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Effects of grape seed supplementation, alone or associated with linseed, on ruminal metabolism in
 Sarda dairy sheep

3 F. Correddu, A. Nudda*, G. Battacone, R. Boe, A.H.D. Francesconi, G. Pulina

4 Dipartimento di Agraria, Sezione di Scienze Zootecniche, University of Sassari, Viale Italia 39,

5 07100, Sassari, Italy

6 * Corresponding author at: Dipartimento di Agraria, University of Sassari, Viale Italia 39, 07100,

7 Sassari, Italy. Tel.: +39 079229390. E-mail address: anudda@uniss.it

8 ABSTRACT

Grape seed is a by-product of the winery and distillery industry which could be used in animal 9 10 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 11 12 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 13 fed as follows: (1) control diet (CON), (2) a diet supplemented with 300 g/d per head of grape seed 14 (GS), (3) a diet supplemented with 220 g/d per head of extruded linseed (LIN), (4) and a diet 15 supplemented with a mix of both grape seed and linseed (300 and 220 g/d per head, respectively) 16 (MIX). Ammonia, pH, volatile fatty acids (VFA) and FA composition were determined in rumen 17 liquor at 3 sampling dates (20, 40 and 60 d). Rumen pH was not influenced by diet (P>0.05). The 18 ammonia content was increased (P<0.05) in GS and MIX compared with LIN and CON. The molar 19 proportions of acetate and propionate and their ratio were not affected by the diet (P>0.05), whereas 20 the molar proportion of butyrate was the lowest in MIX. Rumenic acid (RA; CLA cis-9,trans-11) 21 concentration increased in GS compared with CON (0.78 vs. 0.45 mg/100 mg FA; P<0.05), whereas 22 the percentage of vaccenic acid (VA; C18:1 trans-11) tended to increase (P<0.10) in GS compared 23 with CON. The concentration of VA was higher in MIX than in CON (8.18 vs. 3.77 mg/100 mg FA; 24

P<0.05), whereas RA did not differ between the same groups. The concentration of linoleic acid 25 (LA; C18:2 n-6) decreased and stearic acid (SA; C18:0) increased in all supplemented groups, 26 whereas linolenic acid (LNA; C18:3 n-3) decreased in the two groups receiving grape seed 27 compared with CON and LIN. The concentration of total odd- and branched-chain fatty acids 28 (OBCFA) decreased in all supplemented groups compared with CON (P<0.05), evidencing that 29 grape seed and linseed supplementation influenced the ruminal BH processes. Grape seed was able 30 to increase the accumulation of RA when supplemented alone, and of VA when combined with 31 linseed; however, the rumen accumulation of SA in both groups supplemented with grape seed 32 evidenced that this by-product was not effective in decreasing the BH of dietary polyunsaturated 33 fatty acids (PUFA). 34

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

36 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 agroindustrial 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 49 milk and cheese, by increasing the concentration of healthy FA (Caroprese et al., 2010; Mughetti et 50 al., 2012; Nudda et al., 2013). Lipids from the diet are involved in a sequence of reactions, 51 performed by the rumen microbial population, including hydrolysis of esterified lipids to free FA, 52 whose double bonds can be partly isomerized and hydrogenated. These reactions can be explained 53 by a detoxification mechanism to defend microorganisms from the toxicity of unsaturated fatty 54 acids (UFA) as reported by Dehority (2003) and Maia et al. (2010). Because C18:2 cis-9, cis-12 55 (linoleic acid, LA, n-6 series) and LNA provided with the diet are greatly reduced (by 80% and 56 92%, respectively) in the rumen (Doreau and Ferlay, 1994), several strategies have been tested to 57 protect dietary FA from rumen BH. The encapsulation of lipids in a protein matrix is one of the 58 techniques proposed to protect FA (Tymchuk et al., 1998; Hawkins et al., 2013). However, the 59 occasional lack of efficiency of this method (Petit, 2003), its high costs and the need for 60 61 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 62 (Jenkins, 1998) or to calcium salts (Lundy Iii et al., 2004). The presence of some plant compounds, 63 64 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 65 supplement in ruminant nutrition could be an alternative for the expensive management and 66 disposal of this winery by-product. However, the literature available on the use of grape by-67 products in ruminant nutrition is limited (Moate et al., 2014; Santos et al., 2014) and, to our 68 knowledge, the effects of the dietary supplementation with grape seed on the BH of PUFA in the 69 rumen has not been explored yet. 70

71 To test the hypothesis that grape seed supplementation decreases the BH activity of rumen 72 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
- sheep.
- 75

76 2. Material and methods

77 2.1. Animals, experimental design and treatments

Twenty four Sarda dairy ewes in the first part of lactation (<50 days in milk, DIM) were 78 79 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 80 experimental diets (Table 1): control (CON) diet, a diet supplemented with 300 g/d per head of 81 grape seed (GS), a diet supplemented with 220 g/d per head of extruded linseed (LIN), and a diet 82 supplemented with a mix of 300 g/d per head of grape seed and 220 g/d per head of linseed (MIX). 83 The extruded linseed dose of 220 g/d was used to supply 70 g/d of fat per head. Considering that the 84 total phenolic content of grape seed was 333.3 ± 10.1 mg gallic acid equivalent (GAE)/100 g of dry 85 matter (DM; mean \pm S.E.), the grape seed dose of 300 g/d per head was used to provide 86 approximately 1 g/d per head of total polyphenols (approximately 0.4 g polyphenols/kg DM of 87 diet). The grape seed used was obtained from different red grape varieties after distillation in the 88 winemaking process. Grape seeds were ground before administration. The chemical composition 89 90 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 91 concentrate, dehydrated alfalfa hay and mixed hay. In addition, they received a mixed meal 92 93 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 94 95 mixed hay were offered to each group of six ewes, whereas all other dietary ingredients were 96 provided to each animal by using individual feeders. The commercial concentrate (500 g/d per 97 head) was provided at the two daily milkings (7:30 and 17:30). The mixed meals were provided two 98 hours after each milking, and subsequently beet pulp (400 g/d per head) and dehydrated alfalfa hay 99 (on average 800 g/d per head) were provided. The mixed hay (on average 200 g/d per head) was 100 offered during the night. Both hays were offered at a fixed amount to avoid selection by the

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

106 2.2. Rumen sample collection

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

117 *2.3. Chemical analysis*

Dry matter content of feed ingredients was determined by oven-drying at 105°C for 24 h. Neutral detergent fiber (NDF) and acid detergent lignin (ADL) analyses were performed following the method of Van Soest et al. (1991), using an Ankom 220 fiber analyzer (Ankom[™] technology, Fairport, NY, USA); NDF was measured using heat stable amylase and expressed exclusive of residual ash (aNDFom) and ADL was determined by solubilization of cellulose with sulphuric acid. Crude protein (CP) content was measured according to the Kjeldahl method (proc. 988.05; AOAC, 2000), extract ether (EE) by the Soxhlet method (proc. 920.39; AOAC, 2005) and ash by using a muffle at 550°C (proc. 942.05; AOAC, 2000). Non-fiber carbohydrates (NFC) were calculated according to Weiss (1999) as follows: NFC (g/kg DM) = 100 - (NDF + CP + ash + EE).

To determine the phenolic content of grape seed, the seeds were powdered by a blender and an 127 aliquot of 1.5 g was homogenized with 50 mL of a mixture of acetone/water (70/30, v/v) using an 128 Ultra Turrax homogenizer (Ultra Turrax T25, Janke&Kunkel KG, Germany) at 3000 rpm, for 2 129 min, in a water/ice bath. The homogenate was then centrifuged (6,000 x g, 15 min, 4°C) and the 130 supernatant was filtered through Whatman 541 filter paper (Whatman, Maidstone, England) for 131 determination of phenolic compounds, using the Folin-Ciocalteu method described by Kim et al. 132 (2003) with some modifications. Briefly, 0.5 mL of the extract were added to 1 mL of Folin-133 Ciocalteu phenol reagent (Sigma Chem. Co., St. Louis, MO, USA) and 9.5 mL of distilled water in 134 a 25 mL volumetric flask and shaken. After 7 min, 10 mL of 0.71 M sodium carbonate (Na₂CO₃) 135 were added, and then the mixture was diluted to a volume of 25 mL with distilled water and mixed 136 thoroughly. The mixture was then stored in the dark for 120 min at room temperature. The 137 absorbance was read at 750 nm wavelength versus a blank solution. Total phenolic content, 138 expressed as mg of GAE/100 g DM, was determined following a calibration curve obtained using 139 gallic acid (Sigma Chem. Co., St. Louis, MO, USA) as standard. 140

The FA profiles of the whole diets, grape seed, linseed and rumen liquor were determined using 141 the method of Kramer et al. (1997) with some modifications. Samples kept at -80°C were 142 lyophilized and powdered. After adding 2 mL of sodium methoxide 0.5 M in methanol (Sigma and 143 Aldrich, Spain) to the power, it was placed in a water bath at 50°C for 10 min. After cooling to 144 room temperature, the samples were placed in a water/ice bath; then 3 mL of HCl/methanol (3M), 145 prepared with acetyl chloride and methanol, were added. Subsequently, the samples were heated 146 again in a water bath at 50°C for 10 min and cooled to room temperature; then 1 mL of a solution 147 containing methyl nonadecanoate (C19:0) as internal standard (Sigma Chemical Co., St. Louis, 148 MO, USA) and, subsequently, 7.5 mL of a 0.43 M solution of K₂CO₃ were added. After quick 149

agitation the samples were centrifuged $(1,500 \times g, room temperature, 5 min)$ and each supernatant 150 was kept in a vial for GC analysis. Fatty acid methyl esters (FAME) were determined using a Turbo 151 3400 CX gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame ionization 152 detector (FID) and an automatic injector 8200 (CX Varian Inc., Palo Alto, CA, USA). The column 153 (CP-select CB for FAME; 100 m x 0.32 mm i.d., 0.25 µm film thickness, Varian Inc., Palo Alto, 154 CA, USA) was operated with the following program: 75°C for 1 min, increased at 5°C/min to 155 148°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 156 3°C to 230°C, held for 14 min. Helium (1 mL/min flow rate) was used as carrier gas with a pressure 157 of 37.000 psi. Split ratio was 1:100. The injector and detector temperatures were held at 225 and 158 285°C, respectively. Varian Star 3.4.1 software was used to compute the retention time and area of 159 each individual FAME, identified by comparing their retention times with those of known standards 160 and with published studies as detailed by Nudda et al. (2008). 161

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) 165 method. Briefly, a sample of approximately 2 mL was defrozen and centrifuged (15,000 x g, 10 166 min, 4°C); the supernatant was then withdrawn by syringe and injected into a HPLC system (Varian 167 Inc., Palo Alto, California, USA) after filtration (PTFE 0.45 µm, 13 mm). The HPLC was equipped 168 with an auto sampler (Varian 9300), a degasser (Varian 9012 Q), a UV detector (Varian 906P 169 Polychrom) and an Aminex HPX 87H column (Biorad Laboratories, Hercules, CA, USA). The 170 column was operated at 55°C with 0.008 N H₂SO₄ at 0.6 mL/min as eluent. Concentrations of VFA 171 172 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 173 propionic and butyric acid) obtained by appropriate dilutions of a standard mixture of VFA 174

containing 5.40, 5.76 and 7.02 mg/mL of acetic, propionic and butyric acids, respectively, in H₂SO₄
0.1 N. The concentration of total and single VFA were expressed as mmol/L and mol/100 mol of
total VFA, respectively.

178 2.4. Statistical analysis

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

183 **3. Results**

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

All dairy ewes consumed the whole daily amount of feeds supplied, as a consequence of the fixed amount of diets provided (Table 3). The intake of most FA varied with diets (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*

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

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

(P<0.05) from sampling 2 (14.71 mg/dL) to sampling 3 (17.71 mg/dL), with sampling 1 (15.67 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 the rumen are shown in Table 4. The concentration of total VFA and the molar proportions of 201 acetate and propionate were not affected by diet, whereas the concentration of butyrate was reduced 202 (P<0.05) by the MIX containing both grape seed and linseed. A time effect was observed (P<0.01) 203 for total VFA and individual VFA, with the highest concentrations of propionate, acetate and 204 butyrate being on samplings 1, 2, and 3, respectively (P<0.05; Fig. 1). The acetate:propionate ratio 205 was not affected by diet, but differed (P<0.05) among sampling 1 (3.14), sampling 2 (4.04) and 206 sampling 3 (3.54). This ratio was influenced by group \times sampling interaction (P<0.05), but no 207 208 significant differences between diets within sampling were detected.

209 *3.3. FA composition in rumen liquor*

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

The concentration of total odd- and branched-chain fatty acids (OBCFA) decreased (P<0.05) in all supplemented groups compared with CON. The MIX group had the lowest concentration of 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). Many of the FA measured during the trial were influenced by sampling, but the pattern varied among them. In general, most of the SCFA (C4:0, C5:0 and C6:0) and isomers of C18:1 showed a significant decrease (P<0.05) in the last sampling, whereas most of the MCFA increased (P<0.01) over time. The individual LCFA showed a variable pattern, without a significant sampling effect on total LCFA (P>0.05). The PUFA decreased over time, mainly due to a decrease in PUFA n-6 (LA) (P<0.05). Among OBCFA, *iso* BCFA and *anteiso* BCFA increased after sampling 1, whereas OCFA decreased over time on the last sampling (P<0.05).

252 **4. Discussion**

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

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

could be explained by two hypotheses. The first is that the level of polyphenols in the grape seed 270 used in the present work was too low to influence the activity of some strains of proteolytic 271 bacteria, compared with the doses of polyphenols (values higher than 5 g/kg DM) which influenced 272 rumen bacteria population in other studies (Hervás et al., 2003; Vasta et al., 2010; Anantasook et 273 al., 2014). In addition, in some studies low concentrations of tannins increased the enzymatic 274 activity and growth of some bacteria in vitro (Jones et al., 1994) and in vivo (Vasta et al., 2010), 275 likely because the interaction between proteins and tannins can cause conformational changes in the 276 protein structure, giving more accessible sites for some proteolytic bacteria (Mole and Waterman, 277 1985; Molan et al., 2001). The second hypothesis is that the high concentration of ADL in the grape 278 seed used (410 g/kg DM) reduced the growth of cellulolytic bacteria, considering that high amounts 279 of lignin in ruminant diets can reduce fiber digestion (Hartley, 1972; Jung and Fahey, 1984) by 280 decreasing the growth of this type of bacteria (Akin, 1982), whose favorite substrate for protein 281 282 synthesis is ammonia (Brayant, 1973; Van Soest, 1994), with a consequent accumulation of ammonia in the rumen. 283

The lack of effect of grape seed or extruded linseed, alone or in combination, on the 284 concentration of total VFA and on the proportion of acetate and propionate and their ratio in dairy 285 ewes observed in our study is in accordance with previous studies on dairy sheep fed diets rich in 286 LA and supplemented with 10 or 20 g tannins/kg DM from quebracho (Toral et al., 2011, 2013), on 287 cattle supplemented with quebracho polyphenols (Beauchemin et al., 2007) and on dairy cows 288 supplemented with flax seed (Neveu et al., 2013). Differently, Ivan et al. (2013) found a reduction 289 in acetate in dairy cows fed diets supplemented with oilseeds rich in LA or LNA and an increase in 290 291 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 in accordance with the meta-analysis of Patra (2014) showing a negative effect of increasing levels of fat in the diets of sheep on the proportion of butyrate, likely due to the inhibition of microorganisms (protozoa and *Butyvibrio fibrisolvens*) involved in its production (Hristov et al., 2009).

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

The OBCFA profile has been proposed as diagnostic tool to predict shifts in microbial population associated with the diet variation (Lee et al., 1999; Vlaeminck et al., 2004). In the present work the reduction in the *iso* C17:0 and *anteiso* C17:0 in the rumen liquor of sheep receiving linseed supplementation could be related to a negative effect of this supplement on the protozoa population, which produces a greater proportion of these FA than the bacteria population 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 327 expected and was likely a consequence of the higher concentration of most C18:1 and C18:2 328 isomers, which derive in part from the BH of LNA. This finding is in agreement with previous 329 studies showing that the concentration of C18:1 and C18:2 isomers increased in the rumen of steers 330 fed linseed oil (Shingfield et al., 2011). The greater accumulation of most trans and cis isomers of 331 C18:1 in the MIX group could be mainly a consequence of the BH of OA, before its reduction to 332 SA. The increase in C18:2 trans-9, trans-12 and C18:2 trans-9, cis-12 observed only in the two diets 333 containing linseed is in agreement with an *in vitro* study showing that the concentrations of these 334 geometrical isomers of LA were increased by LNA supplementation, but not by LA 335 supplementation (Jouany et al., 2007). 336

The significantly higher values of VA in MIX, and numerically higher in LIN, compared with 337 GS and CON reflected the pattern of intake of its precursor LNA. The increase in RA with time, 338 although with a different pattern among diets, reflected the observed reduction in its precursor LA 339 340 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-341 342 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 343 of VA to SA (Khiaosa-Ard et al., 2009; Vasta et al., 2009; Rana et al., 2012). The low content of 344

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).

352 **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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 Digestion and body weight change in Tuj lambs receiving oak (*Quercus hartwissiana*) leaves
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- 559
- 560 Abbreviations:
- 561 ADL, acid detergent lignin;
- 562 BCFA, branched-chain fatty acids;

- 563 BH, biohydrogenation;
- 564 CLA, conjugated linoleic acid;
- 565 CON, control diet;
- 566 CP, crude protein;
- 567 DHA, docosahexaenoic acid;
- 568 DM, dry matter;
- 569 DPA, docosapentaenoic acid;
- 570 EE, ether extract;
- 571 EPA, eicosapentaenoic acid;
- 572 FA, fatty acids;
- 573 FAME, fatty acid methyl esters;
- 574 GS, diet supplemented with grape seed;
- 575 LA, linoleic acid;
- 576 LCFA, long-chain fatty acids;
- 577 LIN: diet supplemented with linseed;
- 578 LNA, linolenic acid;
- 579 MCFA, medium-chain fatty acids;
- 580 MIX: diet supplemented with both grape seed and linseed;
- 581 MUFA, monounsaturated fatty acids;
- 582 NDF, neutral detergent fiber;
- 583 NFC, non-fiber carbohydrates;
- 584 OA, oleic acid;
- 585 OBCFA, odd- and branched-chain fatty acids;
- 586 OCFA, odd-chain fatty acids;
- 587 PUFA, polyunsaturated fatty acids;
- 588 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;

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	
Реа	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 <i>cis</i> -7	0.69	0.35	0.36	0.27	
C16:1 <i>cis</i> -9	0.31	0.00	0.13	0.09	
C16:1 <i>cis</i> -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 <i>cis</i> -9 (OA)	22.79	23.52	21.78	21.91	
C18:1 <i>cis</i> -11	0.74	0.81	0.65	0.61	
C18:2 <i>n</i> -6 (LA)	41.53	47.50	23.84	33.46	
C18:3 <i>n</i> -3 (LNA)	8.25	5.04	34.45	24.93	
C20:0	0.67	0.50	0.39	0.38	
C20:1 <i>n</i> -9	0.38	0.36	0.24	0.27	
C20:3 <i>n</i> -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	

^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).

- ⁶ 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,

612 NFC: non-fiber carbohydrates, ADL: acid detergent lignin determined by solubilization of cellulose with 613 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.

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PUFA

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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 <i>cis</i> -9 (OA)	18.0	9.6
C18:2 <i>n</i> -6 (LA)	15.0	74.0
C18:3 <i>n</i> -3 (LNA)	56.3	0.3

^a 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, FAME: fatty acid methyl esters, SA: stearic acid, OA: oleic acid, LA: linoleic acid, LNA: linolenic acid.

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 <i>cis</i> -7	0.30	0.27	0.38	0.37
C16:1 <i>cis</i> -9	0.14	0.00	0.14	0.12
C16:1 <i>cis</i> -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 <i>cis</i> -9 (OA)	10.02	18.63	23.40	30.42
C18:1 <i>cis</i> -11	0.32	0.64	0.70	0.84
C18:2 <i>n</i> -6 (LA)	18.26	37.62	25.62	46.45
C18:3 <i>n</i> -3 (LNA)	3.63	3.99	37.02	34.60
C20:0	0.30	0.40	0.42	0.53
C20:1 <i>n</i> -9	0.17	0.28	0.26	0.37
C20:3 <i>n</i> -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

^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 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.

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638 Effect of experimental diets with grape seed and linseed, alone or in combination, sampling and their

639	interaction on	rumen fermentation	parameters in Sa	rda dairy ewes
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	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	6 <0.001
Butyrate	11.71 ^ª	12.16 ^ª	11.54ª	9.36 ^b	0.37	0.003	3 <0.001
Acetate:Propionate	3.44	3.79	3.31	3.74	0.09	0.422	2 <0.001

640 Means within a row with different superscripts (a, b) are different (P<0.05).

^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.
- ^c D: effect of experimental diet; S: effect of sampling.
- 645 ^d VFA: volatile fatty acids.

648 Fatty acid composition of rumen liquor in Sarda dairy ewes

Fatty acid	Diet ^a				SEM ^b	P-value ^c	
(mg/100 mg 01 FAIVIE)	CON	GS	LIN	MIX		D	S
C4:0	15.15ª	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
<i>iso</i> 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
<i>iso</i> 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 <i>cis-</i> 9	0.00	0.01	0.01	0.03	0.005	0.292	0.109
<i>iso</i> 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
<i>iso</i> 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 <i>cis</i> -7	0.62	0.58	0.73	0.56	0.043	0.649	< 0.001
C16:1 <i>cis-</i> 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 <i>cis-</i> 9 (OA)	7.23ª	5.97 ^a	7.39 ^ª	5.89 ^b	0.157	0.011	0.567
C18:1 <i>trans-</i> 15 + <i>cis-</i> 10	0.46 ^b	0.59 ^b	0.85 ^ª	0.96 ^ª	0.035	<0.001	<0.001
C18:1 <i>cis</i> -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 <i>cis</i> -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 <i>cis</i> -15	0.09 ^c	0.21 ^{bc}	0.26 ^{ab}	0.35ª	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 <i>n</i> -6 (LA)	5.95°	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 <i>n</i> -6	0.29	0.26	0.25	0.24	0.016	0.718	< 0.001
C18:3 <i>n</i> -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 <i>trans-</i> 9, <i>cis-</i> 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	0.12ª	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 <i>n</i> -6	0.02	0.02	0.02	0.02	0.001	0.418	< 0.001
C20:3 n-9	0.03 ^{ab}	0.03ª	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 <i>n</i> -11	0.06 ^ª	0.04 ^b	0.04 ^b	0.03 ^b	0.002	0.003	< 0.001
C20:5 <i>n</i> -3 (EPA)	0.20 ^a	0.15 ^{ab}	0.17 ^{ab}	0.13 ^b	0.007	0.011	< 0.001
C22:2 <i>n</i> -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 <i>cis</i> -15	0.53ª	0.41 ^ª	0.38ª	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	27.65ª	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}	13.87ª	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
<i>n</i> -6	6.44 ^ª	5.09 ^b	4.17 ^b	3.64 ^c	0.160	<0.001	<0.001

n-3	2.03ª	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).

^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 SEM: standard error of the mean.

^c D: effect of experimental diet; S: effect of sampling.

^d FAME: fatty acid methyl esters; SA: stearic acid; VA: vaccenic acid; OA: oleic acid; LA: linoleic acid; LNA: 654 linolenic acid; RA: rumenic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: 655 656 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 reported in this table; LCFA: long-chain fatty acids, sum of the individual fatty acids from C18:0 to DHA 658 reported in this table; OBCFA: odd- and branched-chain fatty acids, sum of iso BCFA, anteiso BCFA, OCFA; iso 659 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: 660 sum of anteiso C13:0, anteiso C15:0, anteiso C17:0; OCFA: odd-chain fatty acids, sum of C5:0, C9:0, C11:0, 661 C13:0, C15:0, C17:0; TFA: trans fatty acids, sum of the individual trans fatty acids reported in this table; SFA: 662 saturated fatty acids, sum of the individual saturated fatty acids reported in this table; MUFA: 663 monounsaturated fatty acids, sum of the individual monounsaturated fatty acids reported in Table 4; PUFA: 664 665 polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids reported in Table 4; n-6: sum of individual *n*-6 fatty acids reported in this table; *n*-3:sum of individual *n*-3 fatty acids reported in this table; CLA: 666 sum of individual conjugated of linoleic acids reported in this table. 667



Fig. 1. Temporal evolution of the molar proportion of acetate, propionate and butyrate in rumen liquor 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.



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