c*10 *t*12 and α-LnA in comparison with the CON diet, while the concentration of C18:2 *t*9 *c*13 and C18:3 *c*6 *c*9 *c*12 was lower in liver from the WPB group compared to CON ($P < 0.05$). The accumulation of some long-chain fatty acids (C20:3 *n*-6, C22:2 *n*-6 and C22:5 *n*-3) in the liver was favoured when the animals were fed with the WPB diet ($P < 0.05$). The CLnA isomers (PA, α-ESO, CA and β-ESO) were detected only in the liver of lambs receiving the WPB diet. The WPB diet reduced the concentration of total OBCFA in the liver compared to the CON treatment ($P < 0.001$). In particular, the concentration of C15:0, C15:0 *iso,* C15:0 *anteiso*¸ C17:0, C17:0 *anteiso* and C21:0 was greater found in liver from lambs fed the CON diet ($P < 0.05$).

**Table 1.4**. Effect of the dietary treatment on total hepatic fat (g/100 g of liver) and fatty acid composition of liver (g/100 g of total FA).

| | Dietary treatment[a] | | SEM[b] | *P*-value |
|---|---|---|---|---|
| | CON | WPB | | |
| Total hepatic fat | 3.67 | 3.85 | 0.064 | 0.173 |
| C12:0 | 0.02 | 0.02 | 0.004 | 0.648 |
| C14:0 | 0.53 | 0.42 | 0.025 | 0.024 |
| C14:0 *iso* | 0.03 | 0.03 | 0.003 | 0.619 |
| C14:1 *c*9 | 0.02 | <0.001 | 0.002 | 0.018 |
| C15:0 | 0.63 | 0.37 | 0.047 | 0.003 |
| C15:0 *iso* | 0.12 | 0.07 | 0.010 | 0.024 |
| C15:0 *anteiso* | 0.23 | 0.11 | 0.022 | 0.005 |
| C16:0 | 13.59 | 12.37 | 0.256 | 0.015 |
| C16:0 *iso* | 0.22 | 0.19 | 0.016 | 0.317 |
| C16:1 *c*7 | 0.40 | 0.32 | 0.013 | 0.001 |
| C16:1 *c*9 | 0.93 | 0.46 | 0.070 | <0.001 |
| C17:0 | 2.33 | 1.36 | 0.153 | <0.001 |
| C17:0 *iso* | 0.54 | 0.40 | 0.039 | 0.079 |
| C17:0 *anteiso* | 0.94 | 0.56 | 0.061 | <0.001 |
| C17:1 *c*9 | 0.79 | 0.32 | 0.063 | <0.001 |
| C18:0 (SA) | 21.08 | 23.16 | 0.486 | 0.033 |
| C18:1 *c*6 | 0.79 | 0.98 | 0.041 | 0.022 |
| C18:1 *c*9 | 14.82 | 12.95 | 0.422 | 0.026 |
| C18:1 *c*11 | 1.53 | 0.95 | 0.080 | <0.001 |
| C18:1 *c*12 | 0.65 | 0.60 | 0.055 | 0.697 |
| C18:1 *c*13 | 0.11 | 0.09 | 0.005 | 0.100 |
| C18:1 *c*14 | 0.30 | 0.41 | 0.017 | 0.001 |
| C18:1 *t*5 | 0.06 | 0.04 | 0.004 | 0.021 |
| C18:1 *t*6 | 0.32 | 0.18 | 0.035 | 0.062 |
| C18:1 *t*9 | 0.46 | 0.62 | 0.033 | 0.015 |
| C18:1 *t*10 | 1.79 | 0.46 | 0.240 | 0.003 |
| C18:1 *t*11 (VA) | 0.85 | 1.85 | 0.204 | 0.012 |
| C18:2 *c*9 *c*12 | 11.28 | 12.59 | 0.274 | 0.015 |
| C18:2 *c*9 *t*11 (RA) | 0.50 | 2.22 | 0.224 | <0.001 |

(*continued*)

**Table 1.4.** (*continued*)

| | Dietary treatment[a] | | SEM[b] | *P*-value |
|---|---|---|---|---|
| | CON | WPB | | |
| C18:2 *c*10 *t*12 | 0.05 | 0.06 | 0.008 | <0.001 |
| C18:2 *t*8 *c*13 | 0.19 | 0.20 | 0.010 | 0.565 |
| C18:2 *t*9 *c*12 | 0.13 | 0.11 | 0.006 | 0.225 |
| C18:2 *t*9 *c*13 | 0.19 | 0.10 | 0.016 | 0.002 |
| C18:3 *c*6 *c*9 *c*12 | 0.32 | 0.24 | 0.017 | 0.018 |
| C18:3 *c*9 *c*12 *c*15 (α-LnA) | 0.50 | 0.66 | 0.027 | 0.002 |
| C18:3 *c*9 *t*11 *c*13 (PA) | n.d. | 0.53 | 0.071 | - |
| C18:3 *c*9 *t*11 *t*13 (α-ESO) | n.d. | 0.12 | 0.015 | - |
| C18:3 *t*9 *t*11 *c*13 (CA) | n.d. | 0.02 | 0.003 | - |
| C18:3 *t*9 *t*11 *t*13 (β-ESO) | n.d. | 0.02 | 0.003 | - |
| C20:0 | 0.09 | 0.10 | 0.003 | 0.430 |
| C20:1 *c*11 | 0.18 | 0.16 | 0.005 | 0.025 |
| C20:1 *t*11 | 0.02 | 0.02 | 0.002 | 0.284 |
| C20:2 *n*-6 | 0.21 | 0.26 | 0.014 | 0.166 |
| C20:3 *n*-3 | 0.03 | 0.03 | 0.004 | 0.735 |
| C20:3 *n*-6 | 1.12 | 1.32 | 0.042 | 0.014 |
| C20:4 *n*-6 | 8.96 | 9.33 | 0.202 | 0.384 |
| C20:5 *n*-3 | 0.91 | 0.89 | 0.031 | 0.754 |
| C21:0 | 0.13 | 0.07 | 0.009 | <0.001 |
| C22:0 | 0.04 | 0.03 | 0.003 | 0.573 |
| C22:1 *c*13 | 0.01 | 0.00 | 0.002 | 0.003 |
| C22:2 *n*-6 | 0.04 | 0.09 | 0.008 | <0.001 |
| C22:4 *n*-6 | 1.82 | 1.54 | 0.071 | 0.052 |
| C22:5 *n*-3 | 2.44 | 2.79 | 0.084 | 0.033 |
| C22:5 *n*-6 | 0.62 | 0.64 | 0.030 | 0.756 |
| C22:6 *n*-3 | 1.35 | 1.49 | 0.067 | 0.339 |
| SFA | 35.40 | 36.15 | 0.407 | 0.384 |
| MUFA | 23.26 | 20.22 | 0.525 | 0.002 |
| PUFA | 31.73 | 36.59 | 0.669 | <0.001 |
| OBCFA | 6.00 | 3.51 | 0.376 | <0.001 |

[a] CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product.

[b] SEM, standard error of the mean.

*3.4. Fatty Acid Composition of Intramuscular Fat*

Table 1.5 reports the effect of the dietary treatment on the individual FA in the intramuscular fat. The dietary treatment did not affect the intramuscular fat (IMF). Similarly, the sum of SFA was not different between groups, whereas the concentration of total OBCFA and MUFA was greater in the intramuscular fat of CON lambs as compared to the WPB group ($P = 0.040$ and $P < 0.001$, respectively). Regarding individual OBCFA, the proportion of C15:0, C15:0 *iso*, C15:0 *anteiso,* C17:0 and C17:0 *anteiso* was lowered by feeding WPB compared to the CON diet ($P < 0.05$). Within the MUFA, the proportion of C16:1 *c*7, C17:1 *c*9, C18:1 *t*5 and C18:1 *t*10 was higher in the muscle of lambs fed the control diet as compared to WPB ($P < 0.05$), while the intramuscular concentration of C18:1 *c*6, C18:1 *c*14, C18:1 *t*9 and C18:1 *t*11 was increased by feeding WPB ($P < 0.05$). The inclusion of WPB in the diet affected the sum of PUFA ($P = 0.017$), whose percentage was greater in the muscle from the WPB lambs in comparison with CON. The proportion of RA was significantly higher in the WPB group as compared to CON ($P < 0.001$), and the same occurred for LA, α-LnA (C18:3 *c*9 *c*12 *c*15) and C20:2 *n*-6 ($P < 0.05$). Similarly to what reported for the rumen and the liver, CLnA isomers (PA, α-ESO, CA and β-ESO) were detected only in the muscle from lambs receiving WPB diet.

**Table 1.5.** Effects of the dietary treatment on total intramuscular fat (g/100 g of fresh muscle) and fatty acid composition of muscle (g/100 g of total FA).

| | Dietary treatment[a] | | SEM[b] | *P*-value |
|---|---|---|---|---|
| | CON | WPB | | |
| Intramuscular fat (IMF) | 1.88 | 2.01 | 0.156 | 0.690 |
| C10:0 | 0.15 | 0.17 | 0.008 | 0.425 |
| C12:0 | 0.11 | 0.10 | 0.010 | 0.714 |
| C14:0 | 2.17 | 2.02 | 0.114 | 0.530 |
| C14:0 *iso* | 0.02 | 0.02 | 0.002 | 0.946 |
| C14:1 *c*9 | 0.06 | 0.06 | 0.006 | 0.838 |
| C15:0 | 0.40 | 0.24 | 0.025 | 0.001 |
| C15:0 *iso* | 0.07 | 0.05 | 0.004 | 0.012 |
| C15:0 *anteiso* | 0.10 | 0.07 | 0.006 | 0.006 |
| C16:0 | 22.68 | 22.11 | 0.497 | 0.588 |
| C16:0 *iso* | 0.13 | 0.13 | 0.005 | 0.768 |
| C16:1 *c*7 | 0.26 | 0.23 | 0.005 | 0.002 |
| C16:1 *c*9 | 1.34 | 1.20 | 0.058 | 0.264 |
| C17:0 | 1.71 | 0.95 | 0.127 | 0.001 |
| C17:0 *iso* | 0.34 | 0.31 | 0.011 | 0.216 |
| C17:0 *anteiso* | 0.56 | 0.34 | 0.033 | <0.001 |
| C17:1 *c*9 | 0.92 | 0.47 | 0.072 | <0.001 |
| C18:0 (SA) | 16.16 | 16.69 | 0.553 | 0.657 |
| C18:1 *c*6 | 0.35 | 0.46 | 0.022 | 0.005 |
| C18:1 *c*9 | 36.59 | 34.75 | 0.498 | 0.069 |
| C18:1 *c*11 | 1.12 | 0.94 | 0.050 | 0.086 |
| C18:1 *c*12 | 0.45 | 0.45 | 0.038 | 0.976 |
| C18:1 *c*13 | 0.10 | 0.11 | 0.004 | 0.439 |
| C18:1 *c*14 | 0.14 | 0.21 | 0.011 | <0.001 |
| C18:1 *t*5 | 0.05 | 0.03 | 0.006 | 0.018 |
| C18:1 *t*6 | 0.16 | 0.12 | 0.011 | 0.124 |
| C18:1 *t*9 | 0.30 | 0.35 | 0.011 | 0.024 |
| C18:1 *t*10 | 1.16 | 0.42 | 0.113 | <0.001 |
| C18:1 *t*11 (VA) | 0.73 | 1.38 | 0.133 | 0.012 |
| C18:2 *c*9 *c*12 | 5.37 | 7.09 | 0.409 | 0.035 |

(*continued*)

**Table 1.5.** (*continued*)

| | Dietary treatment[a] | | SEM[b] | *P*-value |
|---|---|---|---|---|
| | CON | WPB | | |
| C18:2 *c*9 *t*11 (RA) | 0.35 | 0.94 | 0.084 | <0.001 |
| C18:2 *c*10 *t*12 | 0.02 | 0.01 | 0.004 | 0.779 |
| C18:3 *c*6 *c*9 *c*12 | 0.06 | 0.08 | 0.005 | 0.188 |
| C18:3 *c*9 *c*12 *c*15 (α-LnA) | 0.37 | 0.51 | 0.022 | <0.001 |
| C18:3 *c*9 *t*11 *c*13 (PA) | n.d. | 0.42 | 0.055 | - |
| C18:3 *c*9 *t*11 *t*13 (α-ESO) | n.d. | 0.06 | 0.008 | - |
| C18:3 *t*9 *t*11 *c*13 (CA) | n.d. | 0.03 | 0.004 | - |
| C18:3 *t*9 *t*11 *t*13 (β-ESO) | n.d. | 0.01 | 0.002 | - |
| C20:0 | 0.12 | 0.13 | 0.005 | 0.635 |
| C20:1 *c*11 | 0.13 | 0.14 | 0.004 | 0.944 |
| C20:2 *n*-6 | 0.06 | 0.08 | 0.005 | 0.034 |
| C20:3 *n*-3 | 0.01 | 0.01 | 0.003 | 0.847 |
| C20:3 *n*-6 | 0.15 | 0.24 | 0.023 | 0.051 |
| C20:4 *n*-6 | 1.42 | 1.92 | 0.216 | 0.279 |
| C20:5 *n*-3 | 0.13 | 0.20 | 0.021 | 0.086 |
| C21:0 | 0.06 | 0.05 | 0.004 | 0.143 |
| C22:0 | 0.02 | 0.03 | 0.002 | 0.073 |
| C22:1 *c*13 | 0.02 | 0.01 | 0.003 | 0.110 |
| C22:2 *n*-6 | 0.00 | 0.01 | 0.003 | 0.007 |
| C22:4 *n*-6 | 0.16 | 0.18 | 0.018 | 0.593 |
| C22:5 *n*-3 | 0.26 | 0.36 | 0.036 | 0.182 |
| C22:5 *n*-6 | 0.05 | 0.06 | 0.007 | 0.516 |
| C22:6 *n*-3 | 0.09 | 0.11 | 0.016 | 0.618 |
| SFA | 41.43 | 41.25 | 0.769 | 0.912 |
| MUFA | 43.01 | 40.97 | 0.494 | 0.040 |
| PUFA | 8.82 | 12.88 | 0.868 | 0.017 |
| OBCFA | 4.48 | 2.79 | 0.261 | <0.001 |
| PUFA *n*-3 | 0.86 | 1.19 | 0.087 | 0.062 |
| PUFA *n*-6 | 7.29 | 9.67 | 0.665 | 0.080 |
| PUFA *n*-6/*n*-3 | 8.53 | 8.20 | 0.138 | 0.247 |
| PUFA/SFA | 0.22 | 0.32 | 0.025 | 0.051 |

[a] CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product.
[b] SEM, standard error of the mean.

## 4. Discussion

The inclusion of locally available agro-industrial by-products in the animal diet may contribute to improve the sustainability of the food industry (Salami *et al.,* 2019). In particular, the agroindustry could reduce the cost linked to the disposal of waste biomasses, while the farmer could attenuate the cost of animal feeding. Also, replacing conventional feedstuffs, such as cereal grains, with non-human-edible by-products may lead to a lower feed-to-food competition in livestock production. Finally, bioactive compounds could be transferred from the by-products, as such or after modification, to the animal products improving their nutritional and technological properties. In the present study we evaluated the effect of feeding lambs with diets containing pomegranate pomace for 36 days on the growth performances and on the fatty acid composition of rumen digesta, liver and muscle. Such short experimental feeding is coherent with the lamb meat production system typical of some Mediterranean regions, where the farms oriented to dairy production represent the major source of sheep meat. With the aim of exploiting the presence of good quality native pasture for milk production in early autumn and to maximize the profit from the meat selling, lambing is concentrated during late summer. Then, the animals are weaned soon and fattened until December, when the market request, and consequently the prize, for lamb meat is high (Sitzia *et al.,* 2015).

*4.1. Animal performance*

In our experiment the concentration of tannins in the WPB diet was of 17 g (in TA equivalents)/kg. For a long time, tannins in feeds have been considered as antinutritive and/or toxic compounds due to their potentially detrimental effect on feed palatability, intake, digestion and on animal growth performance (Valenti *et al.,* 2019). However, the effect of tannins on animal performances is dependent on a number of factors, among which the dosage, the botanical origin and the chemical class of tannins (i.e. hydrolysable and condensed), as well as the inherent characteristics of the basal diet (Valenti *et al.,* 2019). Shabtay *et al.* (2008) reported that the fresh pomegranate peel (containing 3.35 and 34.4 g/kg DM of hydrolysable and condensed tannins, respectively) is very palatable for beef calves, resulting in a greater feed intake and improved growth performances as compared to a control treatment. On the contrary, Oliveira *et al.* (2010) observed a reduction of grain intake and body weight gain when pre-weaned calves ingested 5 or 10 g/day pomegranate extracts rich in polyphenols (16.9% gallic acid equivalent). Similarly, Hatami *et al.* (2018) reported that feeding a diet including 160 g/kg DM pomegranate marc (containing 3.36% of total tannin in the diet, expressed as TA equivalents) reduced the DMI and ADG of lambs. In the present experiment, despite the tannin concentration was 12-fold higher in the WPB diet compared to the control diet, no differences were found for voluntary DMI, final body weight, carcass weight and ADG between treatments, confirming that effects of tannins on animal performance are still controversial. Our findings are in agreement with those reported by Kotsampasi *et al.,* (2014) who incorporated up to 240 g/kg DM of

ensiled WPB in growing lamb diet, and confirm that the dietary inclusion of high level of WPB in lamb diet could be a strategy to reduce feeding costs and improve environmental sustainability.

*4.2. Fatty acid profiles*

It is commonly recognized that ruminant products are rich in saturated fatty acids because of the biohydrogenation of dietary unsaturated FA occurring in the rumen (Vasta and Bessa, 2012). Therefore, in the last decades, several feeding strategies have been proposed with the aim of improving the fatty acid composition of meat and milk, through enhancing the content of those fatty acids (FA) considered beneficial for human health, such as total PUFA, PUFA *n*-3 and rumenic acid (RA; C18:2 *c*9 *t*11). Also, studies have focused on increasing the content of vaccenic acid (VA; C18:1 *t*11), because this fatty acid is extensively converted to RA in mammal tissues by the $\Delta^9$ desaturase (Palmquist *et al.,* 2004). Feeding fresh forages, such as in pasture-based systems, has proved to effectively increase the content of desirable PUFA in meat (Vahmani *et al.,* 2015). However, in the Mediterranean areas, where pasture availability is strongly limited and erratic, another possibility could be represented by the supplementation of diets with sources of PUFA such as oils or oilseeds. However, this latter strategy does not always reach the desired goals, due to the high efficiency of the microbial biohydrogenation of PUFA in the rumen (Vahmani *et al.,* 2015). Tannins have been proposed as modulators of PUFA biohydrogenation in the rumen thanks to their direct and indirect action on the bacterial and protozoa community involved in the BH process. Although the effect

of tannins is still controversial, it has been generally reported an inhibitory effect on the rumen population, which has been positively associated with the ruminal outflow of PUFA and other desirable fatty acids originating form the biohydrogenation, such as RA and VA (Vasta and Bessa, 2012).

In the light of the above, the present experiment aimed at improving the FA composition of muscle by feeding lambs with WPB, which contains both PUFA and tannins. In order to better understand the effect of WPB we have investigated the fatty acids in ruminal digesta, liver and muscle, which represent three important districts involved in ruminant lipid metabolism. Our results clearly show that the fatty acid composition of muscle was improved by the inclusion of WPB in the diet, with the concentration of PUFA, RA and VA being greater in the muscle of WPB-lambs. The magnitude of this effect was particularly evident for VA and RA, which were found at double and triple concentration in the muscle of WPB-lambs. Interestingly, pomegranate CLnA isomers were detected only in the muscle of lambs of the WPB group, which suggests that some of these molecules were preserved from the complete ruminal BH. In particular, punicic acid was found at the concentration of 0.42 g/100 g of muscle FA. Similar results were found by Emami *et al.* (2015) by feeding lambs with pomegranate seed pulp (100 and 150 g/kg diet DM). To the best of our knowledge, this is the first time that the occurrence of the α-ESO, CA and β-ESO is reported in ruminant products. For the other PUFA in muscle, we found that the dietary inclusion of WPB increased the proportion of α-LnA and tended to increase the PUFA/SFA ratio. Differing from Emami *et al.* (2015), we observed that the PUFA *n*-6 to PUFA *n*-3 ratio in muscle was not

affected by feeding WPB, which can be attributed to the concurrent increase of PUFA *n*-6 and PUFA *n*-3 in muscle from WPB-fed lambs.

Regarding MUFA, the VA (C18:1 *t*11) is the most abundant among the *trans* octadecenoic acids in ruminant meat and milk fat (Wolff, 1995) and it mainly originates from the principal pathway of rumen BH. However, in animals fed a diet characterized by a high starch-to-forage ratio (e.g. concentrate), the BH pathway may be altered causing an accumulation of C18:1 *t*10 at the expense of C18:1 *t*11 in the rumen, known as "*t*-10 shift", which is then reflected in the ruminant products. The *t*10 isomer has been proposed as a risk factor for human health, because of its possible involvement in cardiovascular disease, although this issue is still under debate (Shingfield *et al.,* 2010; Griinari *et al.,* 1998). The occurrence of the "*t*-10 shift" can be evaluated by measuring the C18:1 *t*10/*t*11 ratio, whereby the value of 1 has been proposed as the threshold above which the *t*10-shift can be established (Bessa *et al.,* 2015). In the present experiment, lambs in both treatments were fed with a concentrate-based diet. Therefore, it was interesting to find that the C18:1 *t*10/*t*11 ratio was strongly reduced in the muscle of WPB lambs in comparison with the control group (0.33 and 1.83 for WPB and CON group, respectively; Figure 1.1). The same result was found in the liver and rumen digesta, suggesting that the WPB diet prevented the *t*10-shift. On the one hand, the greater ingestion of fiber (i.e. NDF) in the WPB group might contribute to explain this observation. Indeed, it is well known that a low-fiber diet promotes the *t*10-shift (Alves and Bessa, 2014) mainly by lowering the ruminal pH, with the consequent alteration of the ruminal bacterial species composition (Arrigoni *et al.,* 2016). However, in the present study,

ruminal pH did not differ between the groups. Other possible mechanisms could explain the lower *t*10-*t*11 C18:1 ratio found in the rumen from the WPB-fed lambs. Among these, the BH of CLnA present in WPB might have contributed to increase the formation of VA. Indeed, although the ruminal BH pathways pomegranate CLnA isomers have not been described so far, these fatty acids are likely to be converted to RA and VA in the rumen (Ishlak *et al.,* 2014), mainly after the saturation of one ($\Delta^{13}$) or two ($\Delta^{9}$ and $\Delta^{13}$) double bonds, respectively. In the present study, we estimated the BH rate of PA as reported by Alves *et al.* (2017) and we found that approximately 90% of punicic acid underwent BH. Therefore, it might be supposed that the greater amount of VA produced in the rumen of WPB lambs originated from the BH of punicic acid, with a consequent increase of VA and RA in the liver and muscle. It may also be speculated that the greater intake of vitamin E by lambs in the WPB group contributed to reduce the ruminal production of C18:1 *t*10. Indeed, it has been reported that vitamin E may be involved in preventing the *t*10-shift in the rumen BH, thus reducing the formation and accumulation of C18 *t*10 (Pottier *et al.,* 2006; Juarez *et al.,* 2010). It is still uncertain how vitamin E may affect BH. However, changes involving the rumen population or dynamics could be supposed (Hou *et al.,* 2013). Lastly, pomegranate tannins could have contributed to modulate the ruminal BH. Indeed, on the one hand, it has been reported that different bacterial strains are responsible for the formation of C18:1 *t*10 and *t*11 isomers and, on the other hand, that tannins can promote shifts in the microbial population of rumen (Buccioni *et al.,* 2012). Therefore, it could be supposed that WPB tannins affected the BH by favouring the production of C18:1 *t*11 instead of *t*10. However,

despite total tannins accounted for approximately 90% of total phenols in WPB diet, the effect of non-tannin polyphenols cannot be excluded. Further studies are needed to confirm and to understand how dietary pomegranate by-products may prevent the *t*10-shift in ruminants fed with concentrate-based diets.

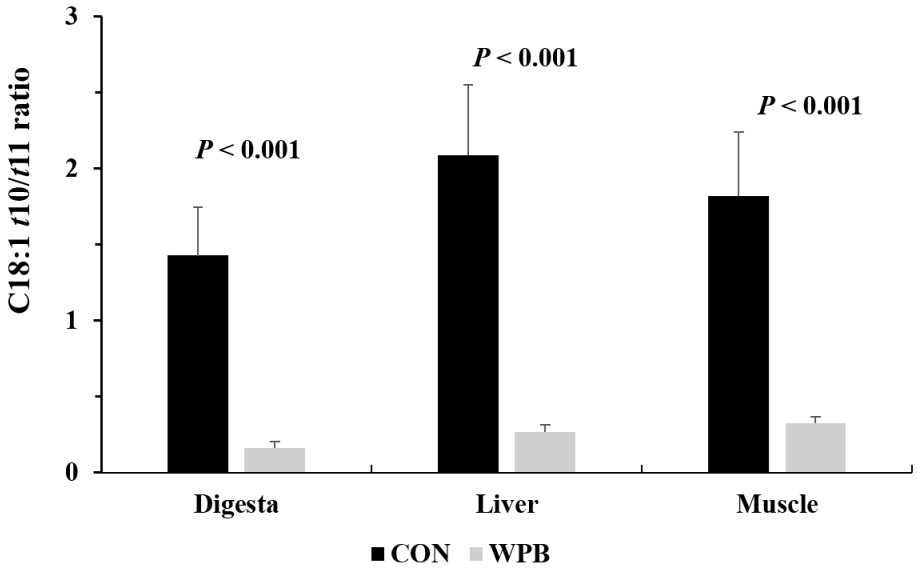


**Figure 1.1.** Effect of dietary inclusion of WPB on C18:1 t10/t11 ratio in rumen digesta, liver and muscle. CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product. Values presented are the least squares means with standard error bars.

Ruminal microorganisms are responsible for the occurrence of odd- and branched-chain fatty acids (OBCFA) in ruminant products (Fievez *et al.,* 2012). Indeed, rumen bacterial membranes contain high proportions of OBCFA, thus the measurement of concentration and composition of these fatty acids could be useful to monitor the rumen functionality (Fievez *et al.,* 2012). In the present study, OBCFA followed a similar trend in the three investigated districts and were strongly

reduced by feeding WPB. This is the first study in which fatty acids were analysed in the rumen digesta from animals fed pomegranate by-products; therefore a comparison with the literature is not possible. However, it could be hypothesized that the reduction of OBFCA in the three districts could be due to an inhibitory effect of dietary WPB on rumen microbial population. This effect likely depends on the tannins contained in the WPB diet, which is consistent with inhibitory effect of tannins on rumen bacteria reported both *in vitro* and *in vivo* (Costa *et al.,* 2017; Castro-Montoya *et al.,* 2016).

Nevertheless, the above hypothesis seems to partially contrast with the results obtained on those ruminal fatty acids involved in BH. Indeed, a depressing effect of the rumen BH is one of the most known effects of dietary tannins, which can determine a general depression of the process or the inhibition of specific steps, such as the conversion of VA to SA (last step of BH) (Costa *et al.,* 2017; Vasta *et al.,* 2009; Khiaosa-Ard *et al.,* 2009; Carreño *et al.,* 2015). As a result, an increase of BH precursors and intermediates and/or a decrease in SA could have been expected in the rumen of WPB lambs. However, our results suggest that the specific limitation of the terminal step of BH does not seem to have occurred, as demonstrated by the similar SA/(SA+VA) ratio in the ruminal digesta between the treatments. Moreover, despite the greater ingestion of PUFA by WPB lambs, the concentration of PUFA in the rumen digesta was not affected by the dietary treatment, which suggests that feeding WPB did not exert a general depression of the BH. This is further supported by the completeness index of biohydrogenation, which was not different between groups. The greater intake of NDF for lambs fed

WPB might partially explain these findings. Indeed, it is well known that the extent of rumen BH is positively correlated with the NDF level of the diet, because of a longer retention time of feedstuff in rumen (Poppi *et al.,* 1981) and a favourable environment for bacteria population (Millen *et al.,* 2016). Another explanation could be linked to the toxicity of PUFA to rumen bacteria, which increases with increasing degree of unsaturation (Maia *et al.,* 2007). Therefore, the BH rate is promoted by feeding high levels of highly unsaturated fatty acids. This could explain why, in spite of the greater intake of highly unsaturated FA (i.e. CLnA) by lambs fed WPB, the proportion of total PUFA in the rumen was not different between the groups. It may be supposed that, in lambs fed the WPB diet, the rumen microorganisms preferentially hydrogenated the highly unsaturated FA from WPB than fatty acids with one or two double bonds. In line with this, no differences between groups were observed for the BH indexes of C18:1 *c*9 and C18:2 *c*9*c*12, while *α*-LnA acid was saturated at a greater extent in the WPB group and more than 90% of dietary PA underwent BH.

In the present study, the fatty acid composition of the liver generally reflected the intramuscular fatty acid profile. However, some fatty acids in liver followed a different trend compared to muscle, probably caused by a different enzymatic activity. Indeed, we found that many of the $\Delta^9$ desaturase enzyme products (i.e. C14:1 *c*9, C16:1 *c*9 and C18:1 *c*9) were higher in the liver of the control group, suggesting that a higher activity occurred in the liver from animals in this treatment. The C17:1 *c*9 is exclusively synthesized endogenously by the $\Delta^9$ desaturase enzyme, as it is almost absent in the feedstuffs and in the rumen bacteria.

Thus, the C17:1 *c*9/(C17:0 + C17:1 *c*9) ratio has been proposed as one of the possible indexes for the indirect estimation of the $\Delta^9$ desaturase enzyme activity (Bessa *et al.,* 2015). In the present study, this desaturation index (C17Di) was different between the groups in the liver but not in muscle (Figure 1.2) and a similar result was obtained even when the desaturation index was calculated for other fatty acids (C14 to C18; data not shown). The greater percentage of PUFA found in the liver of WPB group could contribute to explain these results. Indeed, PUFA have been described as powerful inhibitors of the $\Delta^9$ desaturase activity in liver or adipocytes through a decreased mRNA expression or stability (Ntambi, 1999). Also, Arao *et al.* (2004) reported that the punicic acid has a suppressive effect on the $\Delta^9$ desaturase enzyme activity in rat. Consistently, in the present study we found a negative correlation in liver between the PUFA and the PA with C17Di ($r$ = -0.861, $P < 0.001$; $r$ = -0.740; $P$ = 0.009; for PUFA and PA, respectively). However, a similar result was not observed in muscle suggesting that the influence of PUFA on $\Delta^9$ desaturase enzyme was stronger in the liver than in muscle.

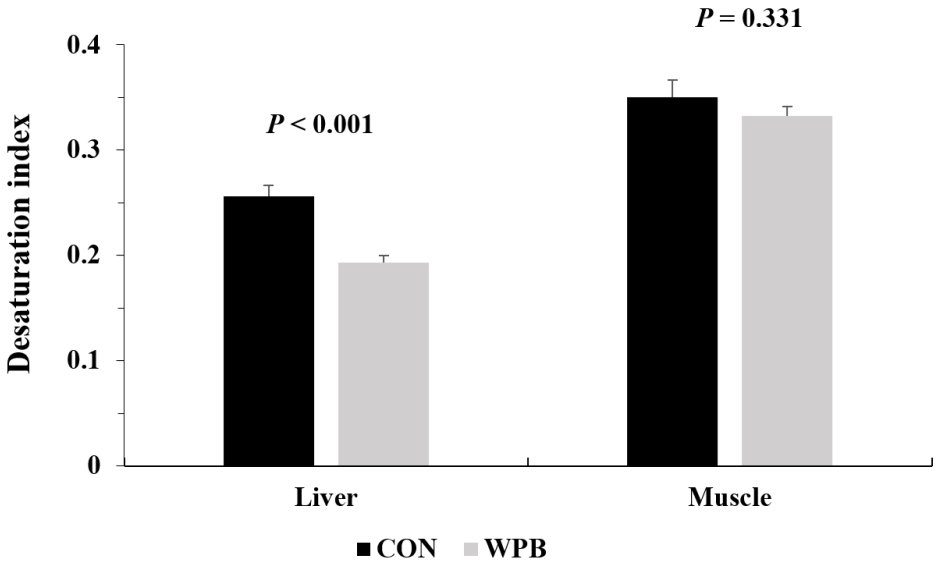


**Figure 1.2.** Effect of dietary inclusion of WPB on desaturase index [C17:1 c9/(C17:0 + C17:1 c9)] in liver and muscle. CON: control barley-corn based concentrate diet. WPB: diet including 20% of whole pomegranate by-product. Values presented are the least squares means with standard error bars.

## 5. Conclusions

In conclusion, the inclusion of whole pomegranate by-product into a concentrate-based diet can be a profitable strategy to improve the fatty acid composition of lamb muscle, without negatively affecting the animal performances. In particular, the proportion of PUFA, RA and VA in muscle was increased by feeding lambs with WPB and the health promoting CLnA of pomegranate were deposited in the intramuscular fat. Moreover, the dietary administration of WPB could be proposed as strategy to prevent the *t*10-shift in intensive concentrate-based feeding systems. Taken together, the results obtained in the ruminal digesta, liver and muscle suggest that the concurrence of both tannins and polyunsaturated fatty acid in WPB would have favoured the deposition of desirable fatty acids in meat.

Further specific studies are needed to better understand the mechanisms and the contribution of each WPB bioactive compound and to investigate potential synergistic effects.

# CHAPTER 6 - Bioactive compounds from pomegranate by-products increase the *in vitro* ruminal accumulation of potentially health promoting fatty acids

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**Abstract**

Increasing demand and production of pomegranate has led to a large amount of by-products that might be used in ruminant feeding. Inclusion of pomegranate by-products in the ovine diet has recently been shown to enrich meat and milk with potentially health-promoting fatty acids (FA). However, it remains unclear whether this effect is due to the action of the bioactive conjugated linolenic acids (CLnA) or of the tannins present in the pomegranate, or perhaps to their interaction. To fill this gap, two *in vitro* experiments were conducted: the first one tested the effects of pomegranate oil and tannins, alone or in combination, on the biohydrogenation process, and the second one compared the ruminal responses to by-products rich in CLnA (pomegranate seeds, PS), in tannins (pomegranate peels and pulp, PPP) or in both bioactive components (i.e., the whole pomegranate by-product; WPB). Three cannulated ewes were used as donors of inocula for batch cultures of rumen microorganisms. Incubations lasted for 12 and 24 h and were repeated on 3 different days (runs). In both experiments, digesta FA profile was examined by gas chromatography. Results from both trials support that pomegranate tannins and CLnA played different roles in modulating ruminal FA composition. Specifically, tannins would favour the accumulation of potentially health-promoting FA present in dietary lipids (e.g., 18:2n-6 or 18:3n-3) and *cis*-9 *trans*-11 conjugated linoleic acid (CLA), whereas the observed increase in *trans*-11 18:1 would mainly derive from the biohydrogenation of CLnA isomers. Results from the second experiment included evident shifts in some minor FA that would support not only direct saturation steps (e.g., increases in *trans*-11 *cis*-13 and *trans*-11 *trans*-13 CLA, and

in *trans*-9, *trans*-11, *cis*-13 and *trans*-13 18:1) but also a putative isomerisation by rumen bacteria (e.g., increases in *trans*-10 *cis*-12 CLA and *trans*-10, *cis*-15, *trans*-15 and *trans*-16 18:1). Changes in ruminal fermentation parameters (i.e., reductions in ammonia concentration and in the proportions of minor volatile FA) showed that pomegranate tannins protected dietary protein from degradation. Nevertheless, a negative impact on *in vitro* ruminal fermentation (i.e., reductions in DM disappearance, gas production and total volatile FA concentrations) was observed when 20% of by-products were included in the diet. Finally, there seem to be no evident synergistic but additive effects between pomegranate bioactive compounds (i.e., tannins and CLnA) on ruminal biohydrogenation or fermentation.

***Key words:*** *conjugated linolenic acid; lipids; phenolic compounds; punicic acid; tannins*

## 1. Introduction

Due to the potential benefits of pomegranate fruits (*Punica granatum* L.) on human health (Lansky and Newman, 2007), and the development of industrial technologies to obtain more appealing products (e.g., ready-to-eat arils or ready-made juices and extracts; Shabtay *et al.,* 2008), there has been a great increase in the demand and production of those fruits. Consequently, the agro-industries yield large amounts of residual biomasses, the whole pomegranate by-product (WPB; constituted of seeds, peels and pulp), being the primary by-product. At present, the disposal of these processing wastes represents a cost, which makes imperative to

find alternatives. In this regard, their use in ruminant feeding would contribute to reduce the amount of cereals fed to the animals, reducing in turn not only the feeding cost of ruminant production but also the feed to food competition (Salami *et al.,* 2019).

Pomegranate by-products contain a number of bioactive compounds: pomegranate peel is a rich source of phenolic compounds, namely flavonoids, anthocyanidins and tannins (Lansky and Newman, 2007), and pomegranate seeds contain about 12-20% lipids, which are characterized by a peculiar composition. Indeed, pomegranate oil is mostly composed of conjugated linolenic acids (CLnA), a mixture of outstanding polyunsaturated fatty acid (PUFA) isomers, with punicic acid (*cis*-9 *trans*-11 *cis*-13 CLnA) being the most abundant (Johanningsmeier and Harris, 2011). Both pomegranate tannins and CLnA have been shown to possess antimicrobial, antioxidant, anti-inflammatory, antitumoral or immunomodulatory properties (Lansky and Newman, 2007; Viuda-Martos *et al.,* 2010).

Several studies have proposed the dietary inclusion of pomegranate by-products as an effective strategy to improve the quality of ruminant products, with particular attention to their health-promoting fatty acid (FA) composition (Ishlak *et al.,* 2014; Razzaghi *et al.,* 2015; Salami *et al.,* 2019). Thus, a greater content of such FA (i.e., total PUFA and rumenic and vaccenic acids) were observed in milk and meat from ruminants fed the whole pomegranate by-product (Kotsampasi *et al.,* 2017; Valenti *et al.,* 2019a) or a by-product containing mostly seeds (Modaresi *et al.,* 2011; Emami *et al.,* 2015; Razzaghi *et al.,* 2015). In a previous work, we found that feeding lambs with 200 g/kg DM of dried WPB resulted in an overall

improvement of the intramuscular fatty acid profile (Natalello *et al.,* 2019). However, we could not discern whether these results were related just to the consumption of the pomegranate PUFA, or to concomitant additive or synergistic effects of the pomegranate tannins, as these phenolic compounds have been reported to be able to modulate the ruminal biohydrogenation (BH) of dietary unsaturated FA (Buccioni *et al.,* 2011; Carreño *et al.,* 2015; Toral *et al.,* 2018).

Therefore, this study was conducted to better understand the mechanisms and contribution of each of these bioactive compounds. To this aim, two *in vitro* experiments were carried out in parallel with the main objectives of assessing the effects of pomegranate oil and tannins on the rumen biohydrogenation of dietary FA (Experiment 1), and evaluating the consequences of dietary inclusion of pomegranate by-products (i.e., seeds, peels with pulp, or both) on the processes of ruminal fermentation and biohydrogenation (Experiment 2).

## 2. Materials and methods

The experiments were conducted at the *Instituto de Ganadería de Montaña* (León, Spain), and all procedures were approved and completed in accordance with the Spanish and EU legislations (Royal Decree 53/2013 and Council Directive 2010/63/EU) for the protection of animals used for experimental purposes.

### *2.1. Pomegranate by-products, oil and phenolic extract*

The pomegranate fruits (*Punica granatum* L. var Wonderful) were halved and manually squeezed with a citrus juicer. Then, the residual part containing peels,

seeds, membranes and little portion of arils was collected and subsequently dried in a ventilated oven at 40 °C until constant weight (approximately 36 h). This product is referred to as “whole pomegranate by-product” (WPB) throughout the article. An aliquot was then deprived manually of seeds to obtain two fractions: “pomegranate peels and pulp” (PPP) and “pomegranate seeds” (PS). The chemical composition of the three by-products is shown in Table 2.1.

The cold-pressed organic pomegranate seed oil (PSO) was purchased from Naissance™ (The Naissance Trading & Innovation Co. Ltd., Neath, UK). Its FA composition, with a noticeable content of punicic acid (*cis*-9 *trans*-11 *cis*-13 CLnA), α-eleostearic acid (*cis*-9 *trans*-11 *trans*-13 CLnA), β-eleostearic acid (*trans*-9 *trans*-11 *cis*-13 CLnA), and catalpic acid (*trans*-9 *trans*-11 *trans*-13 CLnA), is shown in Table 2.2.

Pomegranate phenolic extract (PE) was obtained from WPB using an adaptation of the method by Makkar *et al.* (2003). Briefly, 100 g of milled (1 mm) WPB were mixed with 700 mL petroleum ether and sonicated in a water bath for 15 min. After 2 h of maceration under continuous stirring at 4 °C, petroleum ether was removed by a rotary evaporator system (Rotavapor R-114, Büchi, Flawil, Switzerland), and the leftover solid residue was again sonicated with 1 L of acetone/water (70/30, vol/vol) for 15 more min in a water bath. After an overnight incubation at 4 °C, the liquid phase was collected, and the acetone was removed by rotary evaporation at 40 °C. The remaining water solution was washed twice with an equal volume of hexane in a separating funnel to remove any residual lipids. Then, it was freeze-dried, and the extract obtained was stored at -30 °C.

*2.2. Animals and diet*

Three Merino ewes (body weight = 65 ± 3.2 kg), fitted with a ruminal cannula (40 mm internal diameter), were used as rumen inoculum donors. They were offered a total mixed ration (TMR, forage:concentrate ratio 50:50), based on dehydrated alfalfa hay (particle size >4 cm) and concentrates (in g/kg of fresh matter: whole maize and barley grains, 140 and 100, respectively; soybean meal, 150; sugar beet pulp, 50; molasses, 40, and a vitamin-mineral supplement, 20), at approx. 1.2 times their estimated maintenance energy requirement INRA (2007). The chemical composition of the TMR is given in Table 2.1. Animals had continuous access to clean drinking water.

**Table 2.1.** Chemical composition (g/kg DM) of TMR and pomegranate by-products.[A]

| | TMR | PS | PPP | WPB |
|---|---|---|---|---|
| Organic matter | 907 | 972 | 965 | 967 |
| Crude protein | 184 | 154 | 41.0 | 75.1 |
| Neutral detergent fibre | 272 | 442 | 147 | 238 |
| Acid detergent fibre | 181 | 304 | 104 | 164 |
| Acid detergent lignin | 28.2 | 102 | 19.4 | 42.5 |
| Starch | 151 | 32.2 | 49.7 | 42.0 |
| Ether extract | 15.0 | 153 | 5.70 | 46.3 |
| Total phenols[B] | 5.57 | 8.96 | 215 | 170 |
| Total tannins[B] | 2.39 | 7.34 | 214 | 170 |
| 16:0 | 2.69 | 4.23 | 0.70 | 1.57 |
| 18:0 | 0.48 | 2.53 | 0.23 | 0.93 |
| *cis*-9 18:1 | 2.59 | 6.11 | 0.94 | 2.15 |
| *cis*-11 18:1 | 0.13 | 0.78 | 0.05 | 0.19 |

(*continued*)

**Table 2.1.** (*continued*)

| | TMR | PS | PPP | WPB |
|---|---|---|---|---|
| *cis*-9 *cis*-12 18:2 | 6.34 | 7.77 | 0.80 | 2.62 |
| *cis*-9 *cis*-12 *cis*-15 18:3 | 1.44 | 0.79 | 0.22 | 0.14 |
| *cis*-9 *trans*-11 *cis*-13 CLnA | - | 114 | 2.03 | 33.2 |
| *cis*-9 *trans*-11 *trans*-13 CLnA | - | 4.65 | 0.12 | 1.41 |
| *trans*-9 *trans*-11 *cis*-13 CLnA | - | 2.83 | 0.10 | 0.99 |
| *trans*-9 *trans*-11 *trans*-13 CLnA | - | 0.75 | 0.02 | 0.22 |

[A] TMR, total mixed ration; PS, pomegranate seeds; PPP, pomegranate peels and pulp; WPB, whole pomegranate by-product.
[B] Expressed as tannic acid equivalents.

### *2.3. In vitro experiments*

Both experiments were conducted using batch cultures of rumen microorganisms. After adapting the ewes for 28 days to the experimental diet, rumen fluid inocula were collected via the cannula before feeding, immediately transferred to the laboratory in pre-warmed thermo flasks. The rumen fluids were filtered through a nylon membrane (250 μm; Fisher Scientific S.L., Madrid, Spain) and pooled in equal volume, always under $CO_2$ flushing. The incubations were repeated on 3 different days (runs) for replicates.

#### *2.3.1. Experiment 1*

Four treatments at 2 incubation times (12 and 24 h) were analysed in this experiment. Before describing the treatments, it must be mentioned that the pomegranate PE was a crude (non-purified) extract containing 26.9% of total phenolic compounds (with tannins representing 91.8% of those phenolic

compounds). Therefore, since other constituents of PE might exert some effects on the studied parameters, we decided to include this PE in all treatments and inhibit the effect of tannins, when required, by adding polyethylene glycol (PEG; Makkar *et al.,* 1995).

The incubated substrate was the same TMR used to feed the animals, supplemented with 20 g of linoleic acid (L1376; Sigma–Aldrich, Madrid, Spain)/kg DM diet to facilitate the study of the BH process.

Treatments were therefore as follows:

- Control (C): substrate + PE + PEG,
- Pomegranate tannins (PT): substrate + PE,
- Pomegranate oil (PO): substrate + PE + PEG + PSO,
- Pomegranate tannins and oil (PTO): substrate + PE + PSO.

Both linoleic acid and PSO were dissolved in hexane, and added to the 125-mL serum flasks (at 2% DM diet). The solvent was dried under nitrogen before weighing the substrate (500 mg DM, ground in a hammer mill fitted with a 1-mm screen). One gram of PEG (MW 6000; Fluka Chemie GmbH, Buchs, Switzerland) was weighed in each flask corresponding to treatments C and PO. The phenolic extract was dissolved in lukewarm water and added to the flasks (to provide 20 g of total phenols/kg DM diet) just before the incubation started. Fatty acid composition of the incubated substrates is reported in Table 2.2.

Seventy-two flasks [3 flasks (technical repetitions) × 4 treatments × 2 incubation times × 3 runs (replicates)] were dosed with 50 mL of a mix (1:4) of strained rumen fluid and phosphate bicarbonate buffer (Goering and Van Soest,

1970) and incubated under anaerobic conditions for 12 or 24 h in an incubator set at 39.5 °C. Flasks were individually agitated at 3, 6 and 12 h of incubation. After 12 or 24 h, according to the design, the reaction was stopped by placing the bottles into ice-water, and then the contents of the three flasks per treatment (repetitions) were mixed and stored at −80 °C. Samples were freeze-dried and stored again at –80 ºC until FA analysis.

**Table 2.2.** Fatty acid composition (g/kg DM) of the incubated substrates in Experiment 1.[A]

| | | Treatment | |
|---|---|---|---|
| | PSO | C and PT[B] | PO and PTO[B] |
| 16:0 | 2.47 | 2.64 | 3.13 |
| 18:0 | 1.80 | 0.47 | 0.83 |
| *cis*-9 18:1 | 4.24 | 2.54 | 3.39 |
| *cis*-11 18:1 | 0.41 | 0.13 | 0.21 |
| *cis*-9 *cis*-12 18:2 | 4.70 | 26.2 | 27.2 |
| *cis*-9 *cis*-12 *cis*-15 18:3 | 0.73 | 1.41 | 1.56 |
| *cis*-9 *trans*-11 *cis*-13 CLnA | 61.6 | - | 12.3 |
| *cis*-9 *trans*-11 *trans*-13 CLnA | 6.92 | - | 1.38 |
| *trans*-9 *trans*-11 *cis*-13 CLnA | 9.87 | - | 1.97 |
| *trans*-9 *trans*-11 *trans*-13 CLnA | 1.99 | - | 0.40 |

[A] PSO, pomegranate seed oil; Treatments: C, control; PO, pomegranate oil; PT, pomegranate tannins; PTO, pomegranate tannins and oil.
[B] Data were calculated from individual ingredients.

### *2.3.2. Experiment 2*

Four treatments were studied through incubations at 12 and 24 h: the same TMR used to feed the animals (control, C), and, on a DM basis, 80% TMR plus 20% of whole pomegranate by-product (WPB-20; providing both tannins and oil),

pomegranate seeds (PS-20; providing oil) or pomegranate peels and pulp (PPP-20; providing tannins). Chemical composition of the incubated substrates is reported in Table 2.3.

Six flasks per treatment, incubation time and run were incubated. Three of them were used for the fermentation study and the other 3 for the BH study. Thus, 144 flasks [2 studies × 3 flasks (technical repetitions) × 4 treatments × 2 incubation times × 3 runs (replicates)] were prepared as described previously for the Experiment 1 (500 mg DM of substrate, 50 mL of buffered rumen fluid, etc.).

For the fermentation study, blanks containing buffered rumen fluid without substrate were also incubated under the same conditions (3 flasks per incubation time and run), which made a total of 162 flasks (144, as explained above, + 18 blanks). Accumulated head-space gas pressures were measured with a pressure transducer at 3, 6, 12 and 24 h post-inoculation. Pressure values, corrected for the quantity of substrate DM incubated and gas released from blanks, were used to generate gas volume estimates using a predictive equation derived from earlier simultaneous pressure and volume measurements (Hervás *et al.,* 2005). Once the reaction was stopped, by placing the flasks into ice-water, the pH was measured, and centrifuged samples (at 976 × *g* for 10 min) were collected and stored at −30 °C for ammonia, lactic acid and volatile fatty acid (VFA) analysis, as reported in Carreño *et al.* (2015). Dry matter disappearance (DMD) was estimated by filtering the residues using pre-weighed sintered glass crucibles (100–160 μm; Pyrex, Stone, UK).

The contents of the remaining flasks (i.e., those used to study ruminal BH) were mixed once the reaction was stopped after 12 or 24 h, and then freeze-dried and stored at −80 °C until FA analysis.

**Table 2.3.** Chemical composition (g/kg DM) of the incubated substrates in Experiment 2.[A]

| | C | PS-20 | PPP-20 | WPB-20 |
|---|---|---|---|---|
| Organic matter | 907 | 920 | 918 | 919 |
| Crude protein | 184 | 178 | 156 | 162 |
| Neutral detergent fibre | 272 | 306 | 247 | 265 |
| Acid detergent fibre | 181 | 206 | 166 | 178 |
| Acid detergent lignin | 28.2 | 43.0 | 26.4 | 31.1 |
| Starch | 151 | 127 | 130 | 129 |
| Ether extract | 15.0 | 42.7 | 13.1 | 21.3 |
| Total phenols[B] | 5.57 | 6.25 | 47.4 | 38.5 |
| Total tannins[B] | 2.39 | 3.38 | 44.7 | 35.9 |
| 16:0 | 2.69 | 3.00 | 2.29 | 2.47 |
| 18:0 | 0.48 | 0.89 | 0.43 | 0.57 |
| *cis*-9 18:1 | 2.59 | 3.30 | 2.26 | 2.50 |
| *cis*-11 18:1 | 0.13 | 0.26 | 0.11 | 0.14 |
| *cis*-9 *cis*-12 18:2 | 6.34 | 6.62 | 5.23 | 5.59 |
| *cis*-9 *cis*-12 *cis*-15 18:3 | 1.44 | 1.31 | 1.20 | 1.18 |
| *cis*-9 *trans*-11 *cis*-13 CLnA | - | 22.9 | 0.41 | 6.64 |
| *cis*-9 *trans*-11 *trans*-13 CLnA | - | 0.93 | 0.02 | 0.28 |
| *trans*-9 *trans*-11 *cis*-13 CLnA | - | 0.57 | 0.02 | 0.20 |
| *trans*-9 *trans*-11 *trans*-13 CLnA | - | 0.15 | <0.01 | 0.04 |

[A] C, control TMR; PS-20, 80% DM of control TMR and 20% DM of pomegranate seeds; PPP-20, 80% DM of control TMR and 20% DM of pomegranate peels and pulp; WPB, 80% DM of control TMR and 20% DM of whole pomegranate by-product.
[B] Expressed as tannic acid equivalents.

*2.4. Chemical analysis*

Feed samples were prepared (ISO 6498:2012) and analysed for DM (ISO 6496:1999), ash (ISO 5984:2002), crude protein (ISO 5983-2:2009) and starch (K-TSTA kit; Megazyme International Ireland, Wicklow, Ireland). The neutral and acid detergent fibres (aNDF and ADF) and acid detergent lignin concentrations were sequentially determined using an Ankom$^{2000}$ fiber analyzer (Ankom Technology Methods 13, 12 and 8, respectively; Ankom Technology Corp., Macedon, NY, USA); the former was assayed with sodium sulfite and α-amylase, and both NDF and ADF were expressed with residual ash. Procedure described by AOAC (2006) was used to determine the content of ether extract (AOAC official method 935.38). Total phenolic and tannin contents were assayed following the Folin-Ciocalteu method in combination with polyvinyl-polypyrrolidone, with tannic acid (Merck, Damstadt, Germany) as the reference standard (Makkar, 2003).

Fatty acid methyl esters (FAME) of lipid in samples of TMR, pomegranate by-products and rumen digesta were prepared as detailed in Toral *et al.* (2010), with some modifications to prevent the isomerization of CLnA (i.e., lower methylation temperature and use of anhydrous $Na_2SO_4$ to dry the samples). Briefly, lipid in 200 mg of feed or freeze-dried digesta was extracted in triplicate using 4 mL of a mixture (3:2, vol/vol) of hexane and isopropanol and, in the case of digesta, following the adjustment of sample pH to 2 using 2 M hydrochloric acid (Shingfield *et al.,* 2003). *Cis*-12 13:1 (Larodan Fine Chemicals AB, Solna, Sweden) was used as an internal standard. Organic extracts were dried under nitrogen at 35 °C. Lipid dissolved in 2 mL of hexane was converted to FAME using a base-acid catalysed

transesterification procedure with freshly prepared 0.5 M sodium methoxide in methanol for 5 min at room temperature followed by reaction with 1% (vol/vol) sulfuric acid in methanol at 40 °C for 30 min. The FAME in 5 mg of PSO were prepared by direct base-catalysed transesterification, using the same reagents and conditions applied for the analysis of other feeds and rumen digesta.

Methyl esters were separated and quantified using a gas chromatograph (Agilent 7890A GC System, Santa Clara, CA, USA) equipped with a flame-ionisation detector and a 100-m fused silica capillary column (0.25 mm i.d., 0.2 μm film thickness; CP-SIL 88, CP7489, Varian Ibérica S.A., Madrid, Spain) and hydrogen as the carrier gas (207 kPa, 2.1 mL/min). Total FAME profile in a 2 μL sample volume at a split ratio of 1:50 was determined using a temperature gradient programme (Shingfield *et al.,* 2003). Isomers of 18:1 in rumen digesta samples were further resolved in a separate analysis under isothermal conditions at 170 °C (Shingfield *et al.,* 2003). Peaks were identified based on retention time comparisons with commercially available authentic standards (Nu-Chek Prep., Elysian, MN, USA; Sigma–Aldrich; and Larodan), cross referencing with chromatograms reported in the literature (e.g., Shingfield *et al.,* 2003; Sassano *et al.,* 2009; Toral *et al.,* 2010) and comparison with reference samples for which the FA composition was determined based on gas chromatography analysis of FAME and gas chromatography–mass spectrometry analysis of corresponding 4,4-dimethyloxazoline derivatives (Toral *et al.,* 2017).

As described in Carreño *et al.* (2015), the ammonia was measured spectrophotometrically, using the salicylate method, and VFA were analysed by gas

chromatography, using crotonic acid as the internal standard. Lactic acid concentration was determined colorimetrically after reaction of acidified samples with copper sulphate pentahydrate y *p*-phenylphenol (Taylor, 1996).

*2.5. Statistical analysis*

All statistical analyses were performed using the SAS software package (version 9.4, SAS Institute Inc., Cary, NC, USA). Data were analysed by one-way ANOVA using the MIXED procedure of SAS with a model that included the fixed effect of experimental treatment and the random effect of run. Means were separated through the pairwise differences ("pdiff") option of the least squares means ("lsmeans") statement of the MIXED procedure, and adjusted for multiple comparisons using Bonferroni's method. Differences were declared significant at $P < 0.05$ and considered a trend toward significance at $0.05 \leq P < 0.10$. Least squares means are reported.

## 3. Results

*3.1. Composition of pomegranate by-products and oil and incubated substrates*

The chemical composition of TMR and pomegranate by-products are reported in Table 2.1. Feedstuffs containing pomegranate peels (i.e., PPP and WPB) were rich in phenolic compounds, with tannins representing more than 99% of total phenols. Lipids (ether extract) were much higher in seeds than PPP, and their FA composition was characterized by the occurrence of peculiar CLnA, mainly punicic

acid (*cis*-9 *trans*-11 *cis*-13 18:3). The other CLnA isomers were present at lower concentration in all the three by-products. As shown in Table 2.2, the FA composition of pomegranate oil used in the Experiment 1 was in line with that found for the seeds described above. However, the relative proportion of *cis*-9 *trans*-11 *cis*-13 18:3 over total CLnA was 18% lower in pomegranate oil than in seeds.

The incubated substrates in Experiment 2 slightly differed in the amount of crude protein, which was higher in the control and PS-20 (Table 2.3), whereas the ether extract was at least 2-fold greater in PS-20 than in other substrates. Treatments comprising pomegranate peels (i.e., PPP-20 and WPB-20) contained considerable amounts of total phenols and tannins. Regarding fatty acid profiles, the control (C) and PPP-20 substrates contained manly *cis*-9 *cis*-12 18:2, *cis*-9 18:1, 16:0 and *cis*-9 *cis*-12 *cis*-15 18:3, while punicic acid was predominant in WPB-20 and PS-20.

*3.2. Fatty acid composition of ruminal digesta (Experiment 1)*

Table 2.4 shows the effect of pomegranate oil and pomegranate tannins on the FA composition of the *in vitro* ruminal digesta after 12 or 24 hours of incubation. The concentration of most FA was altered, although the final product of the BH of C18 (i.e., stearic acid) was never significantly affected. Regarding other saturated FA, the concentration of 14:0 was higher in C and PO, while 16:0 was lower in PTO than in control ($P < 0.05$). The proportion of the odd- and branched- chain FA (OBCFA) was generally higher in the control and lower in PTO (e.g., 17:0 and 17:0 *anteiso* at 12 h, 15:0 and 15:0 *anteiso* at 24 h, or 17:0 *iso* at both incubation times; $P < 0.05$), whereas few significant reductions, compared with the control, were

observed for PO (e.g., 15:0 and 17:0) and PT (e.g., 14:0 *iso* and 15:0 *iso*; $P < 0.05$). Treatments containing pomegranate oil (i.e., PO and PTO) presented a higher proportion of *trans*-11 18:1, but these variations were only significant after 24 h of incubation ($P < 0.001$). Neither *cis*-9 18:1 nor *trans*-10 18:1 concentration differed with treatments. Among PUFA, the proportion of linoleic acid (*cis*-9 *cis*-12 18:2) was greater in those treatments containing tannin extract (i.e., PT and PTO) in 24 h incubations ($P < 0.01$). The proportion of rumenic acid (*cis*-9 *trans*-11 conjugated linoleic acid, CLA) was higher in PTO compared with the other treatments at 12 h ($P < 0.001$), and after 24 h its concentration was more than double in PT and PTO than in the control ($P < 0.01$). *Trans*-10 *cis*-12 CLA concentration was always higher in PT than in the other treatments ($P < 0.01$), and that of *trans*-11 *cis*-13 CLA in PTO ($P < 0.01$), except for the comparison with PO at 12 h. Although *trans*-11 *trans*-13 CLA proportion was higher in treatments containing pomegranate oil (i.e., PO and PTO; $P < 0.001$), the sum of other *trans,trans* CLA isomers was more abundant in PT than PO and PTO after 12 h of incubation ($P < 0.01$). The CLnA isomers of pomegranate were only detected in treatments containing pomegranate oil. The higher concentration of *cis*-9 *trans*-11 *cis*-13, *cis*-9 *trans*-11 *trans*-13, and *trans*-9 *trans*-11 *cis*-13 CLnA in PTO at 12 h ($P < 0.05$) disappeared at the longer incubation time.

**Table 2.4.** Fatty acid composition (g/100 g total fatty acids) of the ruminal digesta after 12 or 24 h of *in vitro* incubation with rumen inoculum from sheep (Experiment 1).

| | Time, h | Treatment[A] | | | | SED[B] | *P*-value |
|---|---|---|---|---|---|---|---|
| | | C | PO | PT | PTO | | |
| 14:0 | 12 | 2.35$^{a}$ | 2.07$^{a}$ | 1.43$^{b}$ | 1.38$^{b}$ | 0.141 | 0.001 |
| | 24 | 2.25$^{a}$ | 2.00$^{a}$ | 1.52$^{b}$ | 1.47$^{b}$ | 0.107 | <0.001 |
| 14:0 *iso* | 12 | 0.12 | 0.11 | 0.10 | 0.10 | 0.008 | 0.080 |
| | 24 | 0.17$^{a}$ | 0.16$^{ab}$ | 0.14$^{bc}$ | 0.14$^{c}$ | 0.008 | 0.004 |
| 15:0 | 12 | 0.78 | 0.74 | 0.74 | 0.71 | 0.028 | 0.216 |
| | 24 | 1.09$^{a}$ | 1.01$^{b}$ | 1.02$^{ab}$ | 0.96$^{b}$ | 0.023 | 0.003 |
| 15:0 *iso* | 12 | 0.30 | 0.29 | 0.23 | 0.23 | 0.021 | 0.025[C] |
| | 24 | 0.36$^{a}$ | 0.32$^{ab}$ | 0.27$^{b}$ | 0.24$^{b}$ | 0.024 | 0.009 |
| 15:0 *anteiso* | 12 | 0.84 | 0.78 | 0.64 | 0.63 | 0.064 | 0.041[C] |
| | 24 | 1.00$^{a}$ | 0.89$^{ab}$ | 0.76$^{b}$ | 0.70$^{b}$ | 0.055 | 0.006 |
| 16:0 | 12 | 12.37$^{a}$ | 11.55$^{ab}$ | 11.78$^{ab}$ | 11.37$^{b}$ | 0.276 | 0.031 |
| | 24 | 13.58$^{a}$ | 12.94$^{ab}$ | 12.67$^{ab}$ | 12.23$^{b}$ | 0.278 | 0.015 |
| 16:0 *iso* | 12 | 0.22 | 0.21 | 0.20 | 0.20 | 0.012 | 0.158 |
| | 24 | 0.29 | 0.27 | 0.25 | 0.23 | 0.018 | 0.040[C] |
| 17:0 | 12 | 0.62$^{a}$ | 0.60$^{b}$ | 0.61$^{a}$ | 0.57$^{c}$ | 0.003 | <0.001 |
| | 24 | 0.71$^{a}$ | 0.66$^{b}$ | 0.71$^{ab}$ | 0.67$^{ab}$ | 0.014 | 0.017 |
| 17:0 *iso* | 12 | 0.28$^{a}$ | 0.26$^{ab}$ | 0.27$^{ab}$ | 0.25$^{b}$ | 0.005 | 0.005 |
| | 24 | 0.40$^{a}$ | 0.38$^{ab}$ | 0.38$^{ab}$ | 0.36$^{b}$ | 0.008 | 0.007 |
| 17:0 *anteiso* | 12 | 0.36$^{a}$ | 0.32$^{ab}$ | 0.32$^{ab}$ | 0.30$^{b}$ | 0.015 | 0.032 |
| | 24 | 0.49 | 0.46 | 0.44 | 0.41 | 0.020 | 0.042[C] |
| 18:0 | 12 | 47.17 | 46.51 | 46.89 | 45.29 | 0.729 | 0.149 |
| | 24 | 52.39 | 51.30 | 52.87 | 51.76 | 0.708 | 0.232 |
| *cis*-9 18:1 | 12 | 3.03 | 2.93 | 2.94 | 3.07 | 0.117 | 0.585 |
| | 24 | 1.95 | 2.10 | 1.88 | 1.94 | 0.149 | 0.533 |
| *cis*-11 18:1 | 12 | 0.56$^{a}$ | 0.56$^{ab}$ | 0.47$^{c}$ | 0.50$^{bc}$ | 0.014 | 0.002 |
| | 24 | 0.41$^{ab}$ | 0.42$^{a}$ | 0.33$^{c}$ | 0.34$^{bc}$ | 0.020 | 0.007 |
| *cis*-12 18:1 | 12 | 0.71$^{a}$ | 0.70$^{a}$ | 0.58$^{b}$ | 0.61$^{b}$ | 0.024 | 0.003 |
| | 24 | 0.51 | 0.51 | 0.44 | 0.42 | 0.024 | 0.026[C] |

(*continued*)

**Table 2.4.** (*continued*)

| | Time, h | Treatment[A] | | | | SED[B] | *P*-value |
|---|---|---|---|---|---|---|---|
| | | C | PO | PT | PTO | | |
| *cis*-13 18:1 | 12 | 0.07 | 0.08 | 0.07 | 0.08 | 0.006 | 0.420 |
| | 24 | 0.06 | 0.06 | 0.06 | 0.07 | 0.005 | 0.192 |
| *cis*-15 18:1[D] | 12 | 0.12$^{ab}$ | 0.13$^{a}$ | 0.11$^{b}$ | 0.13$^{ab}$ | 0.005 | 0.026 |
| | 24 | 0.12 | 0.12 | 0.11 | 0.12 | 0.007 | 0.180 |
| *cis*-16 18:1 | 12 | 0.13 | 0.14 | 0.14 | 0.14 | 0.004 | 0.063 |
| | 24 | 0.10$^{b}$ | 0.11$^{ab}$ | 0.11$^{ab}$ | 0.12$^{a}$ | 0.003 | 0.044 |
| *trans*-5 18:1 | 12 | 0.07 | 0.07 | 0.06 | 0.06 | 0.004 | 0.088 |
| | 24 | 0.07 | 0.07 | 0.06 | 0.06 | 0.002 | 0.048[C] |
| *trans*-6, -7, -8 18:1 | 12 | 0.49$^{a}$ | 0.48$^{a}$ | 0.41$^{b}$ | 0.46$^{ab}$ | 0.019 | 0.017 |
| | 24 | 0.43$^{ab}$ | 0.45$^{a}$ | 0.38$^{b}$ | 0.41$^{ab}$ | 0.016 | 0.022 |
| *trans*-9 18:1 | 12 | 0.38$^{a}$ | 0.39$^{a}$ | 0.30$^{b}$ | 0.32$^{ab}$ | 0.021 | 0.011 |
| | 24 | 0.33$^{ab}$ | 0.35$^{a}$ | 0.28$^{b}$ | 0.31$^{ab}$ | 0.016 | 0.016 |
| *trans*-10 18:1 | 12 | 0.75 | 0.70 | 0.70 | 0.71 | 0.045 | 0.647 |
| | 24 | 0.62 | 0.63 | 0.68 | 0.61 | 0.061 | 0.624 |
| *trans*-11 18:1 | 12 | 8.92$^{ab}$ | 9.81$^{a}$ | 7.66$^{b}$ | 9.17$^{ab}$ | 0.365 | 0.006 |
| | 24 | 7.33$^{b}$ | 8.39$^{a}$ | 6.95$^{b}$ | 8.36$^{a}$ | 0.156 | <0.001 |
| *trans*-12 18:1 | 12 | 1.02$^{ab}$ | 1.10$^{a}$ | 0.88$^{b}$ | 1.00$^{ab}$ | 0.044 | 0.008 |
| | 24 | 0.91$^{ab}$ | 1.01$^{a}$ | 0.79$^{b}$ | 0.90$^{ab}$ | 0.055 | 0.027 |
| *trans*-13, -14 18:1 | 12 | 1.13$^{ab}$ | 1.27$^{a}$ | 0.89$^{b}$ | 1.16$^{ab}$ | 0.069 | 0.007 |
| | 24 | 1.13$^{ab}$ | 1.29$^{a}$ | 0.97$^{b}$ | 1.19$^{ab}$ | 0.065 | 0.013 |
| *trans*-15 18:1 | 12 | 0.85$^{a}$ | 0.91$^{a}$ | 0.71$^{b}$ | 0.84$^{a}$ | 0.020 | <0.001 |
| | 24 | 0.75$^{ab}$ | 0.86$^{a}$ | 0.71$^{b}$ | 0.79$^{ab}$ | 0.032 | 0.020 |
| *trans*-16 18:1[E] | 12 | 0.68$^{a}$ | 0.74$^{a}$ | 0.60$^{b}$ | 0.69$^{a}$ | 0.016 | <0.001 |
| | 24 | 0.68$^{ab}$ | 0.74$^{a}$ | 0.62$^{b}$ | 0.70$^{a}$ | 0.017 | 0.003 |
| *cis*-9 *cis*-12 18:2[F] | 12 | 5.96$^{b}$ | 5.78$^{b}$ | 10.23$^{a}$ | 8.42$^{ab}$ | 0.690 | 0.002 |
| | 24 | 2.87$^{b}$ | 2.97$^{b}$ | 5.24$^{a}$ | 4.42$^{a}$ | 0.410 | 0.001 |
| *cis*-9 *trans*-12 18:2 | 12 | 0.03$^{ab}$ | 0.02$^{b}$ | 0.04$^{a}$ | 0.03$^{ab}$ | 0.003 | 0.028 |
| | 24 | 0.02 | 0.01 | 0.02 | 0.02 | 0.003 | 0.172 |
| *trans*-9 *cis*-12 18:2 | 12 | 0.04$^{ab}$ | 0.03$^{b}$ | 0.06$^{a}$ | 0.05$^{ab}$ | 0.005 | 0.012 |
| | 24 | 0.03 | 0.03 | 0.04 | 0.04 | 0.005 | 0.491 |

(*continued*)

**Table 2.4.** (*continued*)

| | Time, h | Treatment[A] | | | | SED[B] | *P*-value |
|---|---|---|---|---|---|---|---|
| | | C | PO | PT | PTO | | |
| *cis*-9 *trans*-13 18:2[G] | 12 | 0.04 | 0.04 | 0.04 | 0.05 | 0.002 | 0.229 |
| | 24 | 0.05[a] | 0.04[ab] | 0.04[b] | 0.04[ab] | 0.003 | 0.037 |
| *trans*-9 *trans*-12 18:2 | 12 | 0.03 | 0.03 | 0.04 | 0.03 | 0.004 | 0.763 |
| | 24 | 0.02 | 0.02 | 0.03 | 0.02 | 0.002 | 0.112 |
| *trans*-11 *cis*-15 + *trans*-10 *cis*-15 18:2 | 12 | 0.13 | 0.13 | 0.14 | 0.13 | 0.007 | 0.378 |
| | 24 | 0.10 | 0.09 | 0.10 | 0.09 | 0.005 | 0.415 |
| *cis*-9 *trans*-11 CLA | 12 | 0.96[b] | 0.95[b] | 1.24[b] | 1.58[a] | 0.076 | <0.001 |
| | 24 | 0.51[b] | 0.62[ab] | 1.03[a] | 1.07[a] | 0.143 | 0.009 |
| *trans*-10 *cis*-12 CLA[H] | 12 | 0.22[b] | 0.12[c] | 0.31[a] | 0.19[b] | 0.018 | <0.001 |
| | 24 | 0.15[b] | 0.11[b] | 0.27[a] | 0.13[b] | 0.023 | 0.002 |
| *trans*-11 *cis*-13 CLA | 12 | 0.09[b] | 0.12[ab] | 0.11[b] | 0.15[a] | 0.008 | 0.002 |
| | 24 | 0.05[c] | 0.08[b] | 0.08[b] | 0.12[a] | 0.008 | <0.001 |
| *trans*-11 *trans*-13 CLA | 12 | 0.03[b] | 0.08[a] | 0.02[b] | 0.07[a] | 0.005 | <0.001 |
| | 24 | 0.02[b] | 0.07[a] | 0.02[b] | 0.08[a] | 0.003 | <0.001 |
| Other *trans trans* CLA[I] | 12 | 0.49[ab] | 0.41[b] | 0.65[a] | 0.34[b] | 0.060 | 0.010 |
| | 24 | 0.39 | 0.34 | 0.45 | 0.54 | 0.109 | 0.329 |
| *cis*-9 *cis*-12 *cis*-15 18:3 | 12 | 0.53 | 0.49 | 0.51 | 0.50 | 0.028 | 0.580 |
| | 24 | 0.37 | 0.33 | 0.26 | 0.23 | 0.043 | 0.061 |
| *cis*-9 *trans*-11 *cis*-13 CLnA | 12 | - | 0.13[b] | - | 0.27[a] | 0.030 | 0.039 |
| | 24 | - | 0.10 | - | 0.08 | 0.014 | 0.282 |
| *cis*-9 *trans*-11 *trans*-13 CLnA | 12 | - | 0.30[b] | - | 0.43[a] | 0.025 | 0.027 |
| | 24 | - | 0.19 | - | 0.21 | 0.015 | 0.221 |
| *trans*-9 *trans*-11 *cis*-13 CLnA | 12 | - | 0.28[b] | - | 0.42[a] | 0.018 | 0.016 |
| | 24 | - | 0.17 | - | 0.20 | 0.021 | 0.231 |
| *trans*-9 *trans*-11 *trans*-13 CLnA | 12 | - | 0.83 | - | 0.87 | 0.027 | 0.275 |
| | 24 | - | 0.53 | - | 0.63 | 0.111 | 0.432 |
| 10-oxo-18:0 | 12 | 0.12[a] | 0.10[ab] | 0.11[ab] | 0.08[b] | 0.011 | 0.032 |
| | 24 | 0.17[a] | 0.15[ab] | 0.17[a] | 0.13[b] | 0.005 | 0.003 |

(*continued*)

**Table 2.4.** (*continued*)

| | Time, h | Treatment[A] | | | | SED[B] | *P*-value |
|---|---|---|---|---|---|---|---|
| | | C | PO | PT | PTO | | |
| 13-oxo-18:0 | 12 | 0.19[a] | 0.18[ab] | 0.20[a] | 0.16[b] | 0.008 | 0.010 |
| | 24 | 0.23 | 0.20 | 0.23 | 0.20 | 0.012 | 0.036[C] |
| 16-oxo-18:0 | 12 | 0.36 | 0.33 | 0.37 | 0.35 | 0.011 | 0.059 |
| | 24 | 0.37[ab] | 0.35[b] | 0.39[a] | 0.36[ab] | 0.010 | 0.038 |

[A] Treatments: C, control; PO, pomegranate oil; PT, pomegranate tannins; PTO, pomegranate tannins and oil.
[B] SED, standard error of the difference.
[C] No significant differences were found for multiple comparisons using Bonferroni's method.
[D] Contains *trans*-10 *trans*-14 18:2 as a minor component.
[E] Coelutes with *cis*-14 18:1 and *trans*-9 *trans*-13 18:2.
[F] Contains *cis*-9 19:1 as a minor component.
[G] Coelutes with 11-cyclohexyl-11:0 and other minor 18:2 isomers.
[H] Coelutes with 21:0.
[I] Sum of *trans*-7 *trans*-9 + *trans*-8 *trans*-10 + *trans*-9 *trans*-11 CLA.
[a,b,c]Within a row, different superscripts indicate significant differences ($P < 0.05$).

### *3.3. Fatty acid composition of ruminal digesta (Experiment 2)*

As shown in Table 2.5, dietary inclusion of pomegranate by-products modified the FA composition of the *in vitro* ruminal digesta. Stearic acid was significantly affected only after 24 h of incubation, with the highest value being observed in PS-20 and the lowest in PPP-20 ($P < 0.001$). The *trans*-11 18:1 was the predominant monounsaturated FA and its proportion was always greater in the treatment containing 20% seeds ($P < 0.001$), with an intermediate value for WPB-20 in 24 h cultures ($P < 0.001$). Pomegranate seeds also increased the proportion of other *trans*-18:1 (e.g., *trans*-9, *trans*-10, and *trans*-12 to *trans*-16 18:1; $P < 0.05$), with intermediate increments being generally found in WPB-20 ($P < 0.05$).

Regarding *cis*-9 *cis*-12 18:2 and *cis*-9 *cis*-12 *cis*-15 18:3, increases with pomegranate peels and pulp (i.e., PPP-20) and decreases with seeds (i.e., PS-20) were found compared with the C ($P < 0.05$), with no effects in WPB-20, except for a modest increase in 18:2n-6 at 24 h ($P < 0.001$). The proportion of *cis*-9 *trans*-11 CLA was greater in PPP-20 than in C and PS-20 at 24 h post-inoculation ($P < 0.01$), but no differences were found at 12 h. *Trans*-11 *cis*-13 CLA and *trans*-11 *trans*-13 CLA concentrations were greater in PS-20, with intermediate values for WPB-20 ($P < 0.001$). The sum of other *trans*,*trans* CLA isomers was always highest in PS-20 ($P < 0.01$). The pomegranate CLnA isomers were absent in the control, the highest proportion was observed in the PS-20 treatment and the lowest in PPP-20 (which contained no detectable levels of *cis*-9 *trans*-11 *cis*-13 CLnA), while values were intermediate in WPB-20 ($P < 0.05$). All the pomegranate by-products decreased the concentrations of oxo FA ($P < 0.001$).

**Table 2.5.** Fatty acid composition (g/100 g total fatty acids) of the ruminal digesta after 12 or 24 h of *in vitro* incubation with rumen inoculum from sheep (Experiment 2).

| | Time, h | Treatment[A] | | | | SED[B] | *P*-value |
|---|---|---|---|---|---|---|---|
| | | C | PS-20 | PPP-20 | WPB-20 | | |
| 14:0 | 12 | 2.01[a] | 1.34[b] | 1.98[a] | 1.80[a] | 0.059 | <0.001 |
| | 24 | 1.93[ab] | 1.54[b] | 2.23[a] | 2.04[a] | 0.122 | 0.007 |
| 14:0 *iso* | 12 | 0.16[ab] | 0.11[c] | 0.16[a] | 0.14[b] | 0.005 | <0.001 |
| | 24 | 0.26[a] | 0.15[b] | 0.25[a] | 0.21[a] | 0.015 | <0.001 |
| 15:0 | 12 | 1.09[a] | 0.79[c] | 1.05[a] | 0.96[b] | 0.013 | <0.001 |
| | 24 | 1.52[a] | 1.05[d] | 1.45[b] | 1.30[c] | 0.011 | <0.001 |

(*continued*)

**Table 2.5.** (*continued*)

| | Time, h | Treatment[A] | | | | SED[B] | *P*-value |
|---|---|---|---|---|---|---|---|
| | | C | PS-20 | PPP-20 | WPB-20 | | |
| 15:0 *iso* | 12 | 0.40[a] | 0.27[c] | 0.38[a] | 0.34[b] | 0.010 | <0.001 |
| | 24 | 0.52[a] | 0.31[d] | 0.47[b] | 0.41[c] | 0.010 | <0.001 |
| 15:0 *anteiso* | 12 | 1.10[a] | 0.74[b] | 1.10[a] | 0.99[a] | 0.036 | <0.001 |
| | 24 | 1.40[a] | 0.84[c] | 1.38[a] | 1.19[b] | 0.019 | <0.001 |
| 16:0 | 12 | 16.09[a] | 11.92[c] | 16.14[a] | 14.56[b] | 0.242 | <0.001 |
| | 24 | 17.88[a] | 13.00[c] | 18.06[a] | 15.97[b] | 0.246 | <0.001 |
| 16:0 *iso* | 12 | 0.28[a] | 0.20[b] | 0.30[a] | 0.27[a] | 0.011 | <0.001 |
| | 24 | 0.39[a] | 0.26[c] | 0.40[a] | 0.35[b] | 0.009 | <0.001 |
| 17:0 | 12 | 0.87[a] | 0.63[c] | 0.85[a] | 0.77[b] | 0.016 | <0.001 |
| | 24 | 1.00[a] | 0.71[d] | 0.94[b] | 0.85[c] | 0.011 | <0.001 |
| 17:0 *iso* | 12 | 0.39[a] | 0.29[c] | 0.38[ab] | 0.34[b] | 0.010 | <0.001 |
| | 24 | 0.61[a] | 0.40[d] | 0.52[b] | 0.47[c] | 0.006 | <0.001 |
| 17:0 *anteiso* | 12 | 0.46[a] | 0.34[c] | 0.46[a] | 0.41[b] | 0.012 | <0.001 |
| | 24 | 0.73[a] | 0.47[c] | 0.69[a] | 0.59[b] | 0.016 | <0.001 |
| 18:0 | 12 | 51.48 | 49.21 | 49.63 | 50.90 | 0.828 | 0.100 |
| | 24 | 52.03[c] | 54.61[a] | 50.80[d] | 53.31[b] | 0.308 | <0.001 |
| *cis*-9 18:1 | 12 | 2.86[b] | 2.87[b] | 3.53[a] | 3.13[ab] | 0.143 | 0.010 |
| | 24 | 2.00[b] | 1.98[b] | 2.50[a] | 2.21[b] | 0.067 | <0.001 |
| *cis*-11 18:1 | 12 | 0.47 | 0.51 | 0.55 | 0.49 | 0.025 | 0.073 |
| | 24 | 0.34[b] | 0.37[ab] | 0.38[a] | 0.39[a] | 0.007 | 0.003 |
| *cis*-12 18:1 | 12 | 0.24[ab] | 0.31[a] | 0.24[ab] | 0.22[b] | 0.023 | 0.028 |
| | 24 | 0.17[b] | 0.20[a] | 0.18[ab] | 0.18[ab] | 0.008 | 0.024 |
| *cis*-13 18:1 | 12 | 0.05[bc] | 0.09[a] | 0.04[c] | 0.06[b] | 0.004 | <0.001 |
| | 24 | 0.04[c] | 0.06[a] | 0.04[bc] | 0.04[b] | 0.002 | <0.001 |
| *cis*-15 18:1[C] | 12 | 0.08[b] | 0.16[a] | 0.07[b] | 0.09[b] | 0.007 | <0.001 |
| | 24 | 0.07[c] | 0.14[a] | 0.07[c] | 0.08[b] | 0.003 | <0.001 |
| *cis*-16 18:1 | 12 | 0.06[b] | 0.13[a] | 0.06[b] | 0.07[b] | 0.004 | <0.001 |
| | 24 | 0.05[b] | 0.11[a] | 0.05[b] | 0.07[b] | 0.005 | <0.001 |

(*continued*)

**Table 2.5.** (*continued*)

| | Time, h | Treatment[A] | | | | SED[B] | *P*-value |
|---|---|---|---|---|---|---|---|
| | | C | PS-20 | PPP-20 | WPB-20 | | |
| *trans*-5 18:1 | 12 | 0.05$^{b}$ | 0.07$^{a}$ | 0.05$^{c}$ | 0.05$^{bc}$ | 0.002 | <0.001 |
| | 24 | 0.05$^{b}$ | 0.06$^{a}$ | 0.05$^{b}$ | 0.05$^{b}$ | 0.002 | <0.001 |
| *trans*-6, -7, -8 18:1 | 12 | 0.28$^{b}$ | 0.43$^{a}$ | 0.24$^{b}$ | 0.29$^{b}$ | 0.021 | <0.001 |
| | 24 | 0.24$^{b}$ | 0.39$^{a}$ | 0.24$^{b}$ | 0.26$^{b}$ | 0.007 | <0.001 |
| *trans*-9 18:1 | 12 | 0.23$^{b}$ | 0.44$^{a}$ | 0.25$^{b}$ | 0.23$^{b}$ | 0.024 | <0.001 |
| | 24 | 0.18$^{b}$ | 0.34$^{a}$ | 0.19$^{b}$ | 0.20$^{b}$ | 0.010 | <0.001 |
| *trans*-10 18:1 | 12 | 0.27$^{b}$ | 0.47$^{a}$ | 0.27$^{b}$ | 0.24$^{b}$ | 0.032 | 0.001 |
| | 24 | 0.25$^{b}$ | 0.36$^{a}$ | 0.22$^{b}$ | 0.21$^{b}$ | 0.022 | 0.002 |
| *trans*-11 18:1 | 12 | 5.27$^{b}$ | 7.72$^{a}$ | 4.94$^{b}$ | 5.64$^{b}$ | 0.333 | <0.001 |
| | 24 | 4.54$^{c}$ | 6.88$^{a}$ | 4.64$^{c}$ | 5.11$^{b}$ | 0.083 | <0.001 |
| *trans*-12 18:1 | 12 | 0.61$^{b}$ | 1.02$^{a}$ | 0.57$^{b}$ | 0.58$^{b}$ | 0.045 | <0.001 |
| | 24 | 0.48$^{b}$ | 0.82$^{a}$ | 0.46$^{b}$ | 0.53$^{b}$ | 0.030 | <0.001 |
| *trans*-13, -14 18:1 | 12 | 0.50$^{b}$ | 1.53$^{a}$ | 0.44$^{b}$ | 0.56$^{b}$ | 0.059 | <0.001 |
| | 24 | 0.42$^{c}$ | 1.39$^{a}$ | 0.41$^{c}$ | 0.60$^{b}$ | 0.035 | <0.001 |
| *trans*-15 18:1 | 12 | 0.56$^{b}$ | 1.03$^{a}$ | 0.57$^{b}$ | 0.59$^{b}$ | 0.037 | <0.001 |
| | 24 | 0.43$^{b}$ | 0.88$^{a}$ | 0.41$^{b}$ | 0.50$^{b}$ | 0.026 | <0.001 |
| *trans*-16 18:1[D] | 12 | 0.38$^{bc}$ | 0.82$^{a}$ | 0.34$^{c}$ | 0.45$^{b}$ | 0.024 | <0.001 |
| | 24 | 0.36$^{c}$ | 0.78$^{a}$ | 0.34$^{c}$ | 0.45$^{b}$ | 0.007 | <0.001 |
| *cis*-9 *cis*-12 18:2[E] | 12 | 3.15$^{b}$ | 2.19$^{c}$ | 4.46$^{a}$ | 3.61$^{ab}$ | 0.221 | <0.001 |
| | 24 | 1.70$^{c}$ | 1.11$^{d}$ | 2.33$^{a}$ | 1.92$^{b}$ | 0.053 | <0.001 |
| *cis*-9 *trans*-12 18:2 | 12 | 0.02 | 0.03 | 0.02 | 0.02 | 0.002 | 0.048[F] |
| | 24 | 0.02 | 0.02 | 0.01 | 0.01 | 0.001 | 0.552 |
| *trans*-9 *cis*-12 18:2 | 12 | 0.03 | 0.03 | 0.03 | 0.03 | 0.003 | 0.094 |
| | 24 | 0.04 | 0.03 | 0.02 | 0.02 | 0.006 | 0.228 |
| *cis*-9 *trans*-13 18:2[G] | 12 | 0.05 | 0.05 | 0.05 | 0.05 | 0.004 | 0.806 |
| | 24 | 0.06$^{ab}$ | 0.05$^{b}$ | 0.06$^{a}$ | 0.06$^{ab}$ | 0.003 | 0.012 |
| *trans*-9 *trans*-12 18:2 | 12 | 0.03 | 0.03 | 0.03 | 0.02 | 0.003 | 0.090 |
| | 24 | 0.03 | 0.03 | 0.03 | 0.02 | 0.002 | 0.533 |

(*continued*)

**Table 2.5.** (*continued*)

| | Time, h | Treatment[A] | | | | SED[B] | *P*-value |
|---|---|---|---|---|---|---|---|
| | | C | PS-20 | PPP-20 | WPB-20 | | |
| *trans*-11 *cis*-15 + *trans*-10 *cis*-15 18:2 | 12 | 0.11[a] | 0.09[c] | 0.11[ab] | 0.10[bc] | 0.004 | 0.002 |
| | 24 | 0.09[a] | 0.06[c] | 0.08[ab] | 0.08[b] | 0.004 | <0.001 |
| *cis*-9 *trans*-11 CLA | 12 | 0.14 | 0.15 | 0.15 | 0.14 | 0.012 | 0.870 |
| | 24 | 0.09[b] | 0.09[b] | 0.16[a] | 0.13[ab] | 0.014 | 0.007 |
| *trans*-10 *cis*-12 CLA[H] | 12 | 0.11[a] | 0.08[c] | 0.10[ab] | 0.10[b] | 0.002 | <0.001 |
| | 24 | 0.09[ab] | 0.08[b] | 0.10[a] | 0.09[ab] | 0.005 | 0.013 |
| *trans*-11 *cis*-13 CLA | 12 | 0.03[c] | 0.60[a] | 0.04[c] | 0.27[b] | 0.025 | <0.001 |
| | 24 | 0.02[c] | 0.37[a] | 0.04[c] | 0.19[b] | 0.011 | <0.001 |
| *trans*-11 *trans*-13 CLA | 12 | 0.02[b] | 0.45[a] | 0.03[b] | 0.12[b] | 0.033 | <0.001 |
| | 24 | 0.02[c] | 0.32[a] | 0.02[c] | 0.13[b] | 0.022 | <0.001 |
| Other *trans trans* CLA[I] | 12 | 0.10[b] | 0.33[a] | 0.09[b] | 0.10[b] | 0.037 | 0.002 |
| | 24 | 0.09[b] | 0.20[a] | 0.09[b] | 0.08[b] | 0.018 | 0.001 |
| *cis*-9 *cis*-12 *cis*-15 18:3 | 12 | 0.58[b] | 0.35[c] | 0.72[a] | 0.61[b] | 0.034 | <0.001 |
| | 24 | 0.38[b] | 0.21[c] | 0.44[a] | 0.38[b] | 0.016 | <0.001 |
| *cis*-9 *trans*-11 *cis*-13 CLnA | 12 | - | 0.93 | - | 0.42 | 0.147 | 0.072 |
| | 24 | - | 0.43[a] | - | 0.22[b] | 0.042 | 0.036 |
| *cis*-9 *trans*-11 *trans*-13 CLnA | 12 | - | 0.98[a] | 0.08[c] | 0.49[b] | 0.075 | <0.001 |
| | 24 | - | 0.43[a] | 0.05[c] | 0.22[b] | 0.022 | <0.001 |
| *trans*-9 *trans*-11 *cis*-13 CLnA | 12 | - | 0.98[a] | 0.04[c] | 0.43[b] | 0.061 | <0.001 |
| | 24 | - | 0.46[a] | 0.04[c] | 0.19[b] | 0.038 | <0.001 |
| *trans*-9 *trans*-11 *trans*-13 CLnA | 12 | - | 2.18[a] | 0.08[b] | 0.92[ab] | 0.470 | 0.027 |
| | 24 | - | 0.96[a] | 0.05[b] | 0.34[b] | 0.123 | <0.001 |
| 10-oxo-18:0 | 12 | 0.15[a] | 0.10[c] | 0.12[b] | 0.09[c] | 0.005 | <0.001 |
| | 24 | 0.23[a] | 0.13[d] | 0.20[b] | 0.17[c] | 0.007 | <0.001 |
| 13-oxo-18:0 | 12 | 0.24[a] | 0.21[ab] | 0.19[bc] | 0.18[c] | 0.009 | 0.002 |
| | 24 | 0.29[a] | 0.24[b] | 0.23[b] | 0.23[b] | 0.010 | 0.003 |
| 16-oxo-18:0 | 12 | 0.53a | 0.36d | 0.46b | 0.42c | 0.008 | <0.001 |
| | 24 | 0.60a | 0.39d | 0.50b | 0.45c | 0.010 | <0.001 |

[A] C, control TMR; PS-20, 80% DM of control TMR and 20% DM of pomegranate seeds; PPP-20, 80% DM of control TMR and 20% DM of pomegranate peels and pulp; WPB, 80% DM of control TMR and 20% DM of whole pomegranate by-product.

[B] SED, standard error of the difference.
[C] Contains *trans*-10 *trans*-14 18:2 as a minor component.
[D] Coelutes with *cis*-14 18:1 and *trans*-9 *trans*-13 18:2.
[E] Contains *cis*-9 19:1 as a minor component.
[F] No significant differences were found for multiple comparisons using Bonferroni's method.
[G] Coelutes with 11-cyclohexyl-11:0 and other minor 18:2 isomers.
[H] Coelutes with 21:0.
[I] Sum of *trans*-7 *trans*-9 + *trans*-8 *trans*-10 + *trans*-9 *trans*-11 CLA.
[a,b,c] Within a row, different superscripts indicate significant differences ($P < 0.05$).

### *3.4. Ruminal fermentation (Experiment 2)*

The effects of the dietary inclusion of pomegranate by-products on rumen fermentation parameters are reported in Table 2.6. Although no differences were observed at 12 h, the control diet presented a higher DMD than PPP-20 and WPB-20 ($P < 0.01$) after 24 h of incubation. Similarly, the gas production was decreased with the inclusion of by-products only in 24 h-incubations ($P < 0.001$). The concentration of ammonia was lower in PPP-20 and WPB-20 treatments at both times, whereas that of lactic acid was higher in PPP-20 than in PS-20 at 24 h ($P < 0.05$). The production of VFA was always decreased in substrates containing pomegranate by-products ($P < 0.05$). Concerning molar proportions, PPP-20 and WPB-20 increased that of acetate, and the opposite occurred for minor VFA (i.e., the sum of isobutyrate, isovalerate, valerate and caproate; $P < 0.001$). The butyrate proportion slightly differed at 12 h, with a greater value in PS-20 than in WPB-20 ($P < 0.05$).

**Table 2.6.** Rumen fermentation parameters after 12 or 24 h of *in vitro* incubation with rumen inoculum from sheep (Experiment 2).

| | Time, h | Treatment[A] | | | | SED[C] | *P*-value |
|---|---|---|---|---|---|---|---|
| | | C | PS-20 | PPP-20 | WPB-20 | | |
| DM disappearance, g/g | 12 | 0.700 | 0.654 | 0.660 | 0.642 | 0.029 | 0.304 |
| | 24 | 0.762[a] | 0.727[ab] | 0.719[b] | 0.712[b] | 0.010 | 0.006 |
| Gas production, mL/g of DM | 12 | 195 | 180 | 187 | 184 | 4.52 | 0.076 |
| | 24 | 246[a] | 222[b] | 227[b] | 228[b] | 2.71 | 0.001 |
| Ammonia, mg/L | 12 | 372[a] | 404[a] | 261[b] | 284[b] | 13.2 | <0.001 |
| | 24 | 549[a] | 546[a] | 412[b] | 425[b] | 15.9 | <0.001 |
| Lactic acid, mg/L | 12 | 5.32 | 5.26 | 6.12 | 5.54 | 0.348 | 0.153 |
| | 24 | 5.86[ab] | 5.45[b] | 6.52[a] | 6.17[ab] | 0.284 | 0.042 |
| Total VFA[B], mmol/L | 12 | 64.3[a] | 60.0[b] | 59.4[b] | 59.6[b] | 0.536 | <0.001 |
| | 24 | 84.7[a] | 78.8[b] | 78.4[b] | 76.5[b] | 1.311 | 0.004 |
| Molar proportions, mol/mol | | | | | | | |
| Acetate | 12 | 0.646[b] | 0.642[b] | 0.660[a] | 0.661[a] | 0.001 | <0.001 |
| | 24 | 0.634[b] | 0.632[b] | 0.650[a] | 0.647[a] | 0.001 | <0.001 |
| Propionate | 12 | 0.203 | 0.203 | 0.202 | 0.203 | 0.002 | 0.939 |
| | 24 | 0.187 | 0.188 | 0.186 | 0.187 | 0.001 | 0.498 |
| Butyrate | 12 | 0.118[ab] | 0.121[a] | 0.120[ab] | 0.117[b] | 0.001 | 0.046 |
| | 24 | 0.129 | 0.128 | 0.130 | 0.130 | 0.001 | 0.541 |
| Others[D] | 12 | 0.033[a] | 0.034[a] | 0.018[b] | 0.019[b] | 0.001 | <0.001 |
| | 24 | 0.050[a] | 0.052[a] | 0.034[c] | 0.036[b] | 0.001 | <0.001 |
| Acetate:propionate ratio | 12 | 3.18 | 3.17 | 3.27 | 3.27 | 0.038 | 0.075 |
| | 24 | 3.41[bc] | 3.37[c] | 3.49[a] | 3.47[ab] | 0.021 | 0.003 |

[A] C, control TMR; PS-20, 80% DM of control TMR and 20% DM of pomegranate seeds; PPP-20, 80% DM of control TMR and 20% DM of pomegranate peels and pulp; WPB, 80% DM of control TMR and 20% DM of whole pomegranate by-product.
[B] VFA, volatile fatty acids.
[C] SED, standard error of the difference.
[D] Calculated as the sum of isobutyrate, isovalerate, valerate and caproate.
[a,b,c] Within a row, different superscripts indicate significant differences ($P < 0.05$).

## 4. Discussion

Inclusion of WPB in the ovine diet has recently been shown to enrich meat and milk with potentially health-promoting FA of dietary and ruminal origin (e.g., 18:2n-6, 18:3n-3, *trans*-11 18:1 and *cis*-9 *trans*-11 CLA; Natalello *et al.,* 2019; Valenti *et al.,* 2019a). However, it remains unclear whether this is due to the presence of bioactive PUFA in the WBP, to its tannin content, or to the interaction between these compounds. To fill this gap, our first experiment tested the effects of pomegranate oil and tannins, alone or in combination, on BH, and the second experiment compared the ruminal responses to pomegranate by-products rich in PUFA, tannins or both bioactive components (i.e., PS, PPP, and the WPB).

### *4.1. Fatty acid composition of ruminal digesta (Experiment 1)*

Tannins are known to be able to modulate the FA composition of ruminant meat and milk (Vasta and Luciano, 2011), but their mechanism of action is still controversial (e.g., Toral *et al.,* 2018). Early publications reported a specific inhibition of the last BH step (i.e., the saturation of *trans* 18:1 to 18:0), which increased *trans*-11 18:1 concentrations in rumen digesta (e.g., Vasta *et al.,* 2009; Khiaosa-ard *et al.,* 2009), while other studies have evidenced a slowdown of the initial steps of the process, which favours the ruminal accumulation of dietary PUFA (Carreño *et al.,* 2015; Alves *et al.,* 2017). This discrepancy may be due, first of all, to tannins' dose and structural and chemical dissimilarities, which make it difficult to generalize on the effects of these phenolic compounds (Vasta and Luciano, 2011; Carreño *et al.,* 2015; Costa *et al.,* 2017). Results observed in the PT treatment (in

particular, the higher concentrations of 18:2n-6 and *cis*-9 *trans*-11 CLA) would support the hypothesis of a general impairment of BH rather than a specific effect on the last step. In addition, data from PTO at 12 h suggest that pomegranate tannins offered a transient protection of CLnA from BH, which would also have potentially positive implications for consumer's health (Lansky and Newman, 2007; Viuda-Martos *et al.,* 2010). Furthermore, we found no evidence of a tannin-induced shift in BH pathways towards the accumulation of the non-desirable *trans*-10 18:1, in agreement with earlier trials (Khiaosa-ard *et al.,* 2009; Carreño *et al.,* 2015; Costa *et al.,* 2017). Nevertheless, comparison with the literature is challenging, because information on the actual content of tannins, either in plants or in extracts, may be highly variable due to the lack of standardisation on the analysis of tannins and the use of different standards to express their concentration (Álvarez del Pino *et al.,* 2005; Kotsampasi *et al.,* 2017; Valenti *et al.,* 2019b). Moreover, tannin-induced effects on BH may largely depend on the basal diet composition (use of concentrate vs. pasture-based diets, inclusion or not of lipid supplements, etc.; Vasta *et al.,* 2009; Alves *et al.,* 2017).

The impact of phenolic compounds on BH seems to be mediated by changes in microbial composition (Buccioni *et al.,* 2015; Carreño *et al.,* 2015; Vasta *et al.,* 2019), which would be consistent with the effect of the pomegranate tannin extract on most OBCFA, given their known microbial origin (Fievez *et al.,* 2012). On the contrary, the limited effects of PO on OBCFA suggest that incomplete BH of its constituent FA would be largely responsible for variations in digesta FA composition, as reported for other plant oils (Shingfield *et al.,* 2008, 2010). Indeed,

even if the specific BH pathways of punicic acid and other CLnA have not been described yet, it is plausible that *trans*-11 *cis*-13 CLA, *trans*-11 *trans*-13 CLA and *trans*-11 18:1 are intermediate products of this process. However, we found no clear evidence of a similar involvement of *cis*-9 *trans*-11 CLA, which contrasts with results by Ishlak *et al.* (2014). The greater level of pomegranate oil inclusion in this latter *in vitro* study (3% diet DM) and the use of rumen inoculum from cows might explain their observed increase not only in *cis*-9 *trans*-11 CLA but also in *trans*-10 18:1, which remained unaffected in our trial, as discussed above. We reported previously (Natalello *et al.,* 2019; Valenti *et al.,* 2019a) that feeding pomegranate by-products did not increase *trans*-10 18:1 in ovine meat and milk, but we are not aware of similar data in bovine. Nevertheless, cattle seem more prone to the *trans*-10 shift than sheep when their diet is supplemented with other PUFA-rich plant lipids (Mele *et al.,* 2006; Shingfield *et al.,* 2010; Correddu *et al.,* 2015).

In general, results from Experiment 1 suggest that the increase of *cis*-9 *trans*-11 CLA and *trans*-11 18:1 previously observed in meat and milk of sheep fed WPB (Natalello *et al.,* 2019; Valenti *et al.,* 2019a) could be due to the simultaneous presence of both pomegranate tannins and oil, which would favour the ruminal accumulation of the CLA and 18:1 isomers, respectively. Although rumenic acid concentrations in PTO at 12 h of incubation might point to a synergistic effect between pomegranate bioactive compounds, it seems that no other data support synergistic but additive effects.

*4.2. Fatty acid composition of ruminal digesta (Experiment 2)*

Results from Experiment 2 support that pomegranate tannins and PUFA played different roles in modulating digesta FA composition. In this trial, more evident changes were detected for some minor FA, which helped to provide new insights into CLnA metabolism.

It is accepted that major BH pathways of *cis*-9 *cis*-12 18:2 and *cis*-9 *cis*-12 *cis*-15 18:3 begin with a *cis*-12 to *trans*-11 isomerisation step that yields a conjugated system (i.e., *cis*-9 *trans*-11), which favours the saturation of the *cis*-9 double bond and produces *trans*-11 18:1 or *trans*-11 *cis*-15 18:2 as intermediate products (Shingfield *et al.,* 2010; Alves and Bessa, 2014). Given the natural occurrence of the *cis*-9 *trans*-11 conjugated system in *cis*-9 *trans*-11 *cis*-13 CLnA (punicic acid), a quick and direct saturation step may explain the extensive disappearance of this abundant FA in pomegranate lipids. This might also be the first BH step of *cis*-9 *trans*-11 *trans*-13 CLnA (α-eleostearic acid). Our hypothesis is supported by the lack of differences in *cis*-9 *trans*-11 CLA and other *cis*-9-containing intermediates in digesta in PS-20, compared with the large increases in the proportion of *trans*-11 *cis*-13 and *trans*-11 *trans*-13 CLA, the putative first BH intermediates of both CLnA. Under this premise, *cis*-9 *trans*-11 CLA would not derive from pomegranate CLnA metabolism, which contrasts, as mentioned before, with the increased rumenic acid accumulation reported by Ishlak *et al.* (2014) in *in vitro* incubations of pomegranate oil with rumen inoculum from cows, and might be due to technical or interspecies differences, among others. In any event, both hypotheses are compatible with *cis*-9 *trans*-11 CLA increments in the meat or milk

of ruminants fed pomegranate by-products (Modaresi *et al.,* 2011; Emami *et al.,* 2015; Natalello *et al.,* 2019), because this FA largely derives from *trans*-11 18:1 desaturation in body tissues (Palmquist *et al.,* 2004; Shingfield *et al.,* 2010). As discussed for Experiment 1, the positive effects of pomegranate tannins on digesta *cis*-9 *trans*-11 CLA content would also contribute to elucidate *in vivo* responses.

The greater accumulation of *trans*-9 *trans*-11 *trans*-13 CLnA in substrates containing pomegranate by-products in comparison with other CLnA, especially *cis*-9 *trans*-11 *cis*-13 CLnA (punicic acid), was unexpected. We speculated that it might be accounted for by a slower saturation rate of *trans* double bonds, because they are less toxic to rumen microbiota than *cis* double bonds (Heipieper *et al.,* 2010). Temperature-induced isomerisation of *cis*-9 *trans*-11 *cis*-13 CLnA to *trans*-9 *trans*-11 *trans*-13 CLnA has been demonstrated during methylation (Chen *et al.,* 2007), but we are not aware if a similar reaction may be caused by the action of ruminal microorganisms. Further research would be needed to elucidate the BH of these CLnA, and confirm whether their metabolism proceeds via direct saturation steps (as supported by the increases in *trans*-11 *cis*-13 and *trans*-11 *trans*-13 CLA, and in *trans*-9, *trans*-11, *cis*-13 and *trans*-13 18:1) or other pathways and isomerisation steps (as suggested by accumulation of *trans*-10, *cis*-15, *trans*-15 or *trans*-16 18:1 and *trans*-10 *cis*-12 CLA).

The detection of some oxylipids in our trial (i.e., 10-, 13- and 16-O-18:0) is in line with the demonstration that hydration and subsequent oxidation of unsaturated C18 FA is an alternative to BH for some ruminal bacteria (Hudson *et al.,* 1998; Jenkins *et al.,* 2006; Alves *et al.,* 2013). However, results from PS-20

treatment and Experiment 1 would indicate that pomegranate CLnA might not be metabolized through this pathway. The lower concentration of 18:2n-6 and other oxylipid precursors in PPP-20 and WPB-20 substrates might be involved in the decrease in oxo-18:0 proportions, as may also be the presence of tannins. However, the few available studies on the action of these phenolic compounds on oxylipids report inconsistent observations, with either decreases, no effects, or even increases, probably due to the different type of tannins and dosage rates (Carreño *et al.,* 2015; Costa *et al.,* 2017). In any event, bacterial populations responsible for oxo-FA production might require a longer adaptation to treatments, as suggested by time-dependent increases in 10-O-18:0 after lipid supplementation in sheep (Toral *et al.,* 2010).

*4.3. Rumen fermentation (Experiment 2)*

Hydroxyl radicals in tannins have a well-known affinity for dietary protein, which inhibits its ruminal degradation and consistently decreases concentrations in rumen fluid of ammonia and minor VFA (i.e., those that originate mostly from deamination of some amino acids) (Frutos *et al.,* 2004; Getachew *et al.,* 2008; Patra and Saxena, 2011). This effect was observed with the two tannin-containing substrates (PPP-20 and WPB-20), and agrees with previous reports on the effects of tannin-rich plants or extracts (e.g., Getachew *et al.,* 2008; Buccioni *et al.,* 2015). However, there probably was some contribution of their lower crude protein content as well.

The presence of tannins at high levels may also explain the slight to moderate detrimental impact of PPP-20 and WPB-20 treatments (i.e., those containing tannins) on ruminal parameters related to fibre fermentation (i.e., DMD, gas production and total VFA concentration), although slight increases in acetate proportion were also observed. Earlier studies have reported negative, positive and no effects of tannins on these parameters, including acetate production and molar proportions (e.g., Frutos *et al.,* 2004; Khiaosa-ard *et al.,* 2009; Costa *et al.,* 2017). Once again, the controversy would be most probably explained by differences in the type and dosage rate of tannins (Getachew *et al.,* 2008; Buccioni *et al.,* 2015; Patra and Saxena, 2011), although the experimental approach may also be of relevance. In our *in vitro* trial, the use of high by-product inclusion rates in a closed system could have favoured the detection of effects on fermentation that might actually have no detrimental repercussion on *in vivo* animal performance, as supported by a recent study including the same level of WPB in the diet of growing lambs (Natalello *et al.,* 2019).

The negative impact of high amounts of oil-rich products in the diet on fibre rumen fermentation (Jenkins, 1993) may be the reason for reductions in gas production and total VFA concentration with the inclusion of 20% of pomegranate seeds in the substrate, whereas smaller amounts would not detrimentally affect rumen function (Shingfield *et al.,* 2008; Toral *et al.,* 2010; Vargas-Bello-Pérez *et al.,* 2016).

## 5. Conclusions

Bioactive compounds in pomegranate by-products favour the ruminal accumulation of potentially health-promoting FA present in dietary lipids (e.g., 18:2n-6 and 18:3n-3) or derived from microbial BH (e.g., *trans*-11 18:1 or *cis*-9 *trans*-11 CLA). The former response and the increase in *cis*-9 *trans*-11 CLA seem to be accounted for by the presence of tannins, whereas *trans*-11 18:1 increments would derive from the BH of FA in pomegranate oil, specifically from CLnA isomers. The rumen metabolism of these latter conjugated FA might be explained by direct saturation steps, although isomerisation by rumen bacteria cannot be ruled out. Pomegranate tannins protect dietary protein from ruminal degradation, but a negative impact on ruminal fermentation may also exist when high levels of pomegranate by-products are included in the diet. There seem to be no evident synergistic but additive effects between pomegranate bioactive compounds (i.e., tannins and PUFA) on ruminal BH or fermentation.

# CHAPTER 7 - Dietary pomegranate by-product improves oxidative stability of lamb meat

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**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.

***Key words:*** *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

of fundamental importance 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 the potential health properties of this fruit. 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 *et al.,* 2010; Johanningsmeier and 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 pioneer study the 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 the animal antioxidant status (Emami *et al.,* 2015a) and meat fatty acid composition (Emami *et al.,* 2015b) in kids. Recently, we also observed a desirable increment of the polyunsaturated fatty acids (PUFA) in meat (Natalello *et al.,* 2019) and milk (Valenti *et al.,* 2019a) 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 a sought-after purpose for the positive effects on human health, the higher susceptibility of PUFA to peroxidation may drastically worsen meat shelf-life (Bekhit *et al.,* 2013). Few studies have investigated the shelf-life of meat from

ruminants fed with pomegranate by-products. Emami *et al.* (2015c) 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, it should be stressed that the concentration of total phenols in pomegranate seed is not high, especially if compared to other portions of the fruits, such as peels (Pande and Akoh, 2009; Orak *et al.,* 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 *et al.,* 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 oxidative deterioration. We hypothesized that the diverse bioactive molecules present in the WPB could help to 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 halving and squeezing mechanically 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 are presented in Table 3.1.

**Table 3.1**. Ingredients, chemical composition and antioxidant capacity of the experimental diets and whole pomegranate by-product.

| | Whole pomegranate by-product | Experimental diet[a] | |
|---|---|---|---|
| | | 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 mix[b] | | 2.1 | 2.1 |
| *Chemical composition, g/100 g DM* | | | |
| Dry matter, g/100 as fed | 90.0 | 88.7 | 89.2 |
| Crude Protein (CP) | 6.52 | 17.6 | 17.8 |
| NDF[c] | 28.8 | 23.3 | 26.3 |

(*continued*)

**Table 3.1**. (*continued*)

| | Whole pomegranate by-product | Experimental diet[a] | |
|---|---|---|---|
| | | CON | WPB |
| ADF[c] | 20.7 | 12.9 | 15.5 |
| ADL[c] | 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[d] | 9.51 | 0.30 | 1.89 |
| Total tannins[d] | 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[e]* | | | |
| Hydrophilic fraction | 684 | 103 | 342 |
| Lipophilic fraction | 27.3 | 21.1 | 31.1 |

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

*2.2. Animals and experimental treatments*

The experiment was approved by the University of Catania (approval: 015CT325) and the animals were raised at the experimental farm of the University of Catania (37°24'35.3''N 15°03'34.9''E), handled by specialized personnel following the European Union Guidelines (2010/63/ EU Directive).

As described by Natalello *et al.* (2019), the trial involved 17 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 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 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 3.1. 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. During the experimental period all the lambs had always free access to fresh water. 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 the 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 then 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 *et al.* (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 in detail 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 *et al.* (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 pending 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 *et al.* (2019a). 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 (10–80 µmol) was mixed with 1 mL of fluorescein (80nM); 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 485nm and 520nm, 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. Here instead, 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 *et al.* (2019b).

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 *et al.* (2019b). 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 *et al.* (2019b). Meat samples (2.5 g) were homogenized with 12.5 mL of distilled water using a Heidolph Diax 900 tissue homogenizer (Heidolph Elektro GmbH & 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 GLM model to test the effect of the dietary treatment. Data on oxidative stability measured in raw and cooked meat were analysed using a GLM 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 adjustment for multiple comparisons. 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 3.1, 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 compared γ-tocopherol in the pomegranate by-product and both compounds were found at a greater concentration in the WPB diet compared to 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 bodyweight gain, voluntary feed intake and feed conversion ratio (Table 3.2). The above differences in the concentration of phenolic compounds between the diets, led to a greater intake of total phenolic compounds and of 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 ($P < 0.001$).

**Table 3.2.** Effect of the dietary treatment on lamb performances and intakes.

| | Dietary treatment[a] | | SEM[b] | *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[c], g/day | 821 | 882 | 19.50 | 0.125 |
| ADG[c], g/day | 234 | 235 | 7.200 | 0.921 |
| FCR[c], 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 |

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

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

As shown in Table 3.3, 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 3.1, the WPB diet increased the antioxidant capacity (ORAC) of the lipophilic fraction of meat ($P < 0.05$).

**Table 3.3.** Effect of the dietary treatment on the muscle pH, myoglobin, antioxidant vitamins and oxidizable fatty acids in the intramuscular fat.

| | Dietary treatment[a] | | SEM[b] | *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[c] | 0.39 | 0.57 | 0.041 | 0.024 |
| Peroxidability index[d] | 2.07 | 2.85 | 0.191 | 0.042 |

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

*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 3.4. 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 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 over storage duration, 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 3.2). Furthermore, raw meat from lambs in the WPB treatment had lower TBARS values compared to the CON group after 7 days of storage ($P < 0.05$). Also, statistically comparable results were observed between fresh meat (day 0) from control group and the WPB meat stored over 7 days ($P > 0.05$).

**Table 3.4**. 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* values | 46.99 | 49.02 | 48.57 | 48.65 | 47.48 | 0.296 | 0.005 | 0.068 | 0.533 |
| a* values | 11.56 | 11.60 | 12.24$^{a}$ | 11.58$^{ab}$ | 10.93$^{b}$ | 0.166 | 0.929 | <0.001 | 0.181 |
| b* values | 11.21 | 11.39 | 10.15$^{b}$ | 11.87$^{a}$ | 11.93$^{a}$ | 0.194 | 0.663 | <0.001 | 0.159 |
| C values | 16.14 | 16.29 | 15.91 | 16.59 | 16.19 | 0.214 | 0.789 | 0.474 | 0.168 |
| $h_{ab}$ values | 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 adjustment for multiple comparisons.

## 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 el al., 2013). Among the pro-oxidant factors, heme iron in myoglobin has been demonstrated to promote the initiation of lipid oxidation (Baron and 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), due to the lower bond dissociation energies necessary to start the oxidative process compared to monounsaturated or saturated FA and the susceptibility of PUFA to oxidation increases with increasing degree of unsaturation (Johnson and Decker, 2015). Therefore, variations in the concentration of highly unsaturated PUFA may affect meat oxidative stability, although generally these fatty acids represent quantitative minor constituents of the intramuscular fat. Particularly, meat from ruminants is well known to contain low concentrations of PUFA in favour of saturated and monounsaturated fatty acids (SFA and MUFA, respectively). Therefore, many viable dietary strategies have been developed to improve the nutritional quality of ruminant meat by increasing the content of PUFA (Scollan, *et al.,* 2014; Toral *et al.,* 2018). However, such feeding strategies may

present the drawback of impairing the oxidative stability of meat if the increase in the unsaturation degree of the intramuscular fat is 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, which was confirmed also *in vitro* (Natalello *et al.,* 2020). As a consequence, as reported by Natalello *et al.* (2019), meat from the WPB-fed lambs contained specific PUFA derived from the whole pomegranate by-product (i.e., conjugated linolenic acid isomers) which were not detected in meat from CON-fed lambs. Also, other PUFA, such as rumenic acid, were more abundant in meat from animals in the WPB treatment compared to the CON group. 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 and 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 *et al.,* 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 *et al.,* 2005). Also, among the heterogeneous class of phenolic compounds, tannins are considered to be poorly bioavailable in animals (Vasta and 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 *et al.,* 2005; Mphahlele *et al.,* 2016; Natalello *et al.,* 2020).

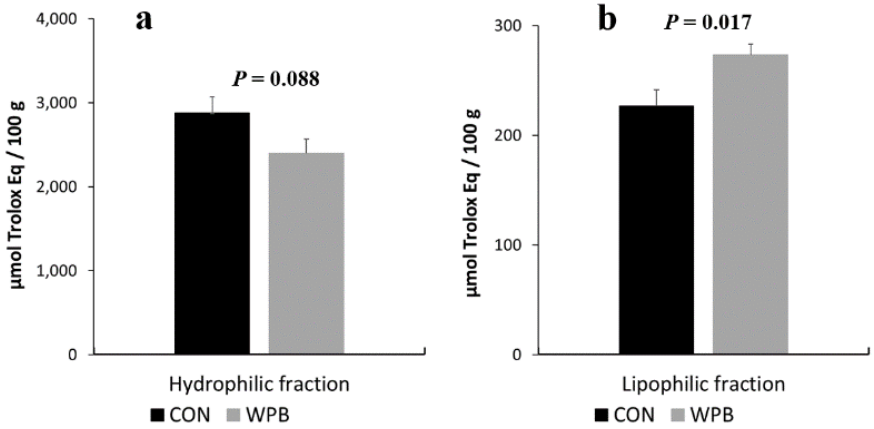


**Figure 3.1.** Effect of the dietary treatment (CON or WPB) on raw meat antioxidant capacity of hydrophilic (a) and lipophilic (b) fraction. Values presented are the estimated least squares means and standard error bars.

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 and 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 *et al.,* 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 *et al.,* 2005; Iglesias *et al.,* 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 *et al.,* 2017; Ortuño *et al.,* 2015; Valenti *et al.,* 2019b). 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 *et al.,* 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 *et al.*

(2015c) 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 and 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 *et al.,* 2015b; Kotsampasi *et al.,* 2014).

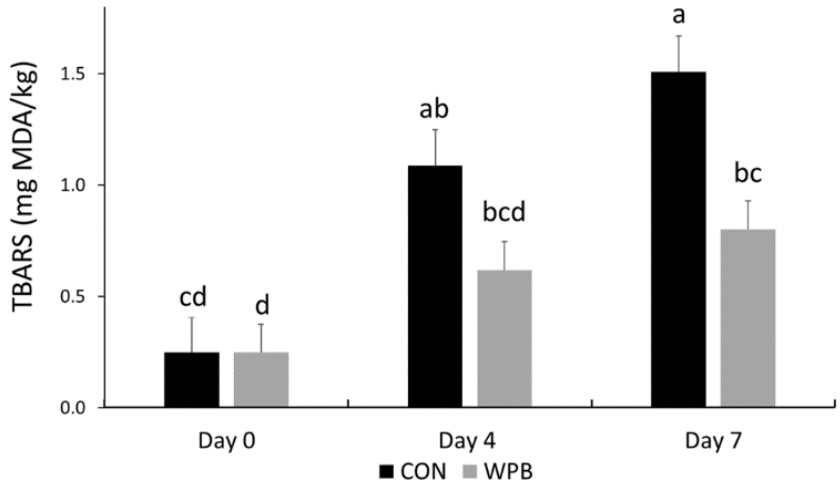


**Figure 3.2**. Effect of the dietary treatment (CON or WPB) and time of storage (days 0, 4 and 7) on TBARS values of raw meat over aerobic storage at 4 °C. Values presented are the estimated least squares means and standard error bars.
[a,b,c]Indicate differences between mean values ($P$ <0.05) tested using the Tukey's adjustment for multiple comparisons.

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 *et al.,* 2000; Priolo *et al.,* 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 and 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 *et al.,* 2019b; 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 *et al.* (2015c) 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 *et al.,* 2010b). 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.

# Concluding remarks

Overall, the results of the studies conducted in the frame of the present thesis encourage the use of whole pomegranate by-product (WPB) as an alternative feedstuff to partially replace the cereals in ruminant feeding and to improve the meat quality, by enriching of potentially health-promoting fatty acids and by delaying the oxidative deterioration of meat stored in retail conditions.

**First.** The proportion of PUFA, rumenic acid (*cis*-9 *trans*-11 CLA) and vaccenic acid (*trans*-11 18:1) in muscle was increased by feeding lambs with WPB and the health-promoting CLnA of pomegranate were deposited in the intramuscular fat. Moreover, the dietary administration of WPB prevented the *t*10-shift in intensive concentrate-based feeding systems. Taken together, the results obtained from the feeding experiment in the ruminal digesta, liver and muscle suggest that the concurrence of both tannins and polyunsaturated fatty acid in WPB would have favoured the deposition of desirable fatty acids in meat. However, in this experiment, it was not possible to understand the mechanisms and the contribution of each WPB bioactive compound and to investigate potential synergistic effects.

**Second.** The *in vitro* study confirmed the findings of the *in vivo* experiment. Indeed, bioactive compounds in pomegranate by-products favour the ruminal accumulation of potentially health-promoting FA. Moreover, from the *in vitro* study it would seem that the increase of 18:2 n-6, 18:3n-3 and *cis*-9 *trans*-11 CLA might

be accounted for by the presence of tannins, whereas the increase of *trans*-11 18:1 would derive from the BH of pomegranate CLnA.

There seem to be no evident synergistic but additive effects between pomegranate bioactive compounds (i.e., tannins and PUFA) on ruminal BH or fermentation. Additionally, pomegranate tannins protect dietary protein from ruminal degradation, but a negative impact on ruminal fermentation may also exist when high levels of pomegranate by-products are included in the diet.

**Third.** Dietary pomegranate has led to a reduction of lipid oxidation in fresh and cooked meat during refrigerated storage, despite the greater concentration of polyunsaturated fatty acids. The formation of metmyoglobin was also slightly reduced. Additionally, the meat antioxidant capacity of the lipophilic fraction was increased by inclusion of pomegranate by-product, whereas the hydrophilic fraction was not affected. These results could be attributed to a direct antioxidant effect of vitamin E, which was found at higher concentration in muscle from animal fed whole pomegranate by-product. These findings add novel information on the antioxidant effect of feeding pomegranate by-products, which so far have been mainly attributed to phenolic compounds.

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