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ABSTRACT

Grape seed is a by-product of the winery and distillery industry which could be used in animal nutrition. To test the hypothesis that dietary supplementation with this by-product can decrease the biohydrogenation (BH) of healthy fatty acids (FA), the present study evaluated the effects of grape seed supplementation, alone or combined with linseed, on ruminal BH processes in dairy sheep. In this 60-d trial, twenty-four lactating Sarda dairy ewes were assigned to 4 homogeneous groups and fed as follows: (1) control diet (CON), (2) a diet supplemented with 300 g/d per head of grape seed (GS), (3) a diet supplemented with 220 g/d per head of extruded linseed (LIN), (4) and a diet supplemented with a mix of both grape seed and linseed (300 and 220 g/d per head, respectively) (MIX). Ammonia, pH, volatile fatty acids (VFA) and FA composition were determined in rumen liquor at 3 sampling dates (20, 40 and 60 d). Rumen pH was not influenced by diet (P>0.05). The ammonia content was increased (P<0.05) in GS and MIX compared with LIN and CON. The molar proportions of acetate and propionate and their ratio were not affected by the diet (P>0.05), whereas the molar proportion of butyrate was the lowest in MIX. Rumenic acid (RA; CLA *cis*-9,*trans*-11) concentration increased in GS compared with CON (0.78 *vs.* 0.45 mg/100 mg FA; P<0.05), whereas the percentage of vaccenic acid (VA; C18:1 *trans*-11) tended to increase (P<0.10) in GS compared with CON. The concentration of VA was higher in MIX than in CON (8.18 *vs.* 3.77 mg/100 mg FA;

P<0.05), whereas RA did not differ between the same groups. The concentration of linoleic acid (LA; C18:2 *n*-6) decreased and stearic acid (SA; C18:0) increased in all supplemented groups, whereas linolenic acid (LNA; C18:3 *n*-3) decreased in the two groups receiving grape seed compared with CON and LIN. The concentration of total odd- and branched-chain fatty acids (OBCFA) decreased in all supplemented groups compared with CON (P<0.05), evidencing that grape seed and linseed supplementation influenced the ruminal BH processes. Grape seed was able to increase the accumulation of RA when supplemented alone, and of VA when combined with linseed; however, the rumen accumulation of SA in both groups supplemented with grape seed evidenced that this by-product was not effective in decreasing the BH of dietary polyunsaturated fatty acids (PUFA).

Keywords: Biohydrogenation, Fatty acid, Grape seed, Linseed, Rumen fermentation, Sheep.

1. Introduction

Grape seed is a by-product derived from the winery and distillery industries. In countries where the wine industry is an important activity, the large production of by-products and wastes is a serious problem, because of the high cost of their management and disposal. As many other agro-industrial by-products, grape seeds are rich in polyphenols (Schieber et al., 2001), especially mono-, oligo- and polymeric proanthocyanidins (Shrikhande, 2000), which are well known for their antioxidant properties (Riceevans et al., 1995; Bagchi et al., 1997).

In the last few decades, a lot of attention has been directed to the content of healthy fatty acids (FA), especially polyunsaturated fatty acids belonging to the family of *n*-3 (PUFA *n*-3), such as alpha-linolenic acid (C18:3 *n*-3, LNA), and conjugated linoleic acid (CLA), such as rumenic acid (CLA *cis*-9, *trans*-11, RA), in ruminant milk and dairy products. A lot of studies have demonstrated that diet composition and rumen microbial biohydrogenation (BH) strongly influence the fatty acid profile of milk and dairy products (Lourenço et al., 2010; Buccioni et al., 2012). Supplementation

with linseed, which is rich in LNA, is often used to improve the FA profile in sheep, goat and cow milk and cheese, by increasing the concentration of healthy FA (Caroprese et al., 2010; Mughetti et al., 2012; Nudda et al., 2013). Lipids from the diet are involved in a sequence of reactions, performed by the rumen microbial population, including hydrolysis of esterified lipids to free FA, whose double bonds can be partly isomerized and hydrogenated. These reactions can be explained by a detoxification mechanism to defend microorganisms from the toxicity of unsaturated fatty acids (UFA) as reported by Dehority (2003) and Maia et al. (2010). Because C18:2 *cis*-9, *cis*-12 (linoleic acid, LA, *n*-6 series) and LNA provided with the diet are greatly reduced (by 80% and 92%, respectively) in the rumen (Doreau and Ferlay, 1994), several strategies have been tested to protect dietary FA from rumen BH. The encapsulation of lipids in a protein matrix is one of the techniques proposed to protect FA (Tymchuk et al., 1998; Hawkins et al., 2013). However, the occasional lack of efficiency of this method (Petit, 2003), its high costs and the need for formaldehyde utilization limit its use. Another technique that has been studied is the decrease in BH by chemical modifications of UFA, such as the transformation of UFA to fatty acyl amides (Jenkins, 1998) or to calcium salts (Lundy Iii et al., 2004). The presence of some plant compounds, such as polyphenols, in the diet of ruminants can also influence the BH process, by inhibiting the activity of rumen microbes (Cabiddu et al., 2009; Vasta et al., 2009). The use of grape seed as a supplement in ruminant nutrition could be an alternative for the expensive management and disposal of this winery by-product. However, the literature available on the use of grape by-products in ruminant nutrition is limited (Moate et al., 2014; Santos et al., 2014) and, to our knowledge, the effects of the dietary supplementation with grape seed on the BH of PUFA in the rumen has not been explored yet.

To test the hypothesis that grape seed supplementation decreases the BH activity of rumen microbes, thus boosting the effect of linseed supplementation, this work investigated the effect of

73 grape seed supplementation, alone or associated with linseed, on rumen BH processes in dairy
74 sheep.

75

2. Material and methods

2.1. Animals, experimental design and treatments

Twenty four Sarda dairy ewes in the first part of lactation (<50 days in milk, DIM) were assigned to 4 groups of 6 animals each, homogeneous for milk production, body weight, DIM, and lactation order. Groups were confined in four boxes and randomly assigned to one of the 4 experimental diets (Table 1): control (CON) diet, a diet supplemented with 300 g/d per head of grape seed (GS), a diet supplemented with 220 g/d per head of extruded linseed (LIN), and a diet supplemented with a mix of 300 g/d per head of grape seed and 220 g/d per head of linseed (MIX). The extruded linseed dose of 220 g/d was used to supply 70 g/d of fat per head. Considering that the total phenolic content of grape seed was 333.3 ± 10.1 mg gallic acid equivalent (GAE)/100 g of dry matter (DM; mean ± S.E.), the grape seed dose of 300 g/d per head was used to provide approximately 1 g/d per head of total polyphenols (approximately 0.4 g polyphenols/kg DM of diet). The grape seed used was obtained from different red grape varieties after distillation in the winemaking process. Grape seeds were ground before administration. The chemical composition and FA profile of the grape seed and linseed are reported in Table 2.

All animals were offered the same basal ration consisting of beet pulp, a commercial concentrate, dehydrated alfalfa hay and mixed hay. In addition, they received a mixed meal composed of corn, soybean, pea, grape seed and linseed, in different proportions depending on the dietary treatments, to obtain isoenergetic and isonitrogenous diets. Dehydrated alfalfa hay and mixed hay were offered to each group of six ewes, whereas all other dietary ingredients were provided to each animal by using individual feeders. The commercial concentrate (500 g/d per head) was provided at the two daily milkings (7:30 and 17:30). The mixed meals were provided two hours after each milking, and subsequently beet pulp (400 g/d per head) and dehydrated alfalfa hay (on average 800 g/d per head) were provided. The mixed hay (on average 200 g/d per head) was offered during the night. Both hays were offered at a fixed amount to avoid selection by the

101 animals. Clean water was always available. Diets were formulated to meet the sheep energy and
102 protein requirements using the Small Ruminant Nutrition Model (Tedeschi et al., 2010). Diets were
103 offered in a fixed amount to ensure constant daily intakes of dietary ingredients and to maintain
104 energy balance. The experiment lasted 10 weeks, with two weeks of adaptation period and 8 weeks
105 of data collection.

106 *2.2. Rumen sample collection*

107 Samples of rumen liquor were collected from all animals on days 20, 40 and 60 of the trial
108 (Samplings 1, 2 and 3), 2 hours after the morning feeding, using a stomach tube and an evacuation
109 pump. The collection of rumen liquor samples was performed by two teams of qualified experience
110 and required approximately 30 min in total. In order to reduce saliva contamination, the first portion
111 of the liquor collected (about 30 mL) was discarded. After sampling and filtering the rumen liquor,
112 the pH value was immediately measured by a pH meter (Orion 250A, Orion Research Inc., Boston,
113 MA, USA), equipped with a glass electrode with Polysolve reference electrolyte (model 238405,
114 Hamilton Company, Reno, NV, USA), and a thermometer. The sample of rumen liquor of each
115 animal was then divided into 3 subsamples, which were immediately stored at −80°C until analysis
116 for ammonia, volatile fatty acids (VFA) and FA.

117 *2.3. Chemical analysis*

118 Dry matter content of feed ingredients was determined by oven-drying at 105°C for 24 h. Neutral
119 detergent fiber (NDF) and acid detergent lignin (ADL) analyses were performed following the
120 method of Van Soest et al. (1991), using an Ankom 220 fiber analyzer (Ankom™ technology,
121 Fairport, NY, USA); NDF was measured using heat stable amylase and expressed exclusive of
122 residual ash (aNDFom) and ADL was determined by solubilization of cellulose with sulphuric acid.
123 Crude protein (CP) content was measured according to the Kjeldahl method (proc. 988.05; AOAC,
124 2000), extract ether (EE) by the Soxhlet method (proc. 920.39; AOAC, 2005) and ash by using a

125 muffle at 550°C (proc. 942.05; AOAC, 2000). Non-fiber carbohydrates (NFC) were calculated
126 according to Weiss (1999) as follows: NFC (g/kg DM) = 100 − (NDF + CP + ash + EE).

127 To determine the phenolic content of grape seed, the seeds were powdered by a blender and an
128 aliquot of 1.5 g was homogenized with 50 mL of a mixture of acetone/water (70/30, v/v) using an
129 Ultra Turrax homogenizer (Ultra Turrax T25, Janke&Kunkel KG, Germany) at 3000 rpm, for 2
130 min, in a water/ice bath. The homogenate was then centrifuged (6,000 x *g*, 15 min, 4°C) and the
131 supernatant was filtered through Whatman 541 filter paper (Whatman, Maidstone, England) for
132 determination of phenolic compounds, using the Folin-Ciocalteu method described by Kim et al.
133 (2003) with some modifications. Briefly, 0.5 mL of the extract were added to 1 mL of Folin-
134 Ciocalteu phenol reagent (Sigma Chem. Co., St. Louis, MO, USA) and 9.5 mL of distilled water in
135 a 25 mL volumetric flask and shaken. After 7 min, 10 mL of 0.71 M sodium carbonate (Na_2CO_3)
136 were added, and then the mixture was diluted to a volume of 25 mL with distilled water and mixed
137 thoroughly. The mixture was then stored in the dark for 120 min at room temperature. The
138 absorbance was read at 750 nm wavelength versus a blank solution. Total phenolic content,
139 expressed as mg of GAE/100 g DM, was determined following a calibration curve obtained using
140 gallic acid (Sigma Chem. Co., St. Louis, MO, USA) as standard.

141 The FA profiles of the whole diets, grape seed, linseed and rumen liquor were determined using
142 the method of Kramer et al. (1997) with some modifications. Samples kept at −80°C were
143 lyophilized and powdered. After adding 2 mL of sodium methoxide 0.5 M in methanol (Sigma and
144 Aldrich, Spain) to the power, it was placed in a water bath at 50°C for 10 min. After cooling to
145 room temperature, the samples were placed in a water/ice bath; then 3 mL of HCl/methanol (3M),
146 prepared with acetyl chloride and methanol, were added. Subsequently, the samples were heated
147 again in a water bath at 50°C for 10 min and cooled to room temperature; then 1 mL of a solution
148 containing methyl nonadecanoate (C19:0) as internal standard (Sigma Chemical Co., St. Louis,
149 MO, USA) and, subsequently, 7.5 mL of a 0.43 M solution of K_2CO_3 were added. After quick

agitation the samples were centrifuged (1,500 x *g*, room temperature, 5 min) and each supernatant was kept in a vial for GC analysis. Fatty acid methyl esters (FAME) were determined using a Turbo 3400 CX gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and an automatic injector 8200 (CX Varian Inc., Palo Alto, CA, USA). The column (CP-select CB for FAME; 100 m x 0.32 mm i.d., 0.25 μm film thickness, Varian Inc., Palo Alto, CA, USA) was operated with the following program: 75°C for 1 min, increased at 5°C/min to 148°C and at 8°C/min to 165°C, held for 35 min; then increased at 5.5°C/min to 210 and, finally, at 3°C to 230°C, held for 14 min. Helium (1 mL/min flow rate) was used as carrier gas with a pressure of 37.000 psi. Split ratio was 1:100. The injector and detector temperatures were held at 225 and 285°C, respectively. Varian Star 3.4.1 software was used to compute the retention time and area of each individual FAME, identified by comparing their retention times with those of known standards and with published studies as detailed by Nudda et al. (2008).

Ammonia content in rumen liquor was determined by colorimetric method, according to Chaney and Marbach (1962) with one modification (the use of salicylate instead of phenol), using a UV-Visible Spectrophotometer (Varian, Inc., Palo Alto, CA, USA).

The VFA analysis was performed by a high-performance liquid chromatography (HPLC) method. Briefly, a sample of approximately 2 mL was defrozen and centrifuged (15,000 x *g*, 10 min, 4°C); the supernatant was then withdrawn by syringe and injected into a HPLC system (Varian Inc., Palo Alto, California, USA) after filtration (PTFE 0.45 μm, 13 mm). The HPLC was equipped with an auto sampler (Varian 9300), a degasser (Varian 9012 Q), a UV detector (Varian 906P Polychrom) and an Aminex HPX 87H column (Biorad Laboratories, Hercules, CA, USA). The column was operated at 55°C with 0.008 N H_2SO_4 at 0.6 mL/min as eluent. Concentrations of VFA were estimated by comparison with a calibration curve obtained by injecting 5 μl of 5 standard solutions (5.6, 11.25, 22.5, 45 and 90 mmol/L of acetic acid, and 5, 10, 20, 40 and 80 mmol/L of propionic and butyric acid) obtained by appropriate dilutions of a standard mixture of VFA

175 containing 5.40, 5.76 and 7.02 mg/mL of acetic, propionic and butyric acids, respectively, in H_2SO_4
176 0.1 N. The concentration of total and single VFA were expressed as mmol/L and mol/100 mol of
177 total VFA, respectively.

178 *2.4. Statistical analysis*

179 Data of pH, ammonia, VFA and FA profile were analyzed with the PROC MIXED procedure of
180 SAS (2002). The model included the diet treatment (CON, GS, LIN and MIX), sampling (3
181 samplings on days 20, 40 and 60 of the trial) and their interaction as fixed effects, and the animal
182 nested within the treatment as random effect. Means were separated using Tukey test ($P<0.05$).

183 **3. Results**

184 The predominant FA of the lipid fraction in grape seed was LA (74 g/100 g FA), followed by
185 oleic acid (OA; C18:1 *cis*-9) and palmitic acid (C16:0) (9.6 and 8.5 g/100 g FA, respectively). The
186 main FA in linseed was LNA, which accounted for 56 g/100 g FA (Table 2).

187 All dairy ewes consumed the whole daily amount of feeds supplied, as a consequence of the
188 fixed amount of diets provided (Table 3). The intake of most FA varied with diets
189 (CON<GS<LIN<MIX; Table 3) and reflected the EE concentration of the diets (Table 1).

190 *3.1. pH and ammonia in rumen liquor*

191 Rumen pH was not influenced by the diets, ranging from 6.68 in LIN to 6.77 in MIX (Table 4).
192 Sampling affected pH, which was lower on sampling 1 (6.58) than on sampling 2 (6.85), and
193 intermediate on sampling 3 (6.75). A diet × sampling interaction ($P<0.01$) occurred, but no
194 significant differences between diets within sampling were detected.

195 Rumen ammonia was affected by diet and sampling date ($P<0.05$, Table 4), being higher in the
196 GS and MIX groups than in the CON and LIN groups. On average, rumen ammonia increased

197 (P<0.05) from sampling 2 (14.71 mg/dL) to sampling 3 (17.71 mg/dL), with sampling 1 (15.67
198 mg/dL) not differing from them.

199 *3.2. Rumen VFA*

200 Concentration of total VFA, molar proportions of individual VFA and acetate:propionate ratio in
201 the rumen are shown in Table 4. The concentration of total VFA and the molar proportions of
202 acetate and propionate were not affected by diet, whereas the concentration of butyrate was reduced
203 (P<0.05) by the MIX containing both grape seed and linseed. A time effect was observed (P<0.01)
204 for total VFA and individual VFA, with the highest concentrations of propionate, acetate and
205 butyrate being on samplings 1, 2, and 3, respectively (P<0.05; Fig. 1). The acetate:propionate ratio
206 was not affected by diet, but differed (P<0.05) among sampling 1 (3.14), sampling 2 (4.04) and
207 sampling 3 (3.54). This ratio was influenced by group × sampling interaction (P<0.05), but no
208 significant differences between diets within sampling were detected.

209 *3.3. FA composition in rumen liquor*

210 The fatty acid profile in the rumen liquor collected from the ewes of the different experimental
211 treatments is given in Table 5. The total concentration of short-chain fatty acids (SCFA) was lower
212 (P<0.05) in all supplemented groups than in CON, mainly due to a decrease in C4:0 and C5:0
213 (P<0.05). The total concentration of medium-chain fatty acids (MCFA) was affected by diet
214 (P<0.01), with a significant reduction in both groups supplemented with linseed (LIN and MIX)
215 compared with CON, mainly due to the lower concentration (P<0.05) of C16:0 and some C16:1
216 isomers in the LIN and MIX groups. The concentration of MCFA and C16:0 in the GS group was
217 intermediate, not differing significantly from CON and LIN.

218 The concentration of total odd- and branched-chain fatty acids (OBCFA) decreased (P<0.05) in
219 all supplemented groups compared with CON. The MIX group had the lowest concentration of
220 OBCFA, even if not significantly different from the LIN group. The reduction in OBCFA caused by

grape seed was mainly due to the reduction ($P<0.05$) in *anteiso* branched-chain fatty acids (BCFA) and odd-chain fatty acids (OCFA), especially *anteiso* C15:0 and C5:0. The reduction in OBCFA caused by linseed was related not only to the reduction in these FA, but also to the decrease ($P<0.05$) in *iso* BCFA, especially isomers of heptadecanoid acid (C17:0, *iso* C17:0 and *anteiso* C17:0). The total concentration of long-chain fatty acids (LCFA) increased with the grape seed and linseed supplementation, alone and in combination, being the highest in the MIX group ($P<0.05$).

The concentration of C18:0 (stearic acid, SA) was higher ($P<0.05$) in all the supplemented groups than in CON and, on average, increased ($P<0.05$) in the last sampling. The diet × sampling interaction was also significant ($P<0.05$). The temporal evolution of this FA (Fig. 2) showed that MIX was higher than CON throughout the study, whereas GS and LIN were higher than CON on sampling 2 ($P<0.05$).

The concentration of LA decreased in all supplemented groups, being the lowest in LIN and MIX ($P<0.05$). The concentration of LNA was higher in the LIN and CON groups than in the GS and MIX groups ($P<0.05$). The concentrations of the geometrical isomers of LA, C18:2 *trans*-9,*trans*-12 and C18:2 *trans*-9,*cis*-12, were higher in LIN and MIX than in GS and CON ($P<0.05$).

The concentration of RA was the highest ($P<0.05$) in the GS group. A significant effect of sampling ($P<0.01$) occurred, with increasing levels of this FA over time (0.11, 0.55 and 0.97 g/100 g FA, respectively, on samplings 1, 2 and 3). A significant diet × sampling interaction occurred, with GS having a higher concentration of this FA than the other supplemented groups on sampling 3 (Fig. 3).

The sum of total *trans* fatty acids (TFA) was influenced ($P<0.01$) by diet, being higher in both groups receiving linseed supplementation (LIN and MIX) than in the CON, with GS not differing from CON and LIN. In particular, the concentration of C18:1 *trans*-11 (vaccenic acid, VA) was the highest in MIX and the lowest in GS and CON, with LIN being intermediate ($P<0.05$).

245 Many of the FA measured during the trial were influenced by sampling, but the pattern varied
246 among them. In general, most of the SCFA (C4:0, C5:0 and C6:0) and isomers of C18:1 showed a
247 significant decrease ($P<0.05$) in the last sampling, whereas most of the MCFA increased ($P<0.01$)
248 over time. The individual LCFA showed a variable pattern, without a significant sampling effect on
249 total LCFA ($P>0.05$). The PUFA decreased over time, mainly due to a decrease in PUFA n-6 (LA)
250 ($P<0.05$). Among OBCFA, *iso* BCFA and *anteiso* BCFA increased after sampling 1, whereas
251 OCFA decreased over time on the last sampling ($P<0.05$).

252 **4. Discussion**

253 During the experiment, the daily amount of feeds supplied individually including those objective
254 of the study, i.e. grape seed and linseed, were completely eaten by the animals of all groups. Group
255 feeding of the forages was chosen because the length of the experiment (10 weeks in total)
256 suggested to avoid the inevitable stress caused by individual confinement of lactating ewes (Hutson
257 et al., 2007). However the group intake of the forages was similar among the groups and mostly
258 complete, with only the less digestible stems refused, suggesting that the inclusion of grape seed,
259 linseed or both in the diet of lactating ewes did not negatively affect their DM intake.

260 The pH values measured in all experimental groups throughout the trial were between 6.5 and
261 7.0, which is a normal range for rumen liquor pH in sheep (5.5-7.0; Dziuk, 1984). This is in
262 agreement with previous studies showing that rumen pH was not markedly affected when sheep
263 were supplemented with grape pomace by-products, as a source of polyphenols (Yidiz et al., 2005;
264 Abarghuei et al., 2010), or with extruded linseed, as a source of PUFA (Mughetti et al., 2007). The
265 increase in rumen ammonia observed in the groups supplemented with grape seed, alone or in
266 combination with linseed, is in contrast with studies showing that the inclusion of polyphenols in
267 the diet was usually associated with a decrease in protein degradation (Abarghuei et al., 2010;
268 Dschaak et al., 2011), because of their ability to bind proteins and reduce the activity of microbial
269 enzymes by decreasing the growth of proteolytic bacteria (Molan et al., 2001). This discrepancy

could be explained by two hypotheses. The first is that the level of polyphenols in the grape seed used in the present work was too low to influence the activity of some strains of proteolytic bacteria, compared with the doses of polyphenols (values higher than 5 g/kg DM) which influenced rumen bacteria population in other studies (Hervás et al., 2003; Vasta et al., 2010; Anantasook et al., 2014). In addition, in some studies low concentrations of tannins increased the enzymatic activity and growth of some bacteria *in vitro* (Jones et al., 1994) and *in vivo* (Vasta et al., 2010), likely because the interaction between proteins and tannins can cause conformational changes in the protein structure, giving more accessible sites for some proteolytic bacteria (Mole and Waterman, 1985; Molan et al., 2001). The second hypothesis is that the high concentration of ADL in the grape seed used (410 g/kg DM) reduced the growth of cellulolytic bacteria, considering that high amounts of lignin in ruminant diets can reduce fiber digestion (Hartley, 1972; Jung and Fahey, 1984) by decreasing the growth of this type of bacteria (Akin, 1982), whose favorite substrate for protein synthesis is ammonia (Brayant, 1973; Van Soest, 1994), with a consequent accumulation of ammonia in the rumen.

The lack of effect of grape seed or extruded linseed, alone or in combination, on the concentration of total VFA and on the proportion of acetate and propionate and their ratio in dairy ewes observed in our study is in accordance with previous studies on dairy sheep fed diets rich in LA and supplemented with 10 or 20 g tannins/kg DM from quebracho (Toral et al., 2011, 2013), on cattle supplemented with quebracho polyphenols (Beauchemin et al., 2007) and on dairy cows supplemented with flax seed (Neveu et al., 2013). Differently, Ivan et al. (2013) found a reduction in acetate in dairy cows fed diets supplemented with oilseeds rich in LA or LNA and an increase in propionate in cows fed the latter supplement.

The reduction in the proportion of butyrate observed only in the rumen liquor of the ewes fed the MIX could be explained by the synergistic effect of the two sources of LA (grape seed) and LNA (linseed) and by the higher value of fat in the MIX diet than in the GS and LIN diets. Our finding is

295 in accordance with the meta-analysis of Patra (2014) showing a negative effect of increasing levels
296 of fat in the diets of sheep on the proportion of butyrate, likely due to the inhibition of
297 microorganisms (protozoa and *Butyvibrio fibrisolvens*) involved in its production (Hristov et al.,
298 2009).

299 Overall, the observed variations in rumen FA profile between diets suggest shifts in rumen
300 microbial population. Considering that OBCFA derive largely from rumen microflora (Fievez et al.,
301 2012), the observed decrease in total OBCFA in all supplemented groups indicates that the diets
302 affected the activity and the growth of ruminal microorganisms. It is well documented that
303 variations in dietary treatments influence the synthesis of OBCFA, by affecting the relative
304 abundance of specific ruminal bacterial population (Vlaeminck et al., 2006). The reduction in total
305 OBCFA by linseed supplementation is in accordance with previous findings on lactating sheep
306 supplemented with sunflower oil (Toral et al., 2012), likely because these two supplements are a
307 source of PUFA, which have detrimental effects on the ruminal microflora (Maia et al., 2007,
308 2010). The decrease in OBCFA caused by grape seed supplementation could be partly explained by
309 the depressive effects of tannins on the microorganism growth (Baah et al., 2007; Vasta et al.,
310 2010), and mainly by the high concentration of LA in this by-product. The lowest value of OBCFA
311 and the highest value of LCFA in the MIX group suggest a combined effect of the two supplements,
312 likely because of the high LA (74% of total FA) in grape seed and high LNA (56% of total FA) in
313 linseed.

314 The OBCFA profile has been proposed as diagnostic tool to predict shifts in microbial
315 population associated with the diet variation (Lee et al., 1999; Vlaeminck et al., 2004). In the
316 present work the reduction in the *iso* C17:0 and *anteiso* C17:0 in the rumen liquor of sheep
317 receiving linseed supplementation could be related to a negative effect of this supplement on the
318 protozoa population, which produces a greater proportion of these FA than the bacteria population
319 in the rumen (Or-Rashid et al., 2007). In fact, several authors found a negative effect of lipid

addition on the growth of the protozoa population (Broudiscou et al., 1994; Doreau and Ferlay, 1995; Ivan et al., 2013). The decrease in *anteiso* C15:0 in GS compared with CON suggests a negative effect of grape seed supplementation on the rumen bacterial population, which is the main producer of this FA in the rumen (Or-Rashid et al., 2007). The decrease in both *anteiso* C17:0 and *anteiso* C15:0 in ewes fed the MIX suggests that the combination of grape seed and linseed influenced both protozoa and bacteria in the rumen, as confirmed by the lowest OBCFA level of this treatment.

The greater proportion of TFA in the groups fed linseed, alone or in the MIX, than in CON was expected and was likely a consequence of the higher concentration of most C18:1 and C18:2 isomers, which derive in part from the BH of LNA. This finding is in agreement with previous studies showing that the concentration of C18:1 and C18:2 isomers increased in the rumen of steers fed linseed oil (Shingfield et al., 2011). The greater accumulation of most *trans* and *cis* isomers of C18:1 in the MIX group could be mainly a consequence of the BH of OA, before its reduction to SA. The increase in C18:2 *trans*-9,*trans*-12 and C18:2 *trans*-9,*cis*-12 observed only in the two diets containing linseed is in agreement with an *in vitro* study showing that the concentrations of these geometrical isomers of LA were increased by LNA supplementation, but not by LA supplementation (Jouany et al., 2007).

The significantly higher values of VA in MIX, and numerically higher in LIN, compared with GS and CON reflected the pattern of intake of its precursor LNA. The increase in RA with time, although with a different pattern among diets, reflected the observed reduction in its precursor LA during the trial. The highest concentration of RA in GS was likely associated mainly with the intake of its precursor (LA) and, to a lesser extent, with the presence of polyphenols in grape seed by-product. Some authors found that the accumulation of RA and VA in the rumen was increased by tannins (Vasta et al., 2010; Buccioni et al., 2011), which can inhibit the last step of the BH process of VA to SA (Khiaosa-Ard et al., 2009; Vasta et al., 2009; Rana et al., 2012). The low content of

polyphenols in the grape seeds used in this trial was probably not high enough to markedly influence this BH process, as supported by the high rumen accumulation of SA and by the decrease in LA and LNA in both groups supplemented with grape seed. The higher content of SA in rumen liquor of all supplemented groups compared with CON could be also a consequence of the high extent of OA biohydrogenation, as evidenced by the low accumulation of OA in the rumen liquor, despite its high intake. As expected, the linseed supplementation did not influence the accumulation of RA, because this FA is not an intermediate of the rumen BH of LNA (Wilde and Dawson, 1966).

5. Conclusions

The rumen metabolism of lactating dairy ewes was markedly influenced by dietary supplementation with grape seed alone or mixed with linseed. Although grape seed caused a decrease in LA and an increase in SA, this by-product determined an accumulation of RA in the rumen. When grape seed was mixed with linseed, it enhanced the BH of LNA, thus promoting a high accumulation of VA in the rumen. Therefore, the use of grape seed obtained after distillation in the winemaking process seems not to be effective in enhancing the effect of linseed supplementation.

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559
560 Abbreviations:
561 ADL, acid detergent lignin;
562 BCFA, branched-chain fatty acids;

BH, biohydrogenation;

CLA, conjugated linoleic acid;

CON, control diet;

CP, crude protein;

DHA, docosahexaenoic acid;

DM, dry matter;

DPA, docosapentaenoic acid;

EE, ether extract;

EPA, eicosapentaenoic acid;

FA, fatty acids;

FAME, fatty acid methyl esters;

GS, diet supplemented with grape seed;

LA, linoleic acid;

LCFA, long-chain fatty acids;

LIN: diet supplemented with linseed;

LNA, linolenic acid;

MCFA, medium-chain fatty acids;

MIX: diet supplemented with both grape seed and linseed;

MUFA, monounsaturated fatty acids;

NDF, neutral detergent fiber;

NFC, non-fiber carbohydrates;

OA, oleic acid;

OBCFA, odd- and branched-chain fatty acids;

OCFA, odd-chain fatty acids;

PUFA, polyunsaturated fatty acids;

RA, rumenic acid;

589 SA, stearic acid;

590 SCFA, short-chain fatty acids;

591 SFA, saturated fatty acids;

592 TFA, *trans* fatty acids;

593 UFA, unsaturated fatty acids;

594 VA, vaccenic acid;

595 VFA, volatile fatty acids;

596

597

598 **Table 1**
599 Ingredients, chemical composition and fatty acid profile of diets

	Diets[a]			
	CON	GS	LIN	MIX
Ingredients (kg/day per head, as fed)				
Corn	0.15	0.17	-	-
Soybean	0.12	0.24	0.04	0.16
Pea	0.25	0.09	0.15	0.02
Grape seed	-	0.30	-	0.30
Linseed	-	-	0.22	0.22
Beet pulp	0.40	0.40	0.40	0.40
Commercial concentrate[b]	0.50	0.50	0.50	0.50
Dehydrated alfalfa hay[c]	0.80	0.80	0.80	0.80
Mixed hay[d]	0.20	0.20	0.20	0.20
Chemical composition[e]				
Dry matter (DM, g/kg)	908	916	912	920
NDF (g/kg DM)	418	428	437	445
NFC (g/kg DM)	334	289	285	242
ADL (g/kg DM)	46	89	50	94
CP (g/kg DM)	180	179	179	179
Ash (g/kg DM)	78	74	81	76
EE (g/kg DM)	20	32	51	58
Major fatty acids (g/100 g of total FAME)[f]				
C16:0	18.98	14.88	11.99	11.50
C16:1 *cis*-7	0.69	0.35	0.36	0.27
C16:1 *cis*-9	0.31	0.00	0.13	0.09
C16:1 *cis*-10	0.21	0.09	0.14	0.15
C17:0	0.00	0.00	0.15	0.12
C18:0 (SA)	3.33	4.47	4.39	4.68
C18:1 *cis*-9 (OA)	22.79	23.52	21.78	21.91
C18:1 *cis*-11	0.74	0.81	0.65	0.61
C18:2 *n*-6 (LA)	41.53	47.50	23.84	33.46
C18:3 *n*-3 (LNA)	8.25	5.04	34.45	24.93
C20:0	0.67	0.50	0.39	0.38
C20:1 *n*-9	0.38	0.36	0.24	0.27
C20:3 *n*-6	0.00	0.12	0.06	0.07
C24:0	0.53	0.45	0.37	0.30
SFA	24.24	20.88	17.75	17.42
MUFA	25.84	26.02	23.70	23.94

PUFA	49.92	53.11	58.55	58.64

[a] CON: control diet, GS: diet supplemented with grape seed, LIN: diet supplemented with linseed, MIX: diet supplemented with both grape seed and linseed.

[b] Commercial concentrate containing the following ingredients: sunflower seed flour, wheat bran, dehydrated alfalfa meal, corn gluten, rice husk, corn flour, soybean hulls, sugar beet molasses, calcium carbonate from powdered calcium rocks, distilled wheat, sodium chloride, plant oil (palm), mineral supplement (ferrous sulfate monohydrate at 106 mg/kg, calcium diiodate at 1.7 mg/kg, manganese oxide at 90 mg/kg, sodium selenite at 0.46 mg/kg, zinc oxide at 87 mg/kg, and sodium molybdate at 2.5 mg/kg), antioxidant (E310 propyl gallate at 4.3 mg/kg) and vitamin supplement (vitamin A at 17,971 IU/kg, vitamin D3 at 3,494 IU/kg, and vitamin E at 60 mg/kg).

[c] Dehydrated alfalfa hay composition: DM 936 g/kg, NDF 387 g/kg DM, CP 196 g/kg DM, EE 44 g/kg DM.

[d] Mixed hay composition: DM 873 g/kg, NDF 551 g/kg DM, CP 116 g/kg DM, EE 11 g/kg DM.

[e] NDF: neutral detergent fiber measured using heat stable amylase and expressed exclusive of residual ash, NFC: non-fiber carbohydrates, ADL: acid detergent lignin determined by solubilization of cellulose with sulphuric acid, CP: crude protein, EE: ether extract.

[f] FAME: fatty acid methyl esters; SA: stearic acid; OA: oleic acid; LA: linoleic acid; LNA: linolenic acid; SFA: saturated fatty acids, sum of the individual saturated fatty acids; MUFA: monounsaturated fatty acids, sum of the individual monounsaturated fatty acids; PUFA: polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids.

621 **Table 2**

622 Chemical composition and FA profile of grape seed and linseed

Chemical composition[a]	Linseed	Grape seed
Dry matter (DM, g/kg)	911	974
NDF (g/kg DM)	240	539
NFC (g/kg DM)	105	231
ADL (g/kg DM)	31	410
CP (g/kg DM)	264	93
Ash (g/kg DM)	44	27
EE (g/kg DM)	347	109
Fatty acid (g/100 g of FAME)		
C16:0	5.5	8.5
C18:0 (SA)	4.5	4.9
C18:1 *cis*-9 (OA)	18.0	9.6
C18:2 *n*-6 (LA)	15.0	74.0
C18:3 *n*-3 (LNA)	56.3	0.3

623 [a] NDF: neutral detergent fiber measured using heat stable amylase and expressed exclusive of residual ash,
624 NFC: non-fiber carbohydrates, ADL: acid detergent lignin determined by solubilization of cellulose with
625 sulphuric acid, CP: crude protein, EE: ether extract, FAME: fatty acid methyl esters, SA: stearic acid, OA: oleic
626 acid, LA: linoleic acid, LNA: linolenic acid.
627

628 **Table 3**

629 Dry matter and fatty acids intake of Sarda dairy ewes

	Diets[a]			
	CON	GS	LIN	MIX
Dry matter intake (kg/d)	2.2	2.5	2.1	2.4
Fatty acid intake (g/d)[b]				
C16:0	8.34	11.79	12.88	15.96
C16:1 *cis*-7	0.30	0.27	0.38	0.37
C16:1 *cis*-9	0.14	0.00	0.14	0.12
C16:1 *cis*-10	0.09	0.07	0.15	0.21
C17:0	0.00	0.00	0.16	0.17
C18:0 (SA)	1.46	3.54	4.72	6.49
C18:1 *cis*-9 (OA)	10.02	18.63	23.40	30.42
C18:1 *cis*-11	0.32	0.64	0.70	0.84
C18:2 *n*-6 (LA)	18.26	37.62	25.62	46.45
C18:3 *n*-3 (LNA)	3.63	3.99	37.02	34.60
C20:0	0.30	0.40	0.42	0.53
C20:1 *n*-9	0.17	0.28	0.26	0.37
C20:3 *n*-6	0.00	0.09	0.06	0.10
C24:0	0.23	0.36	0.40	0.41
SFA	10.66	16.53	19.08	24.17
MUFA	11.36	20.61	25.47	33.23
PUFA	21.94	42.06	62.91	81.39

630 [a] CON: control diet, GS: diet supplemented with grape seed, LIN: diet supplemented with linseed, MIX: diet
631 supplemented with both grape seed and linseed.
632 [b] SA: stearic acid; OA: oleic acid; LA: linoleic acid; LNA: linolenic acid; SFA: saturated fatty acids, sum of the
633 individual saturated fatty acids; MUFA: monounsaturated fatty acids, sum of the individual monounsaturated
634 fatty acids; PUFA: polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids.

635
636

637 **Table 4**

638 Effect of experimental diets with grape seed and linseed, alone or in combination, sampling and their
639 interaction on rumen fermentation parameters in Sarda dairy ewes

	Diet[a]				SEM[b]	P-value[c]	
	CON	GS	LIN	MIX		D	S
Rumen pH	6.75	6.71	6.68	6.77	0.06	0.807	<0.001
Ammonia (mg/dL)	13.66[b]	18.40[a]	13.88[b]	18.18[a]	0.62	0.040	0.014
Total VFA[d] (mmol/L)	57.52	59.18	58.67	54.10	2.30	0.720	<0.001
VFA[d] (mol/100 mol)							
Acetate	67.45	69.22	67.44	71.04	0.54	0.149	<0.001
Propionate	20.84	18.61	21.03	19.60	0.43	0.386	<0.001
Butyrate	11.71[a]	12.16[a]	11.54[a]	9.36[b]	0.37	0.003	<0.001
Acetate:Propionate	3.44	3.79	3.31	3.74	0.09	0.422	<0.001

640 Means within a row with different superscripts (a, b) are different (P<0.05).
641 [a] CON: control diet, GS: diet supplemented with grape seed, LIN: diet supplemented with linseed, MIX: diet
642 supplemented with both grape seed and linseed.
643 [b] SEM: standard error of the mean.
644 [c] D: effect of experimental diet; S: effect of sampling.
645 [d] VFA: volatile fatty acids.
646

647 **Table 5**

648 Fatty acid composition of rumen liquor in Sarda dairy ewes

Fatty acid (mg/100 mg of FAME)[d]	Diet[a]				SEM[b]	P-value[c]	
	CON	GS	LIN	MIX		D	S
C4:0	15.15[a]	9.59[b]	10.25[b]	8.79[b]	0.475	<0.001	<0.001
C5:0	3.01[a]	1.77[b]	1.86[b]	1.73[b]	0.113	0.002	<0.001
C6:0	0.66	0.58	0.41	0.54	0.036	0.398	<0.001
C8:0	0.01	0.01	0.01	0.01	0.0005	0.154	0.207
C10:0	0.02	0.02	0.02	0.01	0.001	0.466	0.034
C11:0	0.02	0.01	0.01	0.01	0.001	0.479	0.008
C12:0	0.34	0.28	0.32	0.25	0.016	0.399	<0.001
iso C13:0	0.02	0.02	0.02	0.02	0.001	0.998	0.009
anteiso C13:0	0.04	0.04	0.03	0.04	0.002	0.542	0.005
C13:0	0.06	0.08	0.09	0.09	0.007	0.413	0.121
iso C14:0	0.19	0.19	0.18	0.17	0.008	0.914	0.009
C14:0	0.69	0.77	0.78	0.64	0.032	0.504	<0.001
C14:1 *cis*-9	0.00	0.01	0.01	0.03	0.005	0.292	0.109
iso C15:0	0.33	0.30	0.27	0.32	0.013	0.437	<0.001
anteiso C15:0	1.40[a]	1.12[b]	1.19[ab]	0.98[b]	0.031	0.002	0.101
C15:0	1.21	1.16	1.10	1.05	0.029	0.352	<0.001
iso C16:0	2.18[a]	2.00[a]	1.67[ab]	1.28[b]	0.060	0.001	<0.001
C16:0	18.34[a]	16.84[ab]	15.68[bc]	14.21[c]	0.310	0.001	<0.001
C16:1 *trans*-8	0.05[a]	0.02[b]	0.02[b]	0.02[b]	0.003	0.001	0.012
C16:1 *trans*-9	0.21[a]	0.12[b]	0.11[b]	0.01[b]	0.010	<0.001	0.053
C16:1 *cis*-7	0.62	0.58	0.73	0.56	0.043	0.649	<0.001
C16:1 *cis*-9	0.06[a]	0.05[ab]	0.05[ab]	0.03[b]	0.002	0.005	0.227
iso C17:0	0.31[a]	0.29[ab]	0.21[b]	0.23[ab]	0.010	0.007	<0.001
anteiso C17:0	0.71[a]	0.58[ab]	0.51[b]	0.47[b]	0.023	0.009	<0.001
C17:0	0.53[a]	0.44[ab]	0.39[b]	0.41[b]	0.014	0.011	0.021
C18:0 (SA)	19.81[b]	31.11[a]	28.02[a]	32.81[a]	0.797	<0.001	0.003
C18:1 *trans*-4	0.03[c]	0.04[bc]	0.05[a]	0.05[ab]	0.002	<0.001	<0.001
C18:1 *trans*-6 + *trans*-8	0.40[b]	0.58[ab]	0.78[a]	0.83[a]	0.030	<0.001	<0.001
C18:1 *trans*-9	0.23[c]	0.36[bc]	0.45[ab]	0.50[a]	0.018	<0.001	<0.001
C18:1 *trans*-10	0.78	0.89	0.91	1.21	0.072	0.485	0.002
C18:1 *trans*-11 (VA)	3.77[b]	4.76[b]	6.44[ab]	8.18[a]	0.302	0.003	0.836
C18:1 *trans*-12	0.25[c]	0.73[b]	0.67[b]	0.91[a]	0.036	<0.001	0.098
C18:1 *cis*-9 (OA)	7.23[a]	5.97[a]	7.39[a]	5.89[b]	0.157	0.011	0.567
C18:1 *trans*-15 + *cis*-10	0.46[b]	0.59[b]	0.85[a]	0.96[a]	0.035	<0.001	<0.001
C18:1 *cis*-11	0.77[c]	0.97[bc]	1.21[ab]	1.22[a]	0.031	<0.001	0.311
C18:1 *cis*-12	0.33[c]	0.61[ab]	0.44[bc]	0.71[a]	0.028	<0.001	0.001

C18:1 *cis*-13	0.03	0.03	0.05	0.05	0.003	0.116	0.639
C18:1 *cis*-14 + *trans*-16	0.08^{b}	0.16^{a}	0.19^{a}	0.20^{a}	0.010	<0.001	0.006
C18:2 *trans*-9,*trans*-12	0.39^{b}	0.50^{b}	0.78^{a}	0.76^{a}	0.024	<0.001	0.007
C18:1 *cis*-15	0.09^{c}	0.21^{bc}	0.26^{ab}	0.35^{a}	0.016	<0.001	0.052
C18:2 *trans*-8,*cis*-13	0.23^{a}	0.20^{ab}	0.16^{bc}	0.14^{c}	0.007	0.001	0.170
C18:2 *trans*-11,*cis*-15	0.03	0.03	0.01	0.02	0.003	0.097	0.138
C18:2 *trans*-9,*cis*-12	0.04^{b}	0.04^{b}	0.20^{a}	0.21^{a}	0.013	<0.001	0.151
C18:2 *n*-6 (LA)	5.95^{a}	4.66^{b}	3.77^{c}	3.26^{c}	0.161	<0.001	<0.001
C18:2 *n*-4	0.16	0.14	0.17	0.11	0.012	0.434	<0.001
C18:3 *n*-6	0.29	0.26	0.25	0.24	0.016	0.718	<0.001
C18:3 *n*-3 (LNA)	1.60^{a}	0.81^{b}	1.29^{a}	0.84^{b}	0.050	<0.001	0.348
CLA *cis*-9,*trans*-11 (RA)	0.45^{b}	0.78^{a}	0.49^{b}	0.47^{b}	0.057	0.009	<0.001
C18:4 *n*-3	0.20^{a}	0.11^{b}	0.09^{b}	0.08^{b}	0.008	<0.001	0.067
CLA *trans*-9,*cis*-11 + C20	0.60	0.61	0.47	0.43	0.024	0.025	0.190
CLA *trans*-10,*cis*-12	0.12^{ab}	0.10^{b}	0.17^{a}	0.15^{ab}	0.007	0.007	<0.001
CLA *trans*-11,*cis*-13	0.03^{b}	0.08^{ab}	0.14^{a}	0.11^{ab}	0.011	0.012	<0.001
CLA *trans*-11,*trans*-13	0.06^{b}	0.07^{b}	0.12^{a}	0.12^{a}	0.005	<0.001	0.062
CLA *trans*-9,11+ C20:1 *n*-9	0.13	0.14	0.14	0.13	0.004	0.763	0.377
C20:2 *n*-6	0.02	0.02	0.02	0.02	0.001	0.418	<0.001
C20:3 *n*-9	0.03^{ab}	0.03^{a}	0.03^{b}	0.03^{ab}	0.001	0.027	0.323
C22:0	0.36^{a}	0.30^{ab}	0.33^{ab}	0.28^{b}	0.009	0.025	0.201
C22:1 *n*-11	0.06^{a}	0.04^{b}	0.04^{b}	0.03^{b}	0.002	0.003	<0.001
C20:5 *n*-3 (EPA)	0.20^{a}	0.15^{ab}	0.17^{ab}	0.13^{b}	0.007	0.011	<0.001
C22:2 *n*-6	0.17^{a}	0.13^{b}	0.13^{b}	0.11^{b}	0.005	0.001	<0.001
C24:0	0.42^{a}	0.34^{b}	0.37^{ab}	0.31^{b}	0.009	0.002	0.131
C24:1 *cis*-15	0.53^{a}	0.41^{a}	0.38^{a}	0.33^{b}	0.020	0.039	0.221
C22:5 *n*-3 (DPA)	0.01	0.01	0.01	0.01	0.002	0.456	0.909
C22:6 *n*-3 (DHA)	0.03	0.02	0.01	0.01	0.002	0.068	0.072
SCFA	18.86^{a}	11.97^{b}	12.56^{b}	11.10^{b}	0.583	<0.001	<0.001
MCFA	27.65^{a}	25.16^{ab}	23.72^{bc}	21.31^{c}	0.463	0.001	<0.001
LCFA	46.37^{c}	57.01^{b}	57.52^{b}	62.22^{a}	0.799	<0.001	0.229
OBCFA	10.01^{a}	8.00^{b}	7.54^{bc}	6.81^{c}	0.164	<0.001	0.029
iso BCFA	3.03^{a}	2.80^{ab}	2.36^{bc}	2.03^{c}	0.074	<0.001	<0.001
anteiso BCFA	2.15^{a}	1.73^{b}	1.73^{b}	1.49^{b}	0.043	<0.001	<0.001
OCFA	4.84^{a}	3.47^{b}	3.46^{b}	3.30^{b}	0.106	<0.001	<0.001
TFA	6.90^{c}	8.86^{bc}	11.53^{ab}	13.87^{a}	0.447	0.001	0.033
SFA	65.82	67.83	63.73	64.77	0.450	0.175	<0.001
MUFA	16.11^{c}	17.12^{bc}	21.18^{ab}	22.30^{a}	0.497	0.005	0.060
PUFA	10.73^{a}	8.94^{b}	8.64^{b}	7.39^{c}	0.171	<0.001	0.017
n-6	6.44^{a}	5.09^{b}	4.17^{b}	3.64^{c}	0.160	<0.001	<0.001

n-3	2.03[a]	1.10[c]	1.57[b]	1.08[c]	0.059	<0.001	0.054
CLA	1.38[b]	1.79[a]	1.52[ab]	1.42[ab]	0.067	0.045	<0.001

649 Means within a row with different superscripts (a, b, c) are different (P<0.05).
650 [a] CON: control diet, GS: diet supplemented with grape seed, LIN: diet supplemented with linseed, MIX: diet
651 supplemented with both grape seed and linseed.
652 [b] SEM: standard error of the mean.
653 [c] D: effect of experimental diet; S: effect of sampling.
654 [d] FAME: fatty acid methyl esters; SA: stearic acid; VA: vaccenic acid; OA: oleic acid; LA: linoleic acid; LNA:
655 linolenic acid; RA: rumenic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA:
656 docosahexaenoic acid; SCFA: short-chain fatty acids, sum of the individual fatty acids from C4:0 to C10:0
657 reported in this table; MCFA: medium-chain fatty acids, sum of the individual fatty acids from C11:0 to C17:0
658 reported in this table; LCFA: long-chain fatty acids, sum of the individual fatty acids from C18:0 to DHA
659 reported in this table; OBCFA: odd- and branched-chain fatty acids, sum of *iso* BCFA, *anteiso* BCFA, OCFA; *iso*
660 BCFA: *iso* branched-chain fatty acids, sum of *iso* C13:0, *iso* C14:0, *iso* C15:0, *iso* C16:0, *iso* C17:0; *anteiso* BCFA:
661 sum of *anteiso* C13:0, *anteiso* C15:0, *anteiso* C17:0; OCFA: odd-chain fatty acids, sum of C5:0, C9:0, C11:0,
662 C13:0, C15:0, C17:0; TFA: *trans* fatty acids, sum of the individual trans fatty acids reported in this table; SFA:
663 saturated fatty acids, sum of the individual saturated fatty acids reported in this table; MUFA:
664 monounsaturated fatty acids, sum of the individual monounsaturated fatty acids reported in Table 4; PUFA:
665 polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids reported in Table 4; *n*-6: sum of
666 individual *n*-6 fatty acids reported in this table; *n*-3:sum of individual *n*-3 fatty acids reported in this table; CLA:
667 sum of individual conjugated of linoleic acids reported in this table.
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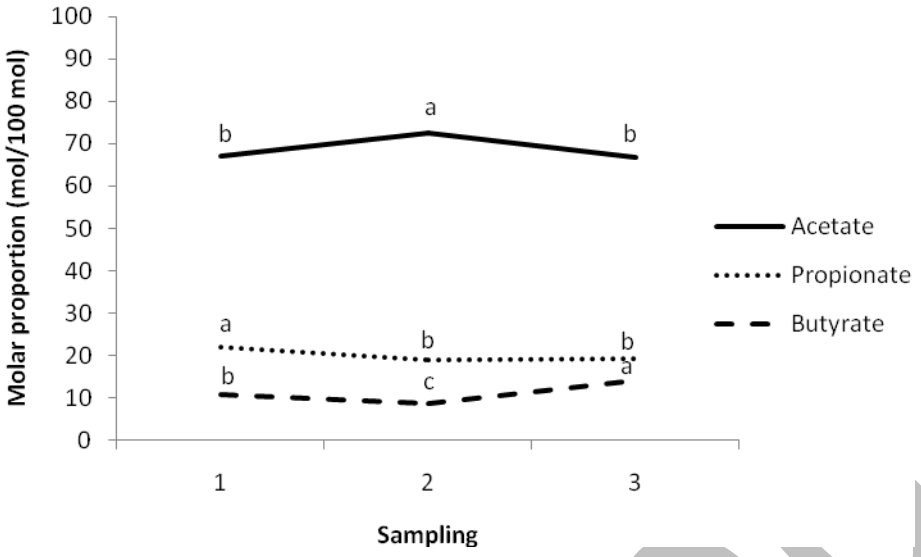
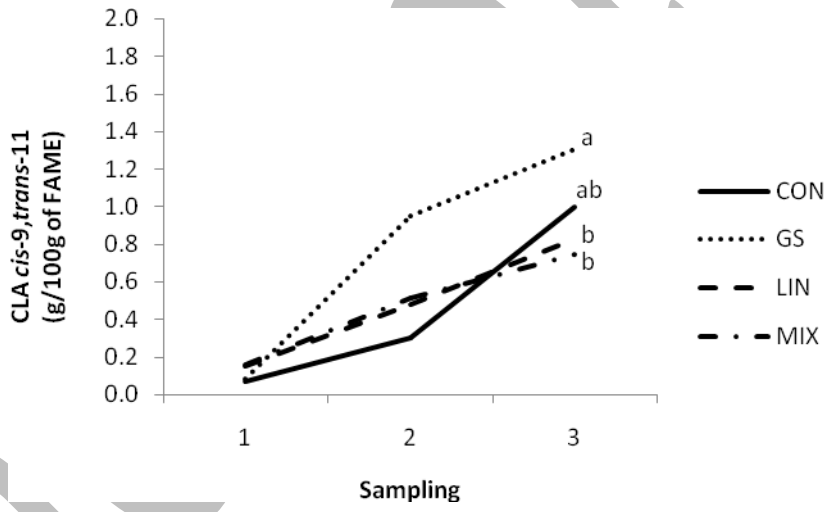


670

671 **Fig. 1.** Temporal evolution of the molar proportion of acetate, propionate and butyrate in rumen liquor
672 on 3 samplings (days 20, 40 and 60) during the trial. Different letters (a, b, c) within the same volatile
673 fatty acid show statistical differences (P<0.05) between the samplings.

674



675

676 **Fig. 3.** Temporal evolution of CLA *cis*-9,*trans*-11 (RA; rumenic acid) in rumen liquor on 3 samplings (days
677 20, 40 and 60) during the trial in dairy ewes fed a control diet (CON), a diet supplemented with grape
678 seed (GS), a diet supplemented with linseed (LIN) and a diet supplemented with both grape seed and
679 linseed (MIX). Different letters (a, b) within the same sampling show statistical differences (P<0.05)
680 between the diets.

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