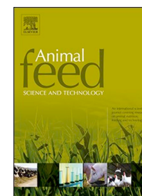
Animal Feed Science and Technology 259 (2020) 114355

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



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

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ARTICLE INFO

Keywords:
Conjugated linolenic acid
Lipids
Phenolic compounds
Punicic acid
Tannins

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,

Abbreviations: ADF, acid detergent fibre; ADL, acid detergent lignin; aNDF, neutral detergent fibre; BH, biohydrogenation; C, control; CLA, conjugated linoleic acid; CLnA, conjugated linolenic acid; DM, dry matter; DMD, dry matter disappearance; FA, fatty acid; FAME, fatty acid methyl ester; OBCFA, odd- and branched-chain fatty acid; PE, phenolic extract; PEG, polyethylene glycol; PO, pomegranate oil treatment; PPP, pomegranate peels and pulp; PS, pomegranate seeds; PSO, pomegranate seed oil; PT, pomegranate tannin treatment; PTO, pomegranate tannins and oil treatment; PUFA, polyunsaturated fatty acid; TMR, total mixed ration; VFA, volatile fatty acid; WPB, whole pomegranate by-product

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https://doi.org/10.1016/j.anifeedsci.2019.114355
Received 31 July 2019; Received in revised form 5 November 2019; Accepted 8 November 2019
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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.

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

Table 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
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.

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 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.

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 1. Animals had continuous access to clean drinking water.

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

Table 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.

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.

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 °C until FA analysis.

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 3.

Table 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.

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.

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 $0.05 \le P < 0.10$. Least squares means are reported.

3. Results

3.1. Chemical composition of pomegranate by-products and oil and incubated substrates

The chemical composition of TMR and pomegranate by-products are reported in Table 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, 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 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 4 shows the effect of pomegranate oil and pomegranate tannins on the FA composition of the in vitro ruminal digesta after 12 or 24 h 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.

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

As shown in Table 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 ($P < 0.001$).

3.4. Ruminal fermentation (Experiment 2)

The effects of the dietary inclusion of pomegranate by-products on rumen fermentation parameters are reported in Table 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

Table 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).

		Treatment[A]					
	Time, h	C	PO	PT	PTO	SED[B]	*P*-value
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]
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
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

(*continued on next page*)

Table 4 (*continued*)

	Time, h	Treatment[A] C	PO	PT	PTO	SED[B]	*P*-value
	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
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,b,c]Within a row, different superscripts indicate significant differences ($P < 0.05$).
[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.

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 ($P < 0.05$).

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).

Table 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
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
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
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

(*continued on next page*)

Table 5 (*continued*)

	Time, h	Treatment[A] C	PS-20	PPP-20	WPB-20	SED[B]	*P*-value
	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	0.53^{a}	0.36^{d}	0.46^{b}	0.42^{c}	0.008	< 0.001
	24	0.60^{a}	0.39^{d}	0.50^{b}	0.45^{c}	0.010	< 0.001

[a,b,c]Within a row, different superscripts indicate significant differences ($P < 0.05$).
[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.

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

Table 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,b,c] Within a row, different superscripts indicate significant differences ($P < 0.05$).

[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.

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.

Declaration of Competing Interest

No conflict of interest.

Acknowledgements

This study was funded by the Instituto de Ganadería de Montaña (CSIC-University of León, Spain) and the University of Perugia (Department DSA3; project “Ricerca di Base – 2015”). P. G. Toral benefits from a ‘Ramón y Cajal’ research contract (MINECO-European Regional Development Fund).

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