Grape seed and linseed, alone and in combination, enhance unsaturated fatty acids in the milk of Sarda dairy sheep

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1   **Grape seed and linseed, alone and in combination, enhance the unsaturated fatty acids in the**

2   **milk of Sarda dairy sheep**

3   *Interpretive Summary.* **Grape seed and linseed, alone and in combination, enhance the**

4   **unsaturated fatty acids in the milk of Sarda dairy sheep. Correddu et al.** Grape seed is a winery

5   by-product which contains a considerable amount of polyphenols and oils. Its use in ruminant

6   nutrition could represent an alternative for their problematic management and disposal, and could be

7   useful to increase the concentration of beneficial fatty acids in sheep milk. The aim of this study was

8   to investigate the effect of dietary grape seed, alone or in combination with linseed (rich in

9   polyunsaturated fatty acids), on milk fatty acid composition in lactating dairy ewes. Grape seed and

10   linseed improve sheep milk quality due to a summative effect on fatty acids profile.

11

12   **GRAPE SEED AND LINSEED FED TO DAIRY EWES**

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20   **ABSTRACT**

21   This study evaluated the effect of the dietary inclusion of grape seed and linseed, alone or in

22   combination, on sheep milk fatty acids (FA) profile using twenty-four Sarda dairy ewes allocated to

23   four isoproductive groups. Groups were randomly assigned to four dietary treatments consisting of a

24   control diet (CON), a diet including 300 g/d per head of grape seed (GS), a diet including 220 g/d per

25   head of extruded linseed (LIN), and a diet including a mix of 300 g/d per head of grape seed and 220

26   g/d per head of extruded linseed (MIX). The study lasted 10 wk, with two wk of adaptation period

27   and 8 wk of experimental period. Milk FA composition was analyzed in milk samples collected in

28   the last four wk of the trial. The milk concentration of saturated fatty acids (SFA) decreased and that

29   of unsaturated, monounsaturated and polyunsaturated fatty acids (UFA, MUFA and PUFA,

30   respectively) increased in GS, LIN and MIX groups compared with CON. The MIX group showed

31   the lowest values of SFA and the highest of UFA, MUFA and PUFA. Milk from ewes fed linseed

32   (LIN and MIX) showed an enrichment of vaccenic acid (VA), oleic acid (OA), α-linolenic acid (LNA)

33   and *cis*-9,*trans*-11 CLA compared with milk from the CON group. The GS group showed a greater

34   content of milk oleic acid (OA) and linoleic acid (LA) and tended to show a greater content of VA

35   and *cis*-9,*trans*-11 CLA than the CON group. The inclusion of grape seed and linseed, alone and in

36   combination, decreased the milk concentration of *de novo* synthesized FA C10:0, C12:0, and C14:0,

37   with the MIX group showing the lowest values. In conclusion, grape seed and linseed could be useful

38   to increase the concentration of FA with potential health benefits, especially when these ingredients

39   are included in combination in the diet.

40   **Key words**: sheep milk, beneficial fatty acids, grape seed, extruded linseed, by-product,

41   multivariate analysis

42

INTRODUCTION

Growing interest in the nutraceutical properties of food has directed the attention of researchers to the improve the quality of dairy products. PUFA, such as PUFA *n*-3, are recognized to be beneficial to human health, by reducing serum triglycerides and low-density lipoprotein cholesterol (Simopoulos, 1991). Ovine milk is a major source of CLA, such as *cis*-9,*trans*-11 CLA (rumenic acid, RA), which has several effects, such as antiatherosclerotic, anticancer, antidiabetic and anti-inflammatory activity (Bhattacharya et al., 2006).

Diet is the most important factor influencing the milk FA composition in dairy ewes. In order to increase the concentration of nutritional FA in milk, sources of unsaturated plant lipids, such as linseed, soybeans, safflower and sunflower can be included in the diet (Nudda et al., 2014). In particular, linseed supplementation resulted in a high concentration of α-linolenic acid (LNA), CLA and vaccenic acid (C18:1 *trans*-11, VA), in milk of sheep, cows and goats (Zhang et al., 2006; Caroprese et al., 2010; Nudda et al., 2013a). Manipulation of ruminal biohydrogenation processes also may influence the milk FA composition. As demonstrated by in vitro and in vivo studies, dietary polyphenols can affect the growth and activity of some strains of bacteria involved in the biohydrogenation pathway of FA, leading to a shift in the ruminal microbial population (Vasta et al., 2009a, 2010). In particular, it has been evidenced that polyphenols can inhibit the proliferation and activity of *Butyrivibrio proteoclasticus*, involved in the last step of biohydrogenation of PUFA, which consists of the enzymatic reduction of VA to stearic acid (C18:0, **SA**) (Vasta et al., 2010; Buccioni et al., 2015). The consequent ruminal accumulation of PUFA and their biohydrogenation intermediates (Vasta et al., 2009b; Khiaosa-Ard et al., 2009) could enhance the extent of rumen escape of these FA and, consequently, could increase their concentration in milk, as demonstrated in studies on dairy cows and ewes (Moate et al., 2014; Buccioni et al., 2015).

Grape seed is a by-product derived from the winery and distillery industries which contains a high amount of polyphenols, mainly proanthocyanidins (Schieber et al., 2001). Therefore, the use of grape

68   seed in ruminant nutrition could be useful to modulate ruminal biohydrogenation of PUFA and could

69   be an alternative for the expensive management and disposal of this by-product. The inclusion of

70   grape residue in the diet of sheep increased rumen accumulation of VA (Correddu et al., 2015); in

71   cows reduced methane emission, improved milk quality, by enhancing milk FA profile (Moate et al.,

72   2014), and increasing antioxidant activity (Santos et al., 2014). Grape seed is also a good source of

73   linoleic acid (C18:2 *n*-6, LA) which could positively affect the milk FA composition in dairy sheep.

74   Principal component analysis (PCA) and hierarchical cluster analysis (HCA) could be helpful

75   methods to simplify the analysis of complex datasets composed of several variables, as the case of

76   FA profile. In the last decades the use of multivariate analysis has become a popular approach to

77   discriminate the effects of dietary treatments throughout the FA composition in meat (Coltro et al.,

78   2005) and milk (Kadegowda et al., 2008) fat.

79   We hypothesized that dietary grape seed could enhance the effectiveness of linseed in increasing

80   the concentration of polyunsaturated fatty acids in sheep milk. Therefore, the main objective of this

81   work was to investigate the effect of the inclusion of grape seed in the diet of lactating ewes, alone

82   or associated with linseed, on milk FA profile. Moreover, the multivariate analysis was used to test

83   the hypothesis that data of milk FA could be useful tool to discriminate between groups of ewes fed

84   diets with a different FA profile.

85   **MATERIAL AND METHODS**

86   The experiment was conducted in a dairy sheep farm located in the north-west of Sardinia from

87   February to April 2013. The sheep management and the chemical analysis of feeds have been

88   previously reported in detail by Correddu et al. (2015). Briefly, 24 Sarda dairy ewes were selected to

89   form four groups balanced for milk production (1.75 ± 0.02 kg/d per head, mean ± SD), body weight

90   (BW 43.2 ± 0.7 kg, mean ± SD), DIM and number of lactation (2-3 lactations). Each group was

91   allocated to one of the following dietary treatments: control diet (CON), a diet containing 300 g/d per

92   head of grape seed (GS), a diet containing 220 g/d per head of extruded linseed (LIN) and a diet

93   containing both 300 g/d of grape seed and 220 g/d of linseed per head (MIX). The ingredients, the

94   chemical composition and the fatty acid profile of the experimental diets are reported in Table 1. All

95   animals were fed a common ration, which included a commercial concentrate, beet pulp, mixed hay

96   and dehydrated alfalfa hay, and a mixed meal, which included corn, soybean, pea, grape seed and

97   linseed at varying amounts depending on the dietary treatment. The quantity of peas, soybeans and

98   corn was calculated in order to make isoproteic diets and to supply the same level of metabolizable

99   energy to each group. Linseeds were offered at the dose of 220 g/d per head in order to provide 70

100   g/d per head of fat. Grape seeds were offered at the dose of 300 g/d per head to provide approximately

101   1 g/d per head of total grape seed polyphenols, considering that the total phenolic content of grape

102   seed was 333.3 ± 10.1 mg gallic acid equivalent (GAE)/100 g of dry matter (DM; mean ± S.E.).

103   ***Milk Samples***

104   Individual morning milk samples were collected weekly and stored at −20°C until analysis. Milk

105   samples collected in the last four wk of the trial were used to analyze the milk FA composition.

106   ***Fatty Acid Composition of Milk***

107   Milk fat extraction and FAME preparation were performed as described by Nudda et al. (2005).

108   The FAME were analyzed using a Turbo 3400 CX gas chromatograph (Varian Inc., Palo Alto, CA),

109   equipped with a flame ionization detector (FID), an automatic injector 8200 CX (Varian Inc.) and a

110   capillary column (CP-select CB for FAME; 100 m x 0.32 mm i.d., 0.25 μm film thickness, Varian

111   Inc.). The temperature program was as follows: 75°C for 1 min, increased at 5°C/min to 148°C and

112   at 8°C/min to 165°C, held for 35 min, then increased at 5.5°C/min to 210 °C and, finally, at 3°C/min

113   to 230°C and held for 14 min. Helium (1 mL/min flow rate) was used as carrier gas with a pressure

114   of 255.10 kPa. Split ratio was 1:100. Injector temperature was set at 225°C and detector temperature

115   was set at 285°C. The FAME peaks were routinely identified by comparing their retention times with

116   those of known standards and with published studies, as detailed in Nudda et al. (2005).Varian Star

117   3.4.1 software was used to compute the retention time and area of each individual FAME.

118   FA were reported as g/100 g of total FAME and groups of FA were calculated as follows: SFA,

119   sum of the individual saturated fatty acids; unsaturated fatty acids (**UFA**), sum of the individual

120   unsaturated fatty acids; MUFA, sum of the individual monounsaturated fatty acids; PUFA, sum of

121   the individual polyunsaturated fatty acids; trans fatty acids (**TFA**) sum of individual trans fatty acids,

122   branched-chain fatty acids (**BCFA**), sum of individual branched-chain fatty acids; odd- and branched-

123   chain fatty acids (**OBCFA**), sum of individual odd- and branched-chain fatty acids; short-chain fatty

124   acids (**SCFA**), sum of the individual fatty acids from C4:0 to C10:0; medium-chain fatty acids

125   (**MCFA**), sum of the individual fatty acids from C11:0 to C17:0; long-chain fatty acids (**LCFA**), sum

126   of the individual fatty acids from C18:0 to C22:6 (DHA); PUFA *n*-3, sum of individual *n*-3 fatty

127   acids; PUFA *n*-6, sum of individual *n*-6 fatty acids; CLA, sum of individual conjugated linoleic acids;

128   Total C18:1, sum of individual C18:1 isomers; Total C18:2, sum of individual C18:2 isomers, Total

129   C18:1-*cis*, sum of individual C18:1-cis isomers; Total C18:1-*trans*, sum of individual C18:1 trans

130   isomers.

131   The nutritional properties of milk fat were estimated by the n-6 to n-3 ratio and three following

132   indices: the atherogenic index (**AI**) and trombogenic index (**TI**) were calculated according to Ulbricht

133   and Southgate (1991) except for the substitution of C18:0 with C12:0, as suggested by Nudda et al.

134   (2013b): $AI = [12:0 + (4 \times 14:0) + 16:0]/[(PUFA) + (MUFA)]$, and $TI = (14:0 + 16:0)/[(0.5 \times MUFA)$

135    $+ (0.5 \times n\text{-}6) + (3 \times n\text{-}3) + (n\text{-}3{:}n\text{-}6)]$; the hypocholesterolemic to hypercholesterolemic ratio (**h:H**)

136   was calculated according to Fernández et al. (2007) as follows: h:H = [(sum of 18:1*cis*-9, 18:1*cis*-11,

137   18:2 *n*-6, 18:3 *n*-6,18:3 *n*-3, 20:3 *n*-6, 20:4 *n*-6, 20:5 *n*-3, 22:4 *n*-6, 22:5 *n*-3 and 22:6 *n*-3)/(14:0 +

138   16:0)].

139   To study the effect of the different diets on the capacity of desaturating SFA to Δ^9- UFA, the Δ^9-

140   desaturase indices (**DI**) were calculated according to Schennink et al. (2008) as follows:

141   C10 index = [C10:1/(C10:0 + C10:1)] × 100;

142   C14 index = [C14:1 *cis*-9/(C14:0 + C14:1 *cis*-9)] × 100;

143   C16 index = [C16:1 *cis*-9/(C16:0 + C16:1 *cis*-9)] × 100;

144   C18 index = [C18:1 *cis*-9/(C18:0 + C18:1 *cis*-9)] × 100;

145   CLA index = [CLA *cis*-9,*trans*-11/(C18:1 *trans*-11 + CLA *cis*-9,*trans*-11)] × 100;

146   Total index = [(C10:1 + C14:1 *cis*-9 + C16:1 *cis*-9 + C18:1 *cis*-9 + CLA *cis*-9,*trans*-11)/(C10:0 +

147   C14:0 + C16:0 + C18:0 + C18:1 *trans*-11+ C10:1 + C14:1 *cis*-9 + C16:1 *cis*-9 + C18:1 *cis*-9 + CLA

148   *cis*-9,*trans*-11)] × 100.

149   ***Statistical Analysis***

150   Milk FA data were analyzed by the PROC MIXED procedure of SAS version 9.2 (SAS Institute

151   Inc., Cary, NC). The model included the fixed effect of diet (D; 4 levels), sampling date (S; 4 levels)

152   and the diet × sampling date interaction (D × S); moreover, to account for individual variability, the

153   random effect of animal was nested within each treatment. The significance of group mean

154   differences was assessed using Tukey Honestly Significant Difference (HSD; $P < 0.05$).

155   A multivariate approach was also adopted to better clarify the effect of the four dietary treatments

156   on the milk FA composition, using a dataset obtained from the average values of four sampling dates

157   per animal. A total of 21 variables were analyzed (17 milk fatty acid groups and 4 nutritional indices)

158   using hierarchical cluster analysis (HCA) and principal component analysis (PCA). HCA was

159   performed on the milk FA profile using the Euclidean distances and the average linkage method. A

160   dendrogram was used to visualize the clustering of the experimental units. Furthermore, the

161   correlation matrix of milk fatty profiles was decomposed by the analysis of principal components

162   (**PC**) as follows:

163    $$PC_j = \alpha_{1j}y_1 + \ldots + \alpha_{ij}y_i + \ldots + \alpha_{(n-1)j}y_{(n-1)} + \ldots + \alpha_{nj}y_n,$$

164   where n represents the number of variables (21), PC_j represents the generic *j*-th linear combination of

165   the observed variables (scores) and α_{ij} the *i*-th coefficients of the eigenvector (loading) of correlation

166   matrix, corresponding to the generic *j*-th eigenvalue (i.e., the variance explained by the *j*-th PC). The

167   process of extraction was stopped when the variance explained by eigenvalues accounted for at least

168 80% of the total variance. Individual PC scores were then used in a one-way ANOVA including the

169 fixed effect of treatments.

170

171 <div align="center">**RESULTS AND DISCUSSION**</div>

172 ***Milk Fatty Acid Profile***

173 The FA composition of milk collected from the ewes of the four experimental treatments is

174 reported in Table 2. The concentration of C4:0 increased in milk of ewes fed grape seed in

175 combination with linseed (MIX) compared with CON ($P < 0.05$). The mean values found for this FA

176 (2.8% of FA) appear to be low when compared with those of other studies, which ranged from 3.1 to

177 4.6% (Gómez-Cortés et al., 2009; Buccioni et al., 2015). This difference could be the consequence of

178 the volatilization of C4:0 during the extraction and methylation processes used in our analyses. The

179 inclusion of linseed, alone or in combination with grape seed, reduced the concentration of FA from

180 C6:0 to C9:0 ($P < 0.05$). A large part of the FA from C10:1 to C17:1 *cis*-9 decreased in the GS, LIN

181 and MIX groups compared with CON ($P < 0.05$), except for *anteiso* C13:0, *iso* C14:0, C16:1 *trans*-

182 6 + *trans*-7 and C16:1 *cis*-7, which did not differ ($P > 0.05$), and C16:1 *trans*-8, which increased in

183 MIX, C16:1 *trans*-9, which increased in LIN and MIX, and C16:1 *cis*-10, which increased in GS,

184 LIN and MIX, compared with CON ($P < 0.05$). These changes resulted in the reduction in SCFA and

185 MCFA concentration in the milk of ewes fed GS, LIN and MIX, in decreasing order, compared with

186 CON ($P < 0.05$). The LCFA concentration was higher in the treated groups than in the CON group

187 ($P < 0.05$), in the following decreasing order: MIX, LIN and GS.

188 The concentration of total SFA decreased and that of UFA, MUFA and PUFA increased in all

189 groups compared with CON ($P < 0.05$), with the lowest SFA and the highest UFA, MUFA and PUFA

190 values being found in the MIX group ($P < 0.05$). The increase in UFA and PUFA, due to the dietary

191 inclusion of grape seed, linseed or both, resulted in a higher UFA to SFA and PUFA to SFA ratios in

192 all treated groups compared with CON ($P < 0.05$). The extent of increase of these ratios, especially

193 those of PUFA:SFA in the milk from the MIX group compared with CON (+ 187.5%) are very

194 interesting, considering that it has been evidenced that replacing dietary SFA with PUFA is likely to

195 reduce the occurrence of coronary heart disease (Mozaffarian et al., 2010).

196   The content of C18:0 increased in GS, LIN and MIX compared with CON ($P < 0.05$). The

197 concentration of most of the C18:1, C18:2 and CLA isomers increased in the milk of sheep fed linseed

198 (LIN and MIX) compared with that of CON ($P < 0.05$), in accordance with other studies on cows

199 (Caroprese et al., 2010; Ferlay et al., 2013), goats (Nudda et al., 2006; 2013a) and ewes (Mughetti et

200 al., 2012) fed linseed. These results were likely due to the fact that linseed is a rich source of C18:3

201 FA (> 55% of FA) and a moderate source of C18:1 and C18:2 (the sum is approximatly 33% of FA).

202 The presence of high concentrations of C18:1 isomers in the LIN and MIX groups can be partly

203 explained by the biohydrogenation of C18:2 and C18:3 FA in the rumen and of the desaturation of

204 SA in the mammary gland (Kennelly, 1996). The concentration of C18:1 *trans*-11 (vaccenic acid,

205 **VA**) increased ($P < 0.05$) in LIN and MIX compared with CON. This is consistent with the high

206 amount of linolenic acid (C18:3 *n*-3, **LNA**) supplied by linseed, considering that this FA is a precursor

207 of VA produced by the ruminal metabolism, and is in accordance with the experiments of Nudda et

208 al. (2006, 2013a) and Mughetti et al. (2012), in which dietary linseed increased the levels of VA in

209 milk of dairy goats and sheep. VA is the precursor of the CLA *cis*-9,*trans*-11 formed in the mammary

210 gland by the Δ^9-desaturase (Griinari and Bauman 1999). In fact, in our study the concentration of

211 CLA *cis*-9,*trans*-11 in the milk of the groups fed linseed (LIN and MIX) was higher ($P < 0.05$) than

212 in those of the CON group. The level of CLA *cis*-9,*trans*-11 concentration in the milk from the LIN

213 group (2.16% of FAME) was comparable to that reported for sheep grazing high-quality pasture

214 (2.20% of FAME, Nudda et al., 2005) or fed a similar dose of linseed (2.33% of FAME, Gómez-

215 Cortés et al., 2009). Interestingly, the concentration of CLA *cis*-9,*trans*-11 (1.73% of FAME) in milk

216 from sheep fed grape seed (GS), which was numerically but not significantly higher than CON, was

217 similar to that reported for sheep fed high amounts of linseed oil (about 40 g/d; Zhang et al., 2006) or

218   fish oil (30 g/d; Mozzon et al., 2002). In the present work, milk from ewes fed grape seed and linseed

219   in combination (MIX) had a high concentration of CLA *cis*-9,*trans*-11 (3.0% of FAME). As reported

220   in the review by Nudda et al. (2014), concentrations of CLA *cis*-9,*trans*-11 higher than 3% of fat have

221   been previously reached by using a very high dose of soybean oil (140 g/d) associated with a high-

222   concentrate diet. Dietary linseed also increased ($P < 0.05$) the concentration of LNA in milk from

223   LIN (1.87% of FAME) and MIX (1.42% of FAME) compared with CON and GS. The extent of

224   enrichment of LNA was consistent with previous studies where linseed was included in the diet of

225   sheep (Mele et al., 2007; Gómez-Cortés et al., 2009)

226   The presence of a moderate concentration of polyphenols in the diet increased the level of

227   beneficial FA, mainly LNA, in milk from ewes (Cabiddu et al., 2009) and cows (Dschaak et al.,

228   2011). This effect has been explained by the capacity of polyphenols to inhibit the activity of some

229   strains of ruminal bacteria involved in the biohydrogenation of UFA (Cabiddu et al., 2009; Vasta et

230   al., 2009a; Minieri et al., 2014). In this work, the inclusion of grape seed, alone or in combination

231   with linseed, increased the concentration of PUFA compared with the CON group ($P < 0.001$).

232   However, this increase was likely due to the high amount of LA in grape seeds (about 75% of FA),

233   considering that GS and MIX also increased LA and, consequently, PUFA *n*-6 in milk compared with

234   CON and LIN ($P < 0.05$). This is in agreement with the findings of Moate et al. (2014) and Santos et

235   al. (2014), who showed increased levels of LA in milk of dairy cows fed grape residue.

236   The concentration of PUFA *n*-3, which was the lowest in CON and GS, was higher in milk from

237   sheep fed linseed alone than in combination with grape seed ($P < 0.001$). This is likely due to the

238   lack of effect of grape seed in reducing the extent of biohydrogenation of LNA, as suggested by the

239   decreased level of LNA in milk of MIX compared with LIN ($P < 0.05$) and the similarly low levels

240   of LNA in CON and GS. The low level of polyphenols in the grape seed used in the present work,

241   compared with those of other studies, could explain the lack of effect of this ingredient in increasing

242   the concentration of LNA in milk of GS compared with CON, but does not explain the reduction in

243 LNA in milk of MIX compared with that of LIN group. Therefore, considering that grape seed

244 contains other compounds that could have affected the biohydrogenation of UFA, we hypothesize

245 that the presence of grape seed might have increased, to some extent, the biohydrogenation of dietary

246 PUFA, as suggested by the higher concentration of VA in MIX than in LIN and in GS than in CON,

247 even though these differences were not statistically significant ($P < 0.10$). Our results are in

248 accordance with the study of Moate et al. (2014), in which the milk concentration of LNA did not

249 increase in lactating cows fed grape marc. The pattern of the concentration of PUFA n-3 mirrored

250 that of LNA, with CON and GS showing lower PUFA n-3 values than LIN, and MIX being

251 intermediate ($P < 0.05$).

252 The inclusion of grape seed and linseed in the diet of sheep, especially when offered in

253 combination (MIX), increased the concentration of milk TFA compared with CON ($P < 0.05$). This

254 result mirrored the increase in most of the individual TFA in those groups compared with CON, likely

255 as a consequence of rumen biohydrogenation of PUFA, whose dietary concentration followed the

256 increasing order CON < GS < LIN < MIX (Table 1). This finding is in agreement with a previous

257 study showing an increased concentration of TFA in milk when extruded linseed was included as

258 source of PUFA in the diet of dairy cows (Livingstone et al., 2015). These results were influenced

259 the most by VA, which accounted for 34.21, 40.42, 43.59 and 46.78% of the total TFA in milk from

260 CON, GS, LIN and MIX, respectively.

261 The total concentration of OBCFA decreased in the milk of the GS, LIN and MIX groups

262 compared with CON, being the lowest in MIX and intermediate in GS and LIN ($P < 0.05$). OBCFA

263 are reported to be mainly derived from the ruminal microflora (Fievez et al., 2012). The decrease in

264 OBCFA in milk of LIN could be explained by the high amount of PUFA, particularly LNA, in linseed,

265 considering that PUFA are reported to be toxic for the growth of ruminal microorganisms (Maia et

266 al., 2007, 2010). Similarly, the high concentration LA in grape seed could explain the reduction in

267 OBCFA in milk from GS compared with CON. Moreover, according with several studies showing

268 the effect of polyphenols on the growth and activity of rumen microbial population (Vasta et al.,

269 2010; Buccioni et al., 2015), the grape seed polyphenols could have contributed to this reduction. The

270 high amount of PUFA, mainly LNA and LA, and the presence of polyphenols in the MIX diet could

271 be the reason for the lowest concentration of OBCFA found in the milk from sheep of this group, as

272 confirmed by the previously reported results of the analysis on rumen liquid FA profile of the ewes

273 of the dietary groups under comparison (Correddu et al., 2015).

274   The inclusion of grape seed and linseed, alone and in combination, decreased ($P < 0.05$) the milk

275 concentration of de novo synthesized FA C10:0, C12:0, and C14:0 compared with the CON group,

276 probably due to the increase in the amount of PUFA in the diet of sheep, in accordance with previews

277 studies in lactating sheep (Zhang et al., 2006), goats (Bernard et al., 2009) and cows (Chilliard et al.,

278 2007). In addition, the concentrations of C10:1, C14:1 *cis*-9, C16:1 *cis*-9 and C17:1 *cis*-9 were also

279 lower in milk of GS, LIN and MIX compared with CON ($P < 0.05$). As suggested by Bernard et al.

280 (2009), an increase in the amount of TFA and PUFA can reduce the activity of stearoyl Co-A

281 desaturase in the mammary gland and, consequently, the extent of Δ^9-desaturation of C10:0, C14:0,

282 C16:0 and C17:0. The analysis of the desaturase indices partly confirmed these results. In particular,

283 CON showed higher values of the C18 and CLA indices ($P < 0.05$) than MIX and the other two

284 groups being intermediate, whereas the C10, C14 and C16 indices were not significantly influenced

285 by the diets ($P > 0.05$). Although the concentration of C18:1 *cis*-9 and CLA *cis*-9,*trans*-11 increased

286 with the inclusion of grape seed and linseed, the DI related to these FA did not follow the same

287 pattern, suggesting that the increase in these FA was not related to an increasing activity of Δ^9-

288 desaturase but, more likely, to the increase in the concentration of their substrates C18:0 and C18:1

289 *trans*-11. The total DI increased in all groups compared with CON ($P < 0.05$), even if the individual

290 DI followed an opposite trend. This is in contrast with the positive correlation between all DI

291 (individual and total) observed by Schennink et al. (2008), and could be explained by differences

292 between these studies in the ratio between C18:1 *cis*-9 and C16:0, which are the most abundant FA

293 in milk. As pointed out by Schennink et al. (2008), the value of total DI mirrors mainly the ratio

294 C18:1 *cis*-9 to C16:0. The opposite trend between individual DI and total DI found in the present

295 work suggests that the total DI is not a reliable indicator of the desaturation activity of stearoyl Co-A

296 desaturase.

297 As shown in Figure 1, the dietary inclusion of grape seed and linseed was effective in reducing the

298 atherogenic and thrombogenic indices, and increasing the h:H ratio compared with CON ($P < 0.05$).

299 Our results are consistent with the fact that dietary sources of PUFA ameliorate cardiac risk factors

300 (Duda et al., 2009, Katare and Saxena, 2013), and with a previous study in which dietary extruded

301 linseed decreased the values of AI and TI and increased the h:H ratio in dairy goats (Nudda et al.,

302 2013a). Similar results were found in Lacaune ewes fed extruded linseed (Casamassima et al., 2014).

303 The effect of the dietary inclusion of grape seed on these indices was likely related to the large

304 decrease in C12:0, C14:0 and C16:0 and increase in MUFA. The values of TI were lower in LIN than

305 in GS ($P < 0.05$), suggesting that the inclusion of linseed is more effective in increasing the

306 concentration of beneficial FA than grape seed. Grape seed and linseed in combination (MIX) led to

307 lower values of the AI and TI indices, and a higher value of h:H than grape seed alone ($P < 0.05$).

308 Moreover, the reduction in AI and TI and the increase in h/H were numerically higher, although not

309 statistically different, in MIX than in LIN, suggesting a summative effect of linseed and grape seed.

310 The substantial improvement in milk FA due to the combined effect of grape seed and linseed is

311 evidenced by the 65.33 and 62.61% decrease in AI and TI, respectively, and by the 125% increase in

312 h:H in MIX compared with CON.

313 Most of the FA measured during the trial were influenced by sampling date, with FA of the same

314 class generally showing a similar pattern (data not shown). In particular, most of the SFA, SCFA and

315 MCFA showed a significant decrease ($P < 0.05$) in the second and third samplings compared with

316 the first and last samplings, whereas most of the UFA, MUFA, PUFA and LCFA showed an opposite

317 trend, with the second and third samplings showing greater values than the first and last samplings

318 (*P* < 0.05). Although many of the FA were significantly influenced by the D × S interaction (*P* <

319 0.05), the few differences observed in the temporal pattern among dietary treatments (data not shown)

320 was not relevant compared with the main effect of the diet on FA concentration.

321 ***Multivariate Analysis***

322   The results of the PCA are shown in Table 3 and Figure 2. Two principal components were retained

323 for subsequent analysis based on the proportion of variance explained by each PC. The first and

324 second principal components accounted for about 90% of the total variability (78% and 12% for PC1

325 and PC2, respectively). Table 3 shows the eigenvalues and eigenvectors of the correlation matrix

326 derived from groups of FA in milk. The PC1 was positively associated with the groups of FA

327 characterized by long and unsaturated chains, whereas it was negatively associated with groups

328 characterized by short- and medium-chain FA and saturated FA. The PC1 was also positively

329 correlated with the sum of C18:1 and C18:2 isomers and, among C18:1, the *trans* isomers showed a

330 greater correlation than the *cis* isomers. According to previous studies on dairy cows, the dietary

331 supplementation with vegetable oils as source of PUFA increased the concentration of long-chain

332 PUFA *n*-3 (Ferlay et al., 2013) or PUFA *n*-6 (Almeida et al., 2013), and decreased the concentration

333 of short- and medium-chain FA in milk. Among PUFA, PC2 loadings were positively correlated with

334 *n*-6 and *n*-6 to *n*-3 ratio, and negatively with *n*-3. Moreover, PC2 negatively discriminated the

335 OBCFA and BCFA. PC1 showed high positive loadings for the h:H ratio and high negative loadings

336 for the AI and TI indices. PC2, to a lesser extent, was positively correlated with the AI and TI and

337 negatively with the h:H ratio.

338   The plot of the first two PC scores allowed the description of the relationship among animals based

339 exclusively on the milk FA profile (Figure 2). Four clusters were identified according to the four

340 dietary treatments, with the CON being the most isolated group and being mainly discriminated by

341 PC1 (negative scores). PC2 scores discriminated GS (positive scores) from LIN (negative scores).

342 We suppose that PC1 was positively associated with the dietary inclusion of PUFA, especially with

343   the PUFA intake (CON < GS < LIN < MIX, as previously reported in Correddu et al., 2015);

344   therefore, PC1 was named "PUFA intake". The PC2 could be related to the different sources of PUFA

345   (grape seed or linseed) and consequently, to the PUFA *n-6* to *n-3* ratio in the diets; thus, PC2 was

346   identified as the "*n-6* to *n-3* ratio". Similar results were reported in the work of Bernard et al. (2009),

347   who investigated the effects of sunflower and linseed oils, characterized by high LA and LNA

348   content, respectively, on goat milk fatty acid composition. In that study, the analysis of principal

349   component, used to clarify the relationship between the oil treatments, forages and milk production

350   and composition, showed that PC1 was related with the lipid supplementation, and PC2 was related

351   to the content of LA and LNA in the diets.

352   The results of the HCA performed in the present study are shown in Figure 3. The dendrogram

353   allowed to group the animals in four clusters, with 72.80% of similarity level. The animals of CON

354   formed a unique cluster, indicating that the chemical composition of milk from this group was

355   different from the milk composition of the other groups. Another unique cluster grouped the animals

356   of GS, indicating that the chemical composition of milk from sheep fed grape seed was different from

357   that of the CON and from those of the sheep fed linseed (LIN and MIX). The animals of the LIN and

358   MIX groups formed two clusters, except for a few cases of incorrect assignation: two animals of the

359   MIX treatment were assigned to the LIN group, and one animal of the LIN treatment was assigned to

360   the MIX group. This suggests that the chemical composition of the milk from sheep fed linseed (LIN

361   and MIX) was different from those of the CON and GS groups. The clustering of animals in the four

362   dietary treatments evidenced by the plot of principal components (Figure 2) and by the dendrogram

363   (Figure 3) was confirmed by the results of the statistical analysis of the relationship between dietary

364   groups and PC1 and PC2 reported in Table 4.

365

366   **CONCLUSIONS**

367   The dietary inclusion of grape seed or linseed, or both, improved the milk FA composition in Sarda

368 dairy ewes and the multivariate approach allowed the detection of differences between dietary

369 treatments based on the milk fatty acid profile. When grape seed was supplied alone, at 300 g/d per

370 head, the milk content of SFA decreased and that of UFA and PUFA increased, mainly due to a high

371 increase of LA, whereas the concentration of RA and VA tended to increase compared to the control

372 group. The inclusion of 200 g/d per head of linseed alone in the diet of lactating ewes increased the

373 concentration of potentially beneficial FA, such as oleic acid, linolenic acid, and CLA *cis*-9,*trans*-11.

374 The inclusion of grape seed and linseed in combination resulted in a major increase of ratios

375 UFA:SFA and PUFA:SFA and of the concentration of CLA *cis*-9,*trans*-11. In conclusion, the use of

376 grape seed in sheep nutrition could be an alternative for the disposal of this by-product, and its

377 combination with linseed could be a successful strategy to enhance PUFA in lactating Sarda ewes.

378

385

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514

515   **Table 1.** Ingredients, chemical composition, dry matter intake and fatty acid profile of diets

	Diet[1]			
	CON	GS	LIN	MIX
Ingredients (kg/day per head, as fed)				
Mixed meal				
Corn	0.15	0.17	–	–
Soybean	0.12	0.24	0.04	0.16
Peas	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	0.50	0.50	0.50	0.50
Dehydrated alfalfa hay	0.80	0.80	0.80	0.80
Mixed hay	0.20	0.20	0.20	0.20
Chemical composition, % of DM (unless otherwise noted)				
DM (%)	90.8	91.6	91.2	92.0
NDF	41.8	42.8	43.7	44.5
NFC	33.4	28.9	28.5	24.2
ADL	4.6	8.9	5.0	9.4
CP	18.0	17.9	17.9	17.9
Ash	7.8	7.4	8.1	7.6
EE	2.0	3.2	5.1	5.8
FA	1.8	2.3	3.7	4.5
ME supplied (Mcal/d)	4.95	4.94	4.97	4.97
Dry Matter intake (kg/d)	2.20	2.47	2.11	2.39
Major fatty acids (g/100 g of total FA)				
C16:0	18.98	14.88	11.99	11.50
C18:0	3.33	4.47	4.39	4.68
C18:1 *cis*-9	22.79	23.52	21.78	21.91
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
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

516   [1]Diet: CON = control diet; GS = diet containing 300 g/d per head of grape seed; LIN = diet containing
517   220 g/d per head of linseed; MIX = diet containing 300 g/d of grape seed and 220 g/d of linseed per
518   head.
519

520 **Table 2.** Fatty acid profile of milk from sheep fed different experimental diets

Fatty acid (g/100 g of FAME)[3]	Diet[1]				SEM	P-value[2]		
	CON	GS	LIN	MIX		D	S	D × S
C4:0	2.58[b]	2.86[ab]	2.83[ab]	2.96[a]	0.034	*	***	*
C6:0	2.10[a]	1.97[ab]	1.65[bc]	1.37[c]	0.043	***	**	***
C8:0	2.20[a]	1.86[ab]	1.43[bc]	1.03[c]	0.058	***	***	**
C9:0	0.05[a]	0.04[b]	0.02[c]	0.02[c]	0.002	***	***	***
C10:0	8.98[a]	6.33[b]	4.63[bc]	3.25[c]	0.251	***	***	*
C10:1	0.35[a]	0.25[b]	0.18[bc]	0.11[c]	0.010	***	***	**
C11:0	0.10[a]	0.05[b]	0.03[bc]	0.02[c]	0.003	***	***	**
C12:0	6.18[a]	3.85[b]	2.96[bc]	2.19[c]	0.166	***	***	ns
iso C13:0	0.06[a]	0.03[b]	0.02[b]	0.02[b]	0.002	***	***	**
anteiso C13:0	0.01	0.01	0.01	0.01	0.001	ns	**	ns
C13:0	0.10[a]	0.07[b]	0.06[bc]	0.04[c]	0.003	***	***	*
iso C14:0	0.10	0.11	0.10	0.09	0.003	ns	ns	*
C14:0	13.35[a]	10.80[b]	9.69[bc]	8.46[c]	0.218	***	***	ns
C14:1 cis-9	0.33[a]	0.22[b]	0.20[b]	0.16[b]	0.009	**	***	**
iso C15:0	0.20[ab]	0.19[ab]	0.21[a]	0.16[b]	0.004	*	ns	ns
anteiso C15:0	0.49[a]	0.44[ab]	0.45[ab]	0.38[b]	0.008	*	ns	**
C15:0	1.26[a]	1.03[b]	1.04[b]	0.89[b]	0.018	***	**	**
isoC16:0	0.29[a]	0.24[ab]	0.25[ab]	0.21[b]	0.005	**	ns	*
C16:0	29.97[a]	23.83[b]	22.45[bc]	20.96[c]	0.393	***	***	ns
C16:1 trans-6 + trans-7	0.06	0.06	0.07	0.07	0.001	ns	ns	**
C16:1 trans-8	0.02[b]	0.04[ab]	0.03[ab]	0.07[a]	0.004	*	ns	**
C16:1 trans-9	0.08[c]	0.25[bc]	0.33[ab]	0.56[a]	0.024	***	**	ns
C16:1 trans-10	0.01[c]	0.01[b]	0.01[b]	0.02[a]	0.001	***	*	ns
C16:1 cis-7	0.28	0.27	0.31	0.29	0.005	ns	***	*
C16:1 cis-9	1.18[a]	0.77[b]	0.73[b]	0.63[b]	0.031	*	***	***
C16:1 cis-10	0.01[c]	0.03[b]	0.02[bc]	0.05[a]	0.002	***	***	**
iso C17:0	0.36[a]	0.32[b]	0.36[a]	0.28[c]	0.005	***	ns	ns
anteiso C17:0	0.50[a]	0.39[bc]	0.43[ab]	0.32[c]	0.008	***	*	*
C17:0	0.65[a]	0.52[bc]	0.60[b]	0.48[c]	0.009	***	***	**
C17:1 cis-9	0.25[a]	0.15[bc]	0.17[b]	0.11[c]	0.006	***	***	ns
C18:0 (SA)	5.43[b]	8.66[a]	9.82[a]	9.95[a]	0.244	***	***	*
C18:1 trans-4	0.03[b]	0.04[ab]	0.05[a]	0.04[ab]	0.002	*	***	ns
C18:1 trans-6 + trans-8	0.20[c]	0.46[b]	0.59[ab]	0.73[a]	0.024	***	***	ns
C18:1 trans-9	0.22[c]	0.48[b]	0.55[b]	0.70[a]	0.021	***	**	ns
C18:1 trans-10	0.52[b]	0.99[ab]	0.85[ab]	1.80[a]	0.092	*	ns	ns
C18:1 trans-11 (VA)	1.03[c]	2.99[bc]	4.06[ab]	6.20[a]	0.253	***	*	ns

C18:1 *cis*-9 + t13 + t14	13.29[b]	17.76[a]	19.51[a]	19.19[a]	0.339	***	***	ns
C18:1 *cis*-10 + t15	0.39[b]	0.45[ab]	0.67[a]	0.69[a]	0.052	**	***	***
C18:1 *cis*-11	0.42[b]	0.59[b]	0.81[a]	0.79[a]	0.021	***	***	***
C18:1 *cis*-12	0.28[d]	0.85[b]	0.61[c]	1.26[a]	0.041	***	***	ns
C18:1 *cis*-13	0.02[b]	0.04[b]	0.07[a]	0.09[a]	0.003	***	ns	ns
C18:1 *cis*-14 + t16	0.16[c]	0.21[c]	0.42[a]	0.34[b]	0.013	***	***	ns
C18:2 *trans*-9,*trans*-12	0.42[c]	0.77[b]	1.24[a]	1.24[a]	0.039	***	ns	ns
C18:1 *cis*-15	0.06[c]	0.08[c]	0.24[a]	0.19[b]	0.009	***	ns	ns
C18:2 *trans*-8,*cis*13	0.02[b]	0.03[b]	0.07[a]	0.07[a]	0.003	***	*	ns
C18:2 *cis*-9,*trans*-12	0.08[c]	0.17[b]	0.27[a]	0.25[a]	0.008	***	ns	ns
C18:2 *trans*-9,*cis*-12	0.15[c]	0.22[ab]	0.19[b]	0.24[a]	0.004	***	*	ns
C18:2 *n*-6 (LA)	2.66[b]	4.62[a]	2.95[b]	4.82[a]	0.119	***	***	ns
C18:3 *n*-6	0.10[a]	0.06[b]	0.02[c]	0.03[c]	0.003	***	*	ns
C18:3 *n*-3 (LNA)	0.74[c]	0.57[c]	1.87[a]	1.42[b]	0.057	***	ns	***
CLA *cis*-9,*trans*-11 (RA)	0.69[c]	1.73[bc]	2.16[ab]	2.99[a]	0.116	***	*	ns
C18:4 *n*-3	0.06[a]	0.04[b]	0.05[ab]	0.06[ab]	0.002	*	***	ns
CLA *trans*-9,*cis*-11+C20:0	0.18[b]	0.19[ab]	0.22[a]	0.21[ab]	0.004	*	***	**
CLA *trans*-10,*cis*-12	0.01[b]	0.02[b]	0.11[a]	0.09[a]	0.006	***	***	***
CLA *trans*-11,*cis*-13	0.01[c]	0.02[c]	0.16[a]	0.13[b]	0.007	***	**	***
CLA *cis*-11,*cis*-13	0.04[b]	0.04[b]	0.10[a]	0.09[a]	0.003	***	***	***
CLA *trans*-11,*trans*-13	0.08[c]	0.10[bc]	0.12[ab]	0.14[a]	0.003	***	***	ns
CLA t9,t11 + C20:1 n-9	0.01	0.01	0.01	0.01	0.001	ns	***	*
C20:2 *n*-6	0.02	0.02	0.02	0.02	0.001	ns	**	ns
C20:3 *n*-9	0.06[a]	0.04[b]	0.06[a]	0.04[b]	0.001	***	***	ns
C20:3 *n*-6	0.03[ab]	0.03[a]	0.02[c]	0.02[bc]	0.001	***	***	**
C20:4 *n*-6	0.15[a]	0.15[a]	0.07[b]	0.07[b]	0.004	***	***	***
C20:3 *n*-3	0.01[bc]	0.01[c]	0.02[a]	0.01[b]	0.001	***	***	ns
C22:0	0.09[ab]	0.07[c]	0.11[a]	0.08[bc]	0.003	***	***	**
C20:4 *n*-3	0.02[ab]	0.01[b]	0.02[a]	0.02[ab]	0.001	**	***	**
C22:1 *n*-11	0.00[b]	0.00[b]	0.01[a]	0.01[ab]	0.001	*	ns	ns
C20:5 *n*-3 (EPA)	0.07[a]	0.03[c]	0.07[a]	0.05[b]	0.002	***	***	*
C22:2 *n*-6	0.04[a]	0.03[b]	0.05[a]	0.03[b]	0.001	***	**	**
C22:4 *n*-6	0.01[ab]	0.01[a]	0.00[b]	0.00[b]	0.001	**	***	***
C24:0	0.02[a]	0.01[b]	0.02[a]	0.02[ab]	0.001	**	ns	***
C22:5 *n*-3 (DPA)	0.07[a]	0.04[b]	0.08[a]	0.05[b]	0.002	***	***	***
C22:6 *n*-3 (DHA)	0.02	0.01	0.02	0.02	0.001	ns	ns	ns
Groups of FA								
SFA	75.07[a]	63.66[b]	59.17[b]	53.17[c]	0.921	***	***	ns
UFA	24.93[c]	36.34[b]	40.83[b]	46.83[a]	0.921	***	***	ns
MUFA	19.20[c]	27.36[b]	30.85[b]	34.71[a]	0.652	***	***	ns

	CON	GS	LIN	MIX	SEM	D	S	D × S
PUFA	5.73[c]	8.98[b]	9.98[ab]	12.12[a]	0.283	***	***	ns
UFA:SFA	0.33[c]	0.58[b]	0.70[b]	0.90[a]	0.025	***	***	*
PUFA:SFA	0.08[c]	0.14[b]	0.17[b]	0.23[a]	0.007	***	***	ns
TFA	2.99[c]	7.07[b]	9.07[ab]	12.93[a]	0.462	***	***	ns
BCFA	2.01[a]	1.73[bc]	1.84[ab]	1.46[c]	0.029	***	ns	*
OBCFA	3.88[a]	3.19[b]	3.35[b]	2.70[c]	0.054	***	ns	**
SCFA	16.26[a]	13.30[b]	10.73[bc]	8.73[c]	0.354	***	***	*
MCFA	55.84[a]	43.68[b]	40.56[b]	36.47[c]	0.795	***	***	ns
LCFA	27.90[d]	43.01[c]	48.71[b]	54.80[a]	1.110	***	***	*
PUFA *n*-3	0.98[c]	0.72[c]	2.14[a]	1.63[b]	0.060	***	*	***
PUFA *n*-6	3.00[b]	4.92[a]	3.13[b]	4.99[a]	0.118	***	***	ns
n-6:*n*-3	3.12[b]	7.01[a]	1.47[c]	3.09[b]	0.227	***	***	***
Total CLA	1.02[c]	2.12[bc]	2.88[ab]	3.66[a]	0.132	***	***	ns
Total C18:1	16.62[c]	25.28[b]	28.76[b]	32.62[a]	0.667	***	***	ns
Total C18:2	4.35[c]	7.92[b]	7.60[b]	10.28[a]	0.260	***	***	ns
Δ^9-desaturase indices								
C10 index	3.75	3.73	3.78	3.26	0.060	ns	**	ns
C14 index	2.38	2.03	200	1.82	0.050	ns	*	**
C16 index	3.73	3.13	3.14	2.91	0.071	ns	*	***
C18 index	71.25[a]	67.27[ab]	66.65[ab]	65.93[b]	0.382	*	*	*
CLA *cis*-9,*trans*-11 index	40.34[a]	37.51[ab]	34.79[b]	33.20[b]	0.444	**	ns	ns
Total index	21.23[b]	28.26[a]	31.01[a]	32.08[a]	0.517	***	***	ns

521 [a-d]Means within a row with different superscripts are different (*P* < 0.05).

522 [1]Diet: CON = control diet; GS = diet containing 300 g/d per head of grape seed; LIN = diet containing

523 220 g/d per head of linseed; MIX = diet containing 300 g/d of grape seed and 220 g/d of linseed per

524 head.

525 [2]P-value: D = effect of diet; S = effect of sampling date; D × S = effect of diet and sampling date

526 interaction; ns indicates *P* > 0.05; **P* < 0.05; ** *P* < 0.01; ****P* < 0.001.

527 [3]FAME = fatty acid methyl esters; SA = stearic acid; VA = vaccenic acid; LA = linoleic acid; LNA

528 = linolenic acid; RA = rumenic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid;

529 DHA = docosahexaenoic acid; SFA = saturated fatty acids, sum of the individual saturated fatty acids

530 reported in this table; UFA = unsaturated fatty acids, sum of the individual unsaturated fatty acids

531 reported in this table; MUFA = monounsaturated fatty acids, sum of the individual monounsaturated

532 fatty acids reported in this table; PUFA = polyunsaturated fatty acids, sum of the individual

533 polyunsaturated fatty acids reported in this table; TFA = *trans* fatty acids, sum of the individuals *trans*

534 fatty acids reported in this table (except CLA isomers); BCFA = branched-chain fatty acids, sum of

535 iso- and anteiso-FA reported in this table; OBCFA = odd- and branched-chain fatty acids, sum of

536 odd-, iso- and anteiso-FA reported in this table; SCFA = short-chain fatty acids, sum of the individual

537 fatty acids from C4:0 to C10:0 reported in this table; MCFA = medium-chain fatty acids, sum of the

538 individual fatty acids from C11:0 to C17:0 reported in this table; LCFA = long-chain fatty acids, sum

539 of the individual fatty acids from C18:0 to DHA reported in this table; PUFA *n*-3 = sum of individual

540 *n*-3 fatty acids reported in this table; PUFA *n*-6 = sum of individual *n*-6 fatty acids reported in this

541 table; CLA = sum of individual conjugated of linoleic acids reported in this table.

542

Table 3. Eigenvectors and eigenvalues of correlation matrix based on groups of milk fatty acids, sorted by decreasing values of the PC1

Item[1]	PC1	PC2
UFA	0.246	−0.027
MUFA	0.244	−0.058
Total C18:1	0.243	−0.058
LCFA	0.243	−0.055
PUFA	0.241	0.044
Total C18:2	0.235	0.154
TFA	0.233	0.055
Total CLA	0.231	−0.004
h:H	0.230	−0.099
Total C18:1-*trans*	0.227	0.085
Total C18:1-*cis*	0.195	−0.188
PUFA *n*-6	0.158	0.455
PUFA *n*-3	0.139	−0.470
n-6:*n*-3	−0.021	0.559
BCFA	−0.174	−0.288
OBCFA	−0.202	−0.249
SCFA	−0.231	0.108
TI	−0.236	0.093
MCFA	−0.238	0.030
AI	−0.238	0.021
SFA	−0.246	0.027
Eigenvalues	16.45	2.51
% variance explained	78.3	11.9

[1]Item: UFA = unsaturated fatty acids, sum of the individual unsaturated fatty acids reported in table 2; MUFA = monounsaturated fatty acids, sum of the individual monounsaturated fatty acids reported in Table 2; LCFA = long-chain fatty acids, sum of the individual fatty acids from C18:0 to DHA reported in table 2; PUFA = polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids reported in Table 2; TFA = *trans* fatty acids, sum of the individuals *trans* fatty acids reported in table 2 (except CLA isomers); Total CLA = sum of individual conjugated of linoleic acids reported in table 2. h:H = hypocholesterolemic to hypercholesterolemic ratio. PUFA *n*-6 = sum of individual *n*-6 fatty acids reported in table 2; PUFA *n*-3 = sum of individual *n*-3 fatty acids reported in table 2; BCFA = branched-chain fatty acids reported in table 2; OBCFA = odd- and branched-chain fatty acids reported in Table 2; SCFA = short-chain fatty acids, sum of the individual fatty acids from C4:0 to C10:0 reported in table 2; TI = trombogenic index; MCFA = medium-chain fatty acids, sum of the individual fatty acids from C11:0 to C17:0 reported in table 2; AI = Atherogenic index; SFA = saturated fatty acids, sum of the individual saturated fatty acids reported in table 2.

559 **Tables 4.** Dietary effects on PC scores of individuals belonging to the different dietary treatments for
560 PC1 (*PUFA intake*) and PC2 (*n-6:n-3*)

| Item | Diets[1] | | | | SEM | *P*-value |
	CON	GS	LIN	MIX		Diet
PC1	−5.7720[d]	−0.2671[c]	1.5283[b]	4.5108[a]	0.5905	< 0.0001
PC2	−0.1499[b]	1.8599[a]	−2.0293[c]	0.3193[b]	0.3105	< 0.0001

561 [a-d]Means within a row with different superscripts are different ($P < 0.05$).
562 [1]Diet: CON = control diet; GS = diet containing 300 g/d per head of grape seed; LIN = diet containing
563 220 g/d per head of linseed; MIX = diet containing 300 g/d of grape seed and 220 g/d of linseed per
564 head.
565

566   **Figure 1.** Effect of experimental diets on milk fat nutritional indices: atherogenic index (AI),

567   thrombogenic index (TI) and hypocholesterolemic to hypercholesterolemic ratio (h:H). CON: control

568   diet, GS: diet containing grape seed, LIN: diet containing linseed, MIX: diet containing both grape

569   seed and linseed.

570   **Figure 2.** Plot of the scores of the first two principal components of individuals belonging to the

571   different experimental diets. CON: control diet, GS: diet containing grape seed, LIN: diet containing

572   linseed, MIX: diet containing both grape seed and linseed.
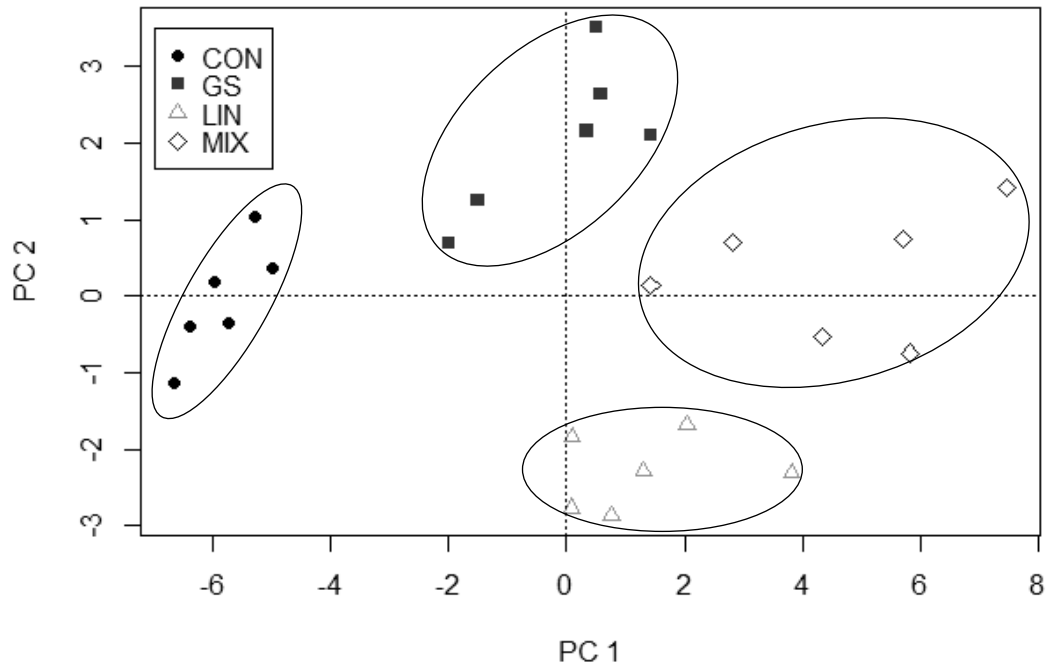
573   **Figure 3.** Hierarchical cluster analysis results for milk of the four dietary treatments. CON: control

574   diet, GS: diet containing grape seed, LIN: diet containing linseed, MIX: diet containing both grape

575   seed and linseed. (Data from groups of FA + nutritional indices).

576

577   Correddu, **Figure 1.**

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580
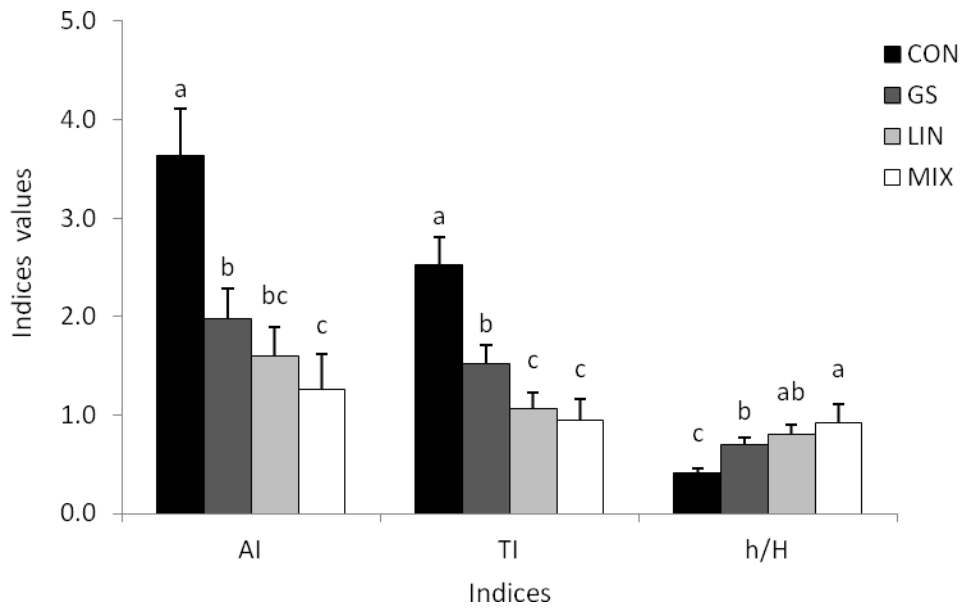
581   Correddu, **Figure 2.**

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585　Correddu, **Figure 3.**

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