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Evaluation of grape pomace from red wine by-product as feed for sheep

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Abstract

BACKGROUND: This work aimed to study the chemical composition and *in vitro* digestibility of seeds and pulp from grape pomace. *In sacco* degradability, rumen fermentation of grape pomace fractions and plasma lipid peroxidation were also studied in sheep fed with or without grape pomace.

RESULTS: Seeds and pulp fractions of grape pomace had different values for cell walls (523 vs. 243 g kg^{-1} dry matter (DM)), crude protein (CP, 104 vs.138 g kg^{-1}DM), ether extract (EE, 99.0 vs. 31.7 g kg^{-1} DM), polyunsaturated fatty acids (69.6 vs. 53.3%) and extractable polyphenols (55.0 vs. 32.1 g kg^{-1} DM). The *in vitro* true digestibility, DM *in sacco* degradability and CP degradability in seeds and pulp were also different (0.51 vs. 0.82; 0.30 vs. 0.45; 0.66 vs. 0.39). Ammonia-N concentration and total volatile fatty acids (VFA) in ruminal liquid were significantly lower, and plasma lipid peroxidation was also numerically lower in sheep that consumed grape pomace.

CONCLUSION: The nutritive value of grape pomace varies, depending on the proportion of seeds and pulp. The interest of this by-product in sheep feeding could be related to its polyphenol and PUFA content, which could improve the meat and milk quality.

Keywords: grape pomace, nutritive value, phenolic compounds, rumen degradation, sheep

INTRODUCTION

Winemaking has great social and economic importance in Spain.[1] Grape pomace, consisting of peel, pulp and seeds, is the main residue produced in the wine industry. These by-products generate a serious environmental and economic problem in relation to their storage, processing and disposal. Red grape pomace undergoes a fermentation-maceration process in which polyphenols are transferred to the wine, although a high proportion still remains in the wine by-products.[2] The phenolic compound content depends on the type of grape, the part of tissue considered, the grape cultivation conditions, the stage of maturity, and the winemaking process.[3]

According to some authors, a major limitation of the use of grape pomace as ruminant feed is the presence of a high level of lignified fibre and secondary compounds, including phenolics such as tannins and anthocyanins,[4] which can have potentially negative effects on digestive nutrient utilization. In contrast, it has been reported that tannins may also improve rumen metabolism by increasing protein supply to the small intestine owing to decreased ruminal degradability and by decreasing methanogenesis.[5] Furthermore, several studies have shown that phenolic compounds possess antioxidant capacity, which could have beneficial effects on animal product quality.[6] In addition, the presence of high amounts of linoleic and oleic acids reported in winery wastes[7] may also have beneficial effects on meat and milk fatty acid profiles.

Evaluation of the chemical composition, phenolic compounds, digestibility and ruminal degradability of seeds and pulp of red grape pomace obtained from winery by-products is essential in order to evaluate the use of these by-products in ruminant feeding.

The objective of the present work was to study the pulp and seeds of grape pomace derived from red wine produced in Castilla y León, by analysing their chemical composition and *in vitro* digestibility and the *in sacco* degradability of their nutrients in sheep.

MATERIALS AND METHODS

Grape pomace samples and chemical composition

Six red wine wineries in Castilla y León (Spain) belonging to the most important Denominations of Origin in this area were selected, and 5 kg of representative fresh grape pomace samples (*Vitis vinifera* sp.) were collected. Samples were dried at 50 °C until constant weight and seeds were manually separated from the pulp plus skin fraction (hereafter referred to as 'pulp'). The samples were ground to pass through a 1 mm screen and frozen at −20 °C until chemical analysis.

The chemical composition (dry matter, DM; organic matter, OM; ether extract, EE; and crude protein, CP) of each grape pomace fraction (seeds and pulp) were determined by standard methods.[8] Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) analyses were performed according to Van Soest et al.,[9] using an ANKOM^{200} fibre analyser (Ankom Technology Corporation, Macedon, NY, USA). Sodium sulphite and heat-stable amylase were used in analysis of NDF, and both NDF and ADF were expressed inclusive of residual ash. Acid detergent insoluble crude protein (ADICP) was determined by Kjeldahl analysis of ADF residues and transformed into CP by multiplying by 6.25.

Lipid extraction from grape pomace (seed and pulp fractions) was performed according to Bligh and Dyer.[10] Lipid extract was methylated following the method of Morrison and Smith,[11] and the methyl esters were quantified using an Agilent 6890 N Network System gas chromatograph (Palo Alto, CA, USA). The gas chromatograph conditions have been described previously by Castro et al.[12]

For determination of phenolic compounds, samples of grape pomace fractions (seeds and pulp) were extracted by shaking at room temperature with methanol–water (50:50 vol/vol, 50 mL g^{-1} of sample for 60 min) and acetone–water (70:30 vol/vol, 50 mL g^{-1} of sample for 60 min). After centrifugation (3000 × *g*, 15 min), supernatants were combined and used to measure the extractable polyphenols (EP), hydrolysable polyphenols (HP), condensed tannins (CT) and total anthocyanins (TA) by colorimetric

methods using a Perkin-Elmer Lambda 25 UV/VIS Spectrometer (Perkin Elmer, MA, USA). Extractable polyphenols were determined by the Folin–Ciocalteu procedure,[13] using gallic acid as standard at 760 nm. To determine the HP content, the methanol–acetone–water extracts were hydrolysed by a methanol–H_2SO_4 treatment following the method of Hartzfeld et al.,[14] and then phenolic content was determined in the hydrolysates using gallic acid at 760 nm.[13] Condensed tannins were analysed by means of a HCl–butanol (5:95 vol/vol, 100 °C for 1.5 h) treatment of the methanol–acetone–water extracts (modified method of Reed et al.[15]). Condensed tannin absorbance was determined at 550 nm and calculated according to Bate-Smith[16] and Ribéreau-Gayon and Stonestreet.[17] Total anthocyanins were determined according to Ribéreau-Gayon and Stonestreet[18] in extract from selected samples, using the bisulphite bleaching method at 520 nm.

Phenolic acids, stilbenes, flavonols and flavanols were quantified according to the methods of Del Álamo et al.[19] and Gallego et al.[20] Extraction of seed and pulp compounds from the previous methanol–acetone–water extracts were carried out using Waters Oasis HLB 200 mg cartridges (Milford, MA, USA). The analyses were performed using a Hewlett-Packard 1100 (LC-DAD) system (Avondale, PA, USA), equipped with Hypersil ODS column (200mm×4.6mm i.d., particle size 5µm). The chromatographic conditions were the following: 10µl injection volume; solvent A was water: acetic acid (98:2) and solvent B was acetic acid:acetonitrile:water (2:20:78) by volume. The flow rate was 1 ml min^{-1}, with 100% A isocratic conditions for 55 min, with a linear gradient from 0 to 70% solvent B over 55 min. Simultaneous detection was allowed at 254, 280 and 340 nm, the UV-Vis spectra (scanning from 190 to 400 nm) were recorded for all peaks. Calibrations were carried out for each phenol from a stock solution with the twelve low molecular weight phenols, by dilution in a solution of synthetic wine (12% ethanol and 3.5 g L^{-1} tartaric acid) to different concentrations. Anthocyanins and other anthocyanin derivatives were determined from the previous methanol-acetone-water seed and pulp extracts. The separation was performed using

a Hypersorb Prontosil ODS column (250 mm x 4.6 mm i.d., particle size 0.45 µm) from Sugelabor (Madrid, Spain). The chromatographic conditions were the following: 30 ml injection volume; 0.8 ml min^{-1} flow-rate; eluent A was methanol; eluent B was methanol:water:formic acid (45:45:10), and eluent C was formic acid:water (15:85). Zero-time conditions were A:B:C (0:25:75); at 25 min the pump was adjusted to A:B:C (0:80:20) and kept at such for 10 min; at 38–43 min the conditions were A:B:C (100:0:0). Detection was allowed at 528 nm and quantification was carried out by means of the external pattern method and by measurement of each peak area. The results are related to malvidin-3-glucoside, in mg L^{-1}.

Degradation kinetics and ruminal fermentation

Eight healthy, non-pregnant, non-lactating Churra ewes (62.7 ± 5.81 kg), each fitted with a permanent rumen cannula (i.d. 35 mm), were used. The animals were housed in individual pens and had free access to water. They were fed at maintenance level (45 g DM kg $LBW^{-0.75}$), in two equal meals at 8:00 and 17:00 h, with a total mixed ration (TMR) consisting of lucerne hay and concentrate in a ratio of 50:50. The ewes (4 animals per treatment) were assigned randomly on the basis of live body weight to two dietary treatments (Table 1): a control treatment (CTRL, without grape pomace), and a grape pomace treatment (GP-75, 75 g kg^{-1} of grape pomace mix from all six red wine wineries, DM basis). The sheep were adapted to the diet for 10 days. Daily intake was recorded and there were no orts. All animal handling practices followed the recommendations of European Council Directive 2010/63/EU. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Valladolid (Spain).

Ruminal degradability of DM, OM and CP of grape pomace fractions (seeds and pulp) was measured in the eight ewes used in the study using the *in sacco* nylon bag technique described by Ørskov et al.[21] Approximately 4 g samples, ground to pass

through a 2 mm screen, were weighed into nylon bags (70 mm × 130 mm, 50 μm pore size). Two bags per sample were incubated in the rumen of each ewe for 0, 3, 6, 12, 24, 48 and 72 h. After each incubation period the nylon bags were rinsed under cold tap water, and then stored at −20 °C for at least 24 h to remove any microbial cells adhering to the particles. After defrosting, the bags were washed with cold water for 20 min and dried for 48 h at 60 ºC. Zero time disappearance was obtained by washing unincubated bags in a similar way. Bag residues were analysed and DM, OM and CP degradation were estimated according to the exponential model of Ørskov and McDonald:[22] $Y = a + b\ (1 - e^{(-ct)})$. The values for disappearance of grape pomace fractions (DM, OM and CP) with time were fitted by non-linear regression using the NLIN procedure in the SAS 9.2 software package. The effective degradability (ED) was calculated assuming passage rates (k) in the rumen of 0.06 (ED6) h^{-1} as: $ED = a + [bc/(c + k)]$.

On days 7 and 9 of the experimental period, rumen content was manually extracted through the cannula of all sheep before first feeding (0 h) and at 1, 3, 6 and 9 h after feeding. Rumen fluid was strained through four layers of cheesecloth, and its pH value was measured immediately. Five mL of the rumen fluid was acidified with 5 mL of 0.2 mol L^{-1} HCl for ammonia-N (NH3-N) determination. A further 0.8 mL of the rumen fluid was added to 0.5 mL of a deproteinising solution (20 g L^{-1} metaphosphoric acid and 4 g L^{-1} crotonic acid in 0.5 mol L^{-1} HCl) for volatile fatty acid (VFA) determination. All samples were stored at −20 ºC until analysis. Ammonia-N concentration in rumen fluid was determined as described by Weatherburn[23] and VFA analyses were performed by gas chromatography,[24] in both cases in centrifuged samples.

In vitro true digestibility

In vitro true digestibility of seed and pulp samples from red grape pomace was determined by the procedure of Tilley and Terry[25] modified by Goering and Van Soest[26] using a Daisy^{II} incubator according to the methodology described by Ankom Technology Corporation (Macedon, NY, USA).[27] The rumen inoculum was withdrawn

from the four Churra sheep that had been fitted with a rumen cannula for the CTRL treatment. Rumen fluid was transferred into pre-warmed (39 °C) thermal bottles and squeezed through four layers of cheesecloth under anaerobic conditions. The inoculum was a mixture (4:1 vol/vol) of rumen liquor and buffer solution consisting of buffer solution A (KH_2PO_4, 10.0 g L^{-1}; $MgSO_47H_2O$, 0.5g L^{-1}; NaCl, 0.5 g L^{-1}; $CaCl_22H_2O$, 0.1 g L^{-1}, and urea, 0.5 g L^{-1}) and buffer solution B (Na_2CO_3, 15.0 g L^{-1} and Na_2S7H_2O, 1.0 g L^{-1}). Portions weighing 0.5 ± 0.05 g were placed in F57 filter bags (Ankom Technology). Three bags per feedstuff were used in each assay. The bags were incubated with the inoculum in the Daisy^{II} incubator at 39 ± 1 °C for 48 h under permanent agitation. After incubation the bags were rinsed thoroughly with cold tap water. Afterwards, the bags were placed in the ANKOM^{200} fibre analyser for determination of NDF in order to remove microbial debris and any remaining soluble fractions. *In vitro* true digestibility was calculated according to Ankom Technology Corporation.[26]

Ewe plasma lipid peroxidation

On days 12 and 14 of the experimental period, ewes were blood sampled by jugular venipuncture 0, 3, 6 and 9 h after feeding in the morning. The blood samples collected in heparin tubes were immediately placed in ice and centrifuged (1600 × *g*, 10 min, 4 °C). Then plasma was separated and stored at −80 °C. Lipid peroxidation was analysed in the plasma samples using the thiobarbituric acid-reactive substances (TBARS) Assay Kit provided by Cayman Chemical (Ann Arbor, MI, USA), according to the manufacturer's instructions.

Statistical analyses

Statistical analysis was performed using the SAS 9.2 software package. Data of the chemical composition of grape pomace fractions, *in vitro* true digestibility, ruminal fermentation and plasma TBARS were subjected to one-way analysis of variance using

the general lineal model (GLM) procedure which considered the effect of the dietary treatment (T; CTRL and GP-75). The LSD test was used to assess the significance between treatment means where the effect was significant. Degradability parameters were analysed according to a factorial model which considered the effect of the samples (S; seeds and pulp), the effect of the dietary treatment of cannulated ewes (T; CTRL and GP-75) and their interaction (S x T). For all statistical procedures, the statistical significance of differences was defined as $P < 0.05$ and trends as $P < 0.10$.

RESULTS AND DISCUSSION

Primary compounds and fatty acid composition

Dry matter content of whole grape pomace ranged from 240 to 637 g kg^{-1} which was close to the values obtained in other works[28,29] and higher than the values reported by Balasan et al.[30] and Baumgärtel et al.[3] for pomace from red grapes. The pressure applied during the processing stage and the moment of collection in the winery affect evaporation losses and could explain differences in the moisture content of grape pomace reported in the literature. The proportion of seeds in the grape pomace ranged from 406 to 564 g kg^{-1} DM. These data are in the range obtained by Spanghero et al.[29] for grape pomace of red wines of other varieties; therefore, the type of red wine produced did not seem to affect the seed proportion..

Grape pomace is of a lignocellulosic nature, with high cell wall (ranging from 851 to 854 g kg^{-1} DM), ADF (ranging from 281 to 343 g kg^{-1} DM) and lignin (ranging from 163 to 232 g kg^{-1} DM) contents. As shown in Table 2, the NDF, ADF and ADL contents in the pulp fraction were 53, 57 and 79% lower than in the seed fraction. However, the *in vitro* true digestibility ($P < 0.05$) in pulp (ranging from 810 to 900 g kg^{-1}) was higher than in seeds (ranging from 500 to 560 g kg^{-1}), supporting the view that digestibility of grape pomace is mainly due to non-structural carbohydrates. The protein content of whole grape pomace ranged from 104 to 134 g kg^{-1} DM, and the proportion

of CP bound to fibre (ADICP) was lower ($P < 0.05$) in seeds than in pulp (104 vs. 138 g kg^{-1} DM and 13.8 and 24.5%, respectively).

The ether extract of whole grape pomace (ranging from 56.4 to 73.3 g kg^{-1} DM) was within the range found in other studies.[7] Grape pomace pulp has a lower content of EE than seeds (31.7 vs. 99.0 g kg^{-1} DM, $P < 0.05$). As shown in Table 2, in seeds the saturated fatty acids (SFA) content was lower (126 vs. 314 g kg^{-1} of total fatty acids, $P < 0.05$) and polyunsaturated fatty acids (PUFA) were higher than in pulp (696 vs. 533 g kg^{-1} of total fatty acids, $P < 0.05$. One of the most commonly employed strategies for increasing the levels of healthy fatty acids in ruminant foods (meat and milk), such as rumenic acid (RA) and n-3 polyunsaturated fatty acids (PUFAs) has been to increase their levels in the rumen by the use of feeds rich in linoleic and linolenic acids.[31] The high PUFA concentration, especially the high proportion of linoleic acid found in the seed fraction (ranging from 673 to 708 g kg^{-1}) and the pulp fractions (ranging from 343 to 427 g kg^{-1}) may suggest potential value for dietary inclusion with the objective of providing substrate for production of bioactive fatty acids in the rumen. In fact, in previous research[32,33] it was reported that the inclusion of a variety of winery industry residues in ruminant feeding could be a good way of multiplying the levels of RA in milk fat. The proportion of C18:3 was higher ($P < 0.05$) in pulp (ranging from 106 to 150 g kg^{-1} of total fatty acids) than seeds (ranging from 5 to 6 g kg^{-1} of total fatty acids). Most seeds are not broken open during eating or rumination, and therefore the fatty acids of pulp fraction could be more useful than seeds to improved fatty acid profile in ruminant products.

Phenolic compounds

Total phenolic levels in whole grape pomace (Table 2) were comparable to reported values obtained by Besharati and Taghizadeh.[34] Extractable polyphenol content was much higher ($P < 0.05$) in seeds (ranging from 49.1 to 58.4 g kg^{-1} DM) than in pulp (ranging from 26.4 to 38.3 g kg^{-1} DM), which is in accordance with

256 Spanghero et al.[29] Hydrolysable polyphenols content was slightly higher (P < 0.05) in
257 seeds (ranging from 8.90 to 9.56 g kg^{-1} DM) than pulp (ranging from 7.0 to 8.5 g kg^{-1}
258 DM) . Some authors[29] have indicated that grape by-products, especially their seeds,
259 present high levels of CT content, which could have potentially negative effects on
260 ruminal fermentation. In contrast, the pulp fraction has a lower level of fibre, with less
261 lignin, and an overall reduction in tannin levels.[4] This statement is in agreement with
262 the present results, since CT content was higher in seeds (ranging from 69.2 to 116.0 g
263 kg^{-1} DM) than in pulp (ranging from 14.1 to 26.7 g kg^{-1}DM). These values are lower
264 than those obtained by Molina-Alcaide et al.[28] However, it should be noted that the
265 samples analysed by those authors came from two different wineries and the authors
266 did not specify the percentages of pulp and seeds in the samples, or the type of wine
267 produced. In the present work, anthocyanin contents were much lower (P < 0.05) in
268 seeds (ranging from 0.91 to 0.41 g kg^{-1} DM) than in pulp (ranging from 3.80 to 9.41 g
269 kg^{-1} DM). The reason for these results is that grapes owe their colour to this pigment,
270 which is mostly in their pulp and skins, colouring wine deep red or purple.[3]
271 The detailed profile of the seed and pulp phenolic compounds is given in Table
272 3. The total content of phenolic acids was lower (P < 0.05) in seeds (ranging from 25.9
273 to 64.1 g kg^{-1} DM) than in pulp (ranging from 82.5 to 206 mg kg^{-1} DM), syringic acid,
274 vanillic acid and gallic acid being the major phenolic acids. *Trans* resveratrol stilbene
275 was only detected in pulp (1.08 mg kg^{-1} DM, ranging from 0 to 3.82 mg kg^{-1} DM). The
276 content of total flavonols did not differ between seeds and pulp (P > 0.05), but there
277 was a notable difference in the content of quercetin flavonol in seeds (ranging from
278 0.37 to 8.74 g kg^{-1} DM) and pulp (ranging from 0.09 to 4.00 mg kg^{-1} DM). The content
279 of total flavanols was higher in seeds (ranging from 10.2 to 16.8 g kg^{-1} DM) than in pulp
280 (ranging from 4.70 to 9.04 g kg^{-1} DM), because the catechin concentration was
281 significantly higher (P < 0.05) in seeds. The main anthocyanins were anthocyanin
282 derivatives, malvidin 3-O glucoside and petunidin 3-O glucoside, which is in agreement
283 with Antoniolli et al.,[35] and they were only detected in the pulp fraction.

The variability of our results is consistent with different studies[29,36] witch quantified secondary compounds in grape seeds and skins. The differences between the contents of secondary compounds in seeds and pulp and the lower fibre level, with less lignin and less CT, suggest that pulp might have potential nutritive value for ruminant nutrition [29] and might also be a potential source of antioxidant polyphenols, such as *trans* resveratrol stilbene and anthocyanins.[2] If the seeds could be separated from the pulp and skin fraction, the latter may have potential as a ruminant feed and a natural source of antioxidants.

Degradation kinetics and ruminal fermentation

The rumen degradation parameters (Table 4) of the whole gra*pe* pomace are within the range obtained by other authors.[28,34] However, the rumen degradation of different fractions of grape pomace (seeds and pulp) has not been reported extensively. In our study, the DM effective degradability (ED) values were higher ($P < 0.05$) in pulp (ranging from 421 to 474 g kg-1) than in seeds (ranging from 274 to 324 g kg-1) probably owing to their lower cell wall content and lignification as it has been reported previously for different lignocellulosic by-products.[37] With regard to the CP degradability, PD and ED were significantly lower ($P < 0.05$) in pulp (ranging from 339 to 447 g kg-1 CP) than in seeds (ranging from 615 to 697 g kg-1 CP) probably attributable to the higher proportion of CP bound to ADF content in the pulp (24.6 %) than in the seeds fraction (13.8 %), as previously mentioned.

The diet fed to the sheep (CTRL vs. GP-75) had minor effects on ruminal degradation parameters of DM, OM and CP. In spite of the presence in the diet of secondary compounds, such as tannins, the rate of protein degradation in the rumen did not decrease.[5] Mean pH values (Figure 1) were lower for the CTRL diet ($P < 0.05$) than for the GP-75 diet (6.35 vs. 6.48). Changes in rumen pH are primarily determined by fermentation products, such as VFA and lactic acid, derived from carbohydrate fermentation, which were higher in CTRL ewes, as detailed later. The ammonia-N

312 concentration in ruminal liquid (Figure 1) was significantly higher ($P < 0.05$) in CTRL
313 sheep. The energy:protein ratio was similar in the two experimental diets, and for the
314 calculated value of non-degradable protein in rumen there were no great differences
315 between treatments (740 vs. 729 g kg^{-1} of CP, for CTRL and GP-75, respectively).
316 Therefore, the lower ruminal ammonia-N content (−33%) in GP-75 animals could be
317 partially attributed to the presence of a high proportion of CP bound to the ADF of
318 grape pomace. Moreover, the addition of tannins and other phenolics, could help to
319 reduce proteolysis, degradation of peptides and deamination of amino acids, with a
320 subsequent increase in amino acid flow to the small intestine, as has been reported
321 when low or moderate levels of tannins are included in ruminant diets.[38] The
322 disappearance of DM, OM and CP did not seem to be negatively affected by the
323 reduced ruminal ammonia concentration, probably because many rumen bacteria use
324 not only ammonia-N but also peptides or amino acids.[39]

325 The experimental treatment (CTRL vs. GP-75) affected ($P < 0.05$) total VFA
326 concentration and molar proportions of acetic acid, propionic acid, isobutyric acid,
327 butyric acid, isovaleric acid and valeric acid in the rumen, with animals in the GP-75
328 group showing lower values (Figure 2). Some authors have reported a reduction in the
329 total VFA proportion in the presence of grape pomace,[34] which could be due to the
330 capacity of tannins to bind to fibre portions of the feed[40] or to their inhibitory effect on
331 microbial activity.[41] There were no significant differences in acetate:propionate ratio
332 between the two experimental treatments for any time.

333

334 **Ewe plasma lipid peroxidation**

335 Inclusion of grape pomace in the diet did not significantly affect ($P > 0.05$)
336 plasma TBARS (Figure 3), which is in agreement with Sgorlon et al.,[42] who did not
337 report differences in lamb plasma TBARS due to supplementation with 5.5 g day^{-1} of
338 grape skin extract. In spite of the lack of significant differences, lower numerical
339 TBARS values as a consequence of grape pomace supplementation accounted for

22%, 25% and 25% 3, 6 and 9 h after feeding compared with CTRL. In this regard, Gladine et al.[43] reported that the inclusion of grape seed and peel extract improved the antioxidant status of sheep and reduced the susceptibility to lipid peroxidation of plasma. These results suggest that grape pomace may be a potential source of antioxidants which could be transferred directly or after metabolic transformation by rumen microbes to ruminant products. *In vivo* trials are needed to test the effect of the inclusion of this winery by-product in practical diets for ruminants on meat and milk quality.

CONCLUSIONS

Chemical composition, *in vitro* true digestibility and ruminal degradability results indicate that the nutritive value of grape pomace from red wine is variable, depending on the proportion of seeds and pulp. The results clearly indicate that pulp had the benefit of lower lignified fibre, higher digestibility and higher content of some polyphenols than seeds. The interest of this by-product in sheep feeding could be related to its polyphenol and linoleic acid contents which could have beneficial effects on meat and milk quality

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365 **REFERENCES**

366 1 FAOSTAT. http://faostat3.fao.org [15th July 2015] (2012)

367 2 Alonso AM, Guillén DA, Barroso CG, Puertas B and García A, Determination of
368 antioxidant activity of wine byproducts and its correlation with polyphenolic content.
369 *J Agr Food Chem* **50:**5832–5836 (2012).

370 3 Baumgärtel T, Kluth H, Epperlein K and Rodehutscord M, A note on digestibility and
371 energy value for sheep of different grape pomace. *Small Ruminant Res* **67:**302–
372 306 (2007).

373 4 Makris DP, Boskou G and Andrikopoulos NK, Polyphenolic content and in vitro
374 antioxidant characteristics of wine industry and other agri-food solid waste
375 extracts. *J Food Composit Anal* **20:**125–132 (2007).

376 5 Patra AK and Saxena J, A review of the effect and mode of action of saponins on
377 microbial population and fermentation in the rumen and ruminant production. *Nutr*
378 *Res Rev* **22:**204–219 (2009).

379 6 Vasta V and Luciano G, The effects of dietary consumption of plants secondary
380 compounds on small ruminals' products quality. *Small Ruminan Res* **101:**150–159
381 (2011).

382 7 Yi C, Shi J, Kramer J, Xue S, Jiang Y, Zhang M, Ma Y and Pohorly J, Fatty acid
383 composition and phenolic antioxidants of winemaking pomace powder. *Food*
384 *Chem* **114:**570–576 (2009).

385 8 AOAC, Official Methods of Analysis (19th ed). Association of Official Analytical
386 Chemists Inc., Arlington, VA, USA (2012).

387 9 Van Soest PJ, Robertson JB and Lewis BA, Methods for dietary fiber, neutral
388 detergent fiber and non-starch polysaccharides in relation to animal nutrition. *J*
389 *Dairy Sci* **74:**3583–3597 (1991).

390 10 Bligh EG and Dyer WJ, A rapid method for total lipid extraction and purification. *Can*
391 *J Biochemi Physiol* **37:**911–917 (1959).

11 Morrison WR and Smith LM, Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *J Lipid Res* **5:**600–608 (1964).

12 Castro T, Manso T, Mantecón AR, Guirao J and Jimeno V, Fatty acid composition and carcass characteristic of growing lambs fed diet containing palm oil supplements. *Meat Sci* ***69:****757–764* (2005).

13 Ribéreau-Gayon P, Le dosage des composés phénoliques totaux dans les vins rouges. *Chim Anal* **52:**627–631 (1970).

14 Hartzfeld PW, Forkner R, Hunter MD and Hagerman AE, Determination of hydrolyzable tannins (gallotannins and ellagitannins) after reaction with potassium iodate. *J Agr Food Chem* **50:**1785–1790 (2002).

15 Reed JD, McDowell RE, Van Soest PJ and Horvath PJ, Condensed tannins: A factor limiting the use of cassava forage. *J Sci Food Agr* **33:**213–220 (1982).

16 Bate-Smith EC, Astringent tannins of the leaves of Geranium species. *Phytochemistry* **20:**211–216 (1981).

17 Ribéreau-Gayon P, and Stonestreet E, Dosage des tanins du vin rouge et détermination de leur structure. *Chim Anal* **48:**188–196 (1966).

18 Ribéreau-Gayon P and Stonestreet E, Le dosage des anthocyanes dans le vin rouge. *Bull Société Chimie* **9:**2649–2652 (1965).

19 Del Álamo M, Nevares I and García S, Influence of different aging systems and oak woods on aged wine color and anthocyanin composition. *Eur Food Res Technol* **219:**124–132 (2004).

20 Gallego L, Nevares I, Fernández JA and Del Álamo M, Determination of low-molecular mass phenols in red wines: The influence of chips, staves and micro-oxygenation aging tank. *Food Sci and Technol Int* **17:**429–438 (2011).

21 Ørskov ER, Hovell FDDeB, and Mould F, The use of the nylon bag technique for the evaluation of feedstuffs. *Trop Anim Health Pro* **5:**195–213 (1980).

419 22 Ørskov ER and McDonald I, The estimation of protein degradability in the rumen
420 from incubation measurements weighted according to rate of passage. *J Agr Sci*
421 **92:**499–503 (1979).
422 23 Weatherburn MW, Phenol-hypochlorite reaction for determination of ammonia. *Anal*
423 *Chem* **39:**971–974 (1967).
424 24 Carro MD, Lebzien P and Rohr K, Influence of yeast culture on the in vitro
425 fermentation (Rusitec) of diets containing variable portions of concentrates. *Anim*
426 *Feed Sci Technol* **37:**209–220 (1992).
427 25 Tilley JMA and Terry RA, A two-stage technique for the *in vitro* digestion of forage
428 crops. *J Brit Grassl Soc* **18:**104–111 (1963).
429 26 Goering HK and Van Soest PJ, Forage fiber analysis apparatus, reagents,
430 procedures, and some applications). USDA Agricultural Research Service.
431 Agriculture Handbook No. 379 (1970).
432 27 Ankom, Procedures for fibre and in vitro analysis. [Online]. Available:
433 http://www.ankom.com [20-may-2016].
434 28 Molina-Alcaide E, Moumen A and Martín-García I, By-products from viticulture and
435 the wine industry: potential as sources of nutrients for ruminants. *J Sci Food Agr*
436 **88:**597–604 (2008).
437 29 Spanghero M, Salem AZM and Robinsond PH, Chemical composition, including
438 secondary metabolites, and rumen fermentability of seeds and pulp of Californian
439 (USA) and Italian grape pomaces. *Anim Feed Sci Technol* **152:**243–255 (2009).
440 30 Basalan M, Gungor T, Owens FN and Yalcinkaya I, Nutrient content and in vitro
441 digestibility of Turkish grape pomaces. *Anim Feed Sci Technol* **16:**194–198 (2011).
442 31. Manso T, Bodas R, Vieira C, Mantecón AR and Castro T. feeding vegetable oils to
443 lactating ewes modifies the fatty acid profile of suckling lambs. *Animal* **5:**1659-
444 1667 (2011).

445 32 Moate PJ, Williams SRO, Torok VA, Hannah MC, Ribaus BE, Tavendale MH,
446 Eckard RJ, Jacobs JL, Auldist MJ and Wales WJ, Grape marc reduces methane
447 emissions when fed to dairy cows. *J Dairy Sci.* **97:**5073–5087 (2014).
448 33 Correddu F, Nudda A, Battacone G, Boe R, Francesconi AHD and Pulina G. Effects
449 of grape seed supplementation, alone or associated with linseed, on ruminal
450 metabolism in Sarda dairy sheep. *Anim Feed Sci Technol* **199**:61–72 (2015).
451 34 Besharati M and Taghizadeh A. Evaluation of dried grape by-product as a
452 tanniniferous tropical feedstuff. *Anim Feed Sci Technol* **152:**198–203 (2009).
453 35 Antoniolli A, Fontana AR, Piccoli P and Bottini R, Characterization of polyphenols
454 and evaluation of antioxidant capacity in grape pomace of the cv. Malbec. *Food*
455 *Chem* **178:**172–178 (2015).
456 36 Monagas LM, Garrido I, Bartolome B and Gómez-Cordoves, C, Chemical
457 characterization of commercial dietary ingredients from Vitis vinifera. *Anal. Chim.*
458 *Acta* **563**:401–410 (2006).
459 37 Lopez S, Davies DR, Giráldez FJ, Dhanoa MS, Dijkstra J and France J. assessment
460 of nutritive value of cereal and legume straws based on chemical composition and
461 in vitro digestibility. *J Sci Food Agric* **85:**1550-1557 (2005).
462 38 Frutos P, Hervás G, Giráldez FJ and Mantecón AR, Review. Tannins and ruminant
463 nutrition. *Span J Agric Res* 2:191–202 (2004).
464 39 Fernández B, Bodas R, López-Campos O, Andrés S, Mantecón AR and Giráldez
465 FJ, Vinasse added to dried sugar beet pulp: preference rate, voluntary intake, and
466 digestive utilization in sheep. *J Anim Sci* **87:**2055–2063 (2009).
467 40 McSweeney CS, Palmer B, McNeill DM and Krause DO, Microbial interactions with
468 tannins: nutritional consequences for ruminants. *Anim Feed Sci Technol* **91:**83–93
469 (2001).
470 41 Jones GA, McAllister TA, Cheng KJ and Muir AD, Effect of sainfoin (*Onobrychris*
471 *viciifolia* Scop.) condensed tannins on growth and proteolysis by 4 strains of rumen
472 bacteria. *Appl. Environ. Microb*. **60:**1374–1378 (1994).

42 Sgorlon S, Stradaioli G, Zanin D and Stefanon B, Biochemical and molecular responses to antioxidant supplementation in sheep. *Small Ruminant Res* **64:**143–151 (2006).

43 Gladine C, Rock E, Morand C, Bauchart D and Durand D, Bioavailability and antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose, in sheep made highly susceptible to lipoperoxidation. *Brit J Nutr* **98:**691–701 (2007).

482 Table 1. Ingredients and chemical composition of the experimental ewe diets.

483

	Treatments[1]	
	CTRL	GP-75
Ingredients (g kg^{-1} DM)		
Lucerne hay	499	462
Barley	202	187
Oats	202	187
Soybean meal	78.3	72.5
Vitamin-mineral premix	16.4	15.1
Sodium bicarbonate	2.20	2.00
Grape pomace from red wine	-	75.0
Composition (g kg^{-1} DM)		
Dry matter (DM)	899	797
Organic matter	918	919
Neutral detergent fibre	361	362
Acid detergent fibre	238	244
Crude protein	184	179
Ether extract	28.6	31.9

484

485 [1] Treatments: CTRL, without grape pomace; GP-75, with 75 g kg^{-1} of grape pomace from red wine, DM
486 basis.
487

Table 2. Chemical composition and *in vitro* true digestibility of seeds and pulp of grape pomace from red wine.

	Seeds	Pulp	RSD[1]	*P*. value
	Mean	Mean		
Chemical composition (g kg^{-1} DM)				
Organic matter	927	811	21.6	<0.001
Neutral detergent fibre	523	243	47.2	<0.001
Acid detergent fibre	454	193	32.8	<0.001
Acid detergent lignin	353	74.7	24.77	<0.001
Hemicellulose [2]	68.9	50.0	21.72	0.163
Cellulose [3]	101	118	12.1	0.033
Neutral detergent-soluble carbohydrates[4]	201	399	18.4	<0.001
Crude protein	104	138	14.6	0.003
Acid detergent insoluble crude protein	14.4	33.9	5.36	<0.001
Ether extract	99.0	31.7	6.6ME5	<0.001
Fatty acid composition (g kg^{-1} of identified fatty acids)				
C8:0	-	1.2	0.29	<0.001
C10:0	-	5.2	0.53	<0.001
C12:0	0.2	8.4	1.36	<0.001
C14:0	1.0	7.4	1.83	<0.001
C14:1	-	1.2	0.29	<0.001
C15:0	-	2.0	0.00	<0.001
C16:0	80.7	193	11.36	<0.001
C16:1	2.0	17.0	3.63	<0.001
C17:0	1.0	2.3	0.37	<0.001
C17:1	1.2	1.0	1.13	0.804
C18:0	39.7	56.2	2.57	<0.001
C18:1	172	127	12.0	<0.001
C18:2	690	402	24.6	<0.001
C18:3	5.2	125	11.0	<0.001
C20:0	2.0	13.8	1.30	<0.001
C20:1	3.2	7.2	5.69	0.251
C20:2	0.7	1.8	0.47	0.002
C20:3	-	3.0	0.00	<0.001
C20:4	-	1.3	0.37	<0.001
C22:0	1.0	15.7	1.39	<0.001
C22:1	-	0.5	0.39	0.049
C23:0	-	3.0	0.44	<0.001
C24:0	-	6.3	0.73	<0.001
Ratios [5]				
SFA	126	314	17.6	<0.001
MUFA	178	153	16.1	0.023
PUFA	696	533	26.4	<0.001
PUFA:SFA	5.55	1.71	0.253	<0.001
Phenolic compounds (g kg^{-1} DM)				
Extractable polyphenols	55.0	32.1	3.73	<0.001
Hydrolysable polyphenols	9.18	8.11	0.447	0.002
Condensed tannins	93.5	19.4	14.97	<0.001
Total anthocyanins	0.28	7.63	1.406	<0.001
In vitro true digestibility (g kg^{-1} DM)	510	820	110.0	0.001

[1] RSD: residual standard deviation.
[2] Hemicellulose: NDF–ADF; [3] Cellulose: ADF–ADL; [4] Neutral detergent-soluble carbohydrates (NDSC): OM–EE–CP–NDF.
[5] SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Table 3. Concentration of phenolic compounds in seeds and pulp of grape pomace from red wine.

	Seeds	Pulp	RSD[1]	*P. value*
	Mean	Mean		
Non-flavonoids				
- Phenolic acids				
. Cinnamic acids				
Caffeic acid	-	-	-	-
Chlorogenic acid	-	6.75	5.330	0.053
p-Coumaric acid	-	-	-	-
Sinapic acid	-	-	-	-
Ferulic acid	-	-	-	-
. Benzoic acids				
Gallic acid	4.94	23.5	16.61	0.081
Gentisic acid	-	-	-	-
Ellagic acid	0.48	2.61	2.098	0.109
Syringic acid	16.8	54.7	7.93	<0.001
Vanillic acid	0.24	30.0	6.90	<0.001
Protocatechuic acid	12.3	4.45	12.358	0.299
Total phenolic acids	34.7	122	33.20	0.001
- Stilbene				
. *Trans* resveratrol	-	1.08	1.130	0.130
Flavonoids				
- Flavonols				
. Quercetin *	3.62	1.10	2.973	0.173
. Kaempferol	0.11	1.67	2.131	0.233
. Myricetin	-	5.09	5.499	0.140
. Total flavonols *	3.62	1.11	2.973	0.174
- Flavanols				
. Catechin *	12.3	6.79	2.204	0.002
. Epicatechin	2.18	9.79	2.775	0.001
. Total flavanols *	12.3	6.80	2.202	0.002
- Anthocyanins				
. Delphinidin 3-O-glucoside	-	217	74.9	0.001
. Cyanidin 3-O-glucoside	-	13.2	8.82	0.027
. Petunidin 3-O-glucoside	-	269	78.8	<0.001
. Peonidin 3-O-glucoside	0.34	60.0	24.89	<0.001
. Malvidin 3-O-glucoside	13.2	977	256.4	<0.001
. ∑ Anthocyanin derivatives *	-	3.36	1.409	0.002
. Total anthocyanins *	0.01	4.90	1.695	0.001

* g kg^{-1} DM.
[1] RSD: residual standard deviation.

Table 4. Effect of experimental ewe diets on ruminal degradation profiles of grape pomace from red wine.

	Treatments[1]				RSD[2]	*P.* value[3]		
	CTRL		GP-75					
	Seeds	Pulp	Seeds	Pulp		S	T	S x T
Dry matter								
a (g kg^{-1})	199	401	191	381	5.0	<0.001	0.024	0.280
b (g kg^{-1})	197	301	214	296	46.0	0.079	0.900	0.815
c (h^{-1})	0.06	0.01	0.07	0.03	0.010	0.001	0.299	0.700
PD (g kg^{-1})	396	702	405	677	41.8	<0.001	0.852	0.695
ED6 (g kg^{-1})	299	446	306	449	10.9	<0.001	0.685	0.887
Organic matter								
a (g kg^{-1})	186	347	178	324	6.5	<0.001	0.038	0.277
b (g kg^{-1})	202	317	221	249	29.5	0.041	0.426	0.175
c (h^{-1})	0.07	0.01	0.07	0.04	0.010	0.003	0.225	0.387
PD (g kg^{-1})	388	665	399	572	24.7	<0.001	0.136	0.071
ED6 (g kg^{-1})	292	400	297	410	11.5	<0.001	0.527	0.836
Crude protein								
a (g kg^{-1})	372	320	246	269	81.9	0.862	0.311	0.659
b (g kg^{-1})	431	478	594	326	86.3	0.235	0.948	0.106
c (h^{-1})	0.11	0.01	0.14	0.05	0.020	0.001	0.129	0.723
PD (g kg^{-1})	804	698	840	595	58.5	0.017	0.587	0.267
ED6 (g kg^{-1})	655	379	658	408	17.2	<0.001	0.399	0.470

[1] Treatments: CTRL, without grape pomace; GP-75, with 75 g kg^{-1} of grape pomace from red wine, DM basis.

[2] RSD: residual standard deviation.

[3] Probability of significant effects due to sample, seeds or pulp (S), dietary treatment (T) and interaction effects (S x T).

a, soluble fraction; *b*, potentially degradable insoluble fraction; *c*, rate of degradation of *b* fraction; PD, potential degradability; ED6, effective degradability = $a + [bc/(c + k)]$, where k = 0.06 h^{-1}.

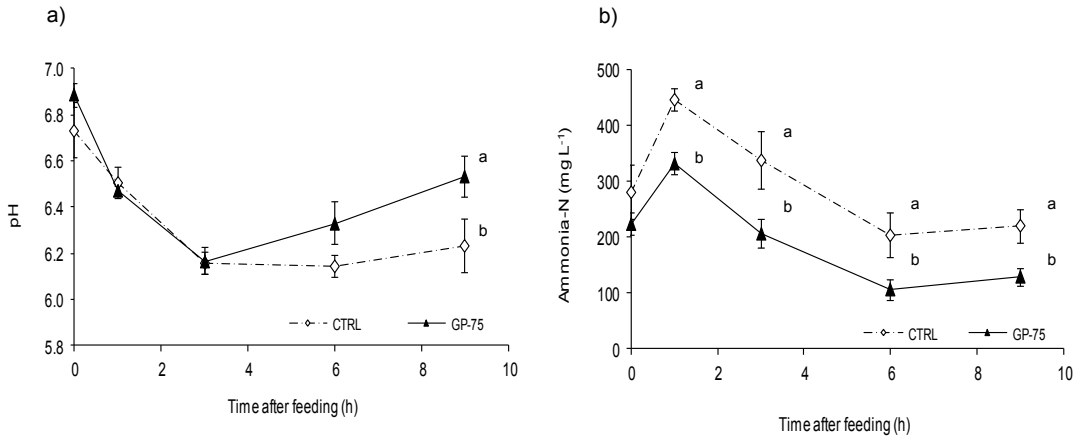

Figure 1. Effect of experimental ewe diets (CTRL, without grape pomace; GP-75, with 75 g kg^{-1} of grape pomace from red wine, DM basis) on ruminal pH (a) and ammonia-N concentration (b) at different times after feeding. Different small letters mean significant differences ($P < 0.05$) between treatments within time. The error bars represent standard error.

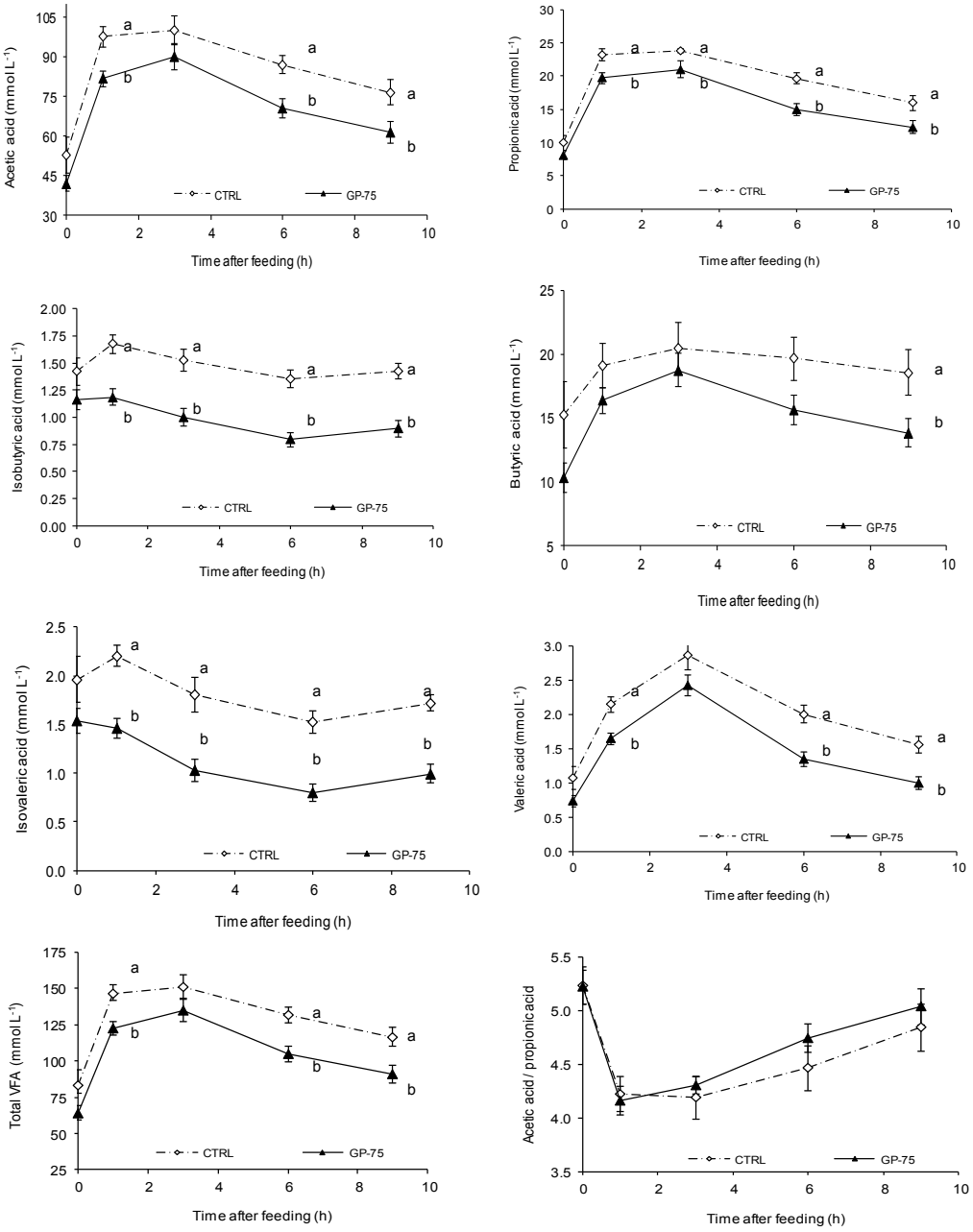

Figure 2. Effect of experimental ewe diets (CTRL, without grape pomace; GP-75, with 75 g kg^{-1} of grape pomace from red wine, DM basis) on volatile fatty acid concentrations at different times after feeding. Different small letters mean significant differences ($P < 0.05$) between treatments within time. The error bars represent standard error.

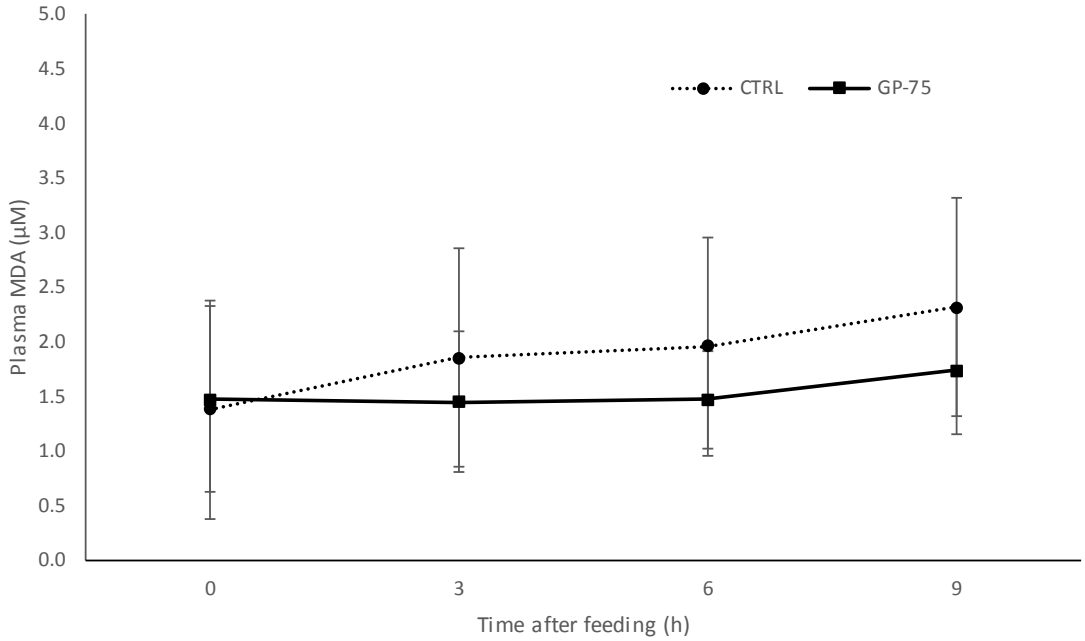

Figure 3. Effect of experimental ewe diets (CTRL, without grape pomace; GP-75, with 75 g kg^{-1} of grape pomace from red wine, DM basis) on plasma MDA (µM) at different times after feeding. The error bars represent standard error.