Milk production, composition, and milk fatty acid profile from grazing sheep fed diets supplemented with chestnut tannin extract and extruded linseed.

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Abstract

Tannins are bioactive compounds able to interfere with protein and lipid metabolism in the rumen, by forming undegradable complexes with dietary proteins and by modulating several bacterial activities, including the biohydrogenation of polyunsaturated fatty acids. The aim of this trial was to study the effect of dietary supplementation with chestnut hydrolysable tannin extract on ewes milk yield and quality. Ninety-six multiparous Sarda ewes in their mid-lactation phase were allotted to two homogeneous groups (control group, C group; group fed concentrate supplemented with chestnut tannin extract, CHE group), each of 48 animals, for a feeding trial. Animals of both groups grazed 8 hours per day on the same pasture based on a mixture of *Lolium multiflorum, Avena sativa* and *Trifolium repens* (1:1:1). The two diets differed only in their concentrate supplement. The control group received 450 g/head per day of a concentrate feed without chestnut tannin extract, whereas the CHE group received 500 g/head per day of a concentrate feed formulated with the same ingredients of the control concentrate plus 80.0 g/kg DM of chestnut tannin extract. The amounts of concentrate

offered to the animals of both groups were calculated in order to obtain isoproteic and isoenergetic dietary treatments considering the expected DM intake of animals. The inclusion of chestnut tannin in the concentrate resulted in a greater production of milk (+18.64%; P<0.001). Moreover, no differences in casein fraction profile between milks from both groups were found while the casein index was greater (P=0.034) in milk from ewes fed CHE than milk from ewes fed C. As regard fatty acid composition, milk from CHE group had a greater concentration of omega-3 fatty acids if compared to milk from C group (alpha-linolenic acid: 2.18 *vs* 2.57 g/100g of total lipids in C and CHE group, respectively), whereas the percentage of CLA and of C18:1 *trans*11 in milk fat from CHE group was smaller (CLA: 2.20 *vs* 1.85 g/100g of total lipids, in C and CHE group, respectively with P=0.001; C18:1 *trans*11: 3.89 *vs* 3.57 g/ 100g of total lipids in C and CHE group, respectively with P=0.001). The use of practical doses of CHT in the diet of grazing ewes may improve the response to dietary linseed supplementation, resulting in milk with a greater concentration of alpha-linolenic acid.

Keywords: dairy ewe, chestnut tannins, milk yield, fatty acids.

Acronyms: ADF, acid detergent fibre; **ADL**, acid detergent lignin; **NDF**, neutral detergent fibre assayed with heat stable amylase and expressed inclusive of residual ash; **BH**, biohydrogenation; **C**, control concentrate; **CHE**, experimental concentrate; **CHT**, chestnut tannin; **C group**, control group; **CHE group**, experimental group; **CI**, casein index; **CLA**, conjugated linoleic acid; **DM**, dry matter; **DMI**, dry matter intake**; FA,** fatty acid; **FCM**, fat corrected milk; **α-LNA**, alpha-linolenic acid; **LA**, linoleic acid; **PUFA**, polyunsaturated fatty acids.

1. Introduction.

In the Mediterranean area, during the early spring, the diet of lactating ewes is almost exclusively based on pasture, which is particularly rich in highly fermentable carbohydrates and proteins. In some

53 cases, this feeding regimen is associated with an increase of urinary nitrogen losses and of elevate
54 concentrations of urea in milk and blood, which, in turn, may be associated with decreased health
55 status of sheep (Morgante, 2004). On the other hand, grazing is also usually associated with great
56 contents of conjugated linoleic acid (CLA) and alpha-linolenic acid (α-LNA) in milk fat, especially
57 in the early vegetative phase of pasture (Mele et al., 2009; Nudda et al., 2005). However, the
58 concentrations of CLA and α-LNA in milk fat may vary according to the pasture plant composition
59 and to the length of the grazing activity (Cabiddu et al., 2005). In order to obtain milk with a stable
60 fatty acid profile suitable for the production of cheese with proven positive effect on human health
61 (Pintus et al., 2013), dietary lipid supplementation is also considered an effective feeding strategy. In
62 particular, the inclusion of extruded linseed in the diet of dairy ewes resulted in an increase of CLA
63 and α-LNA concentration in milk fat similar or greater than that reported for grazing ewes (Gomez-
64 Cortes et al., 2009; Mele et al., 2011). The use of extruded linseed during the grazing period should
65 promote an additive effect between the α-LNA contained in the fresh herbage and that contained in
66 linseed. It could result in greater concentrations of CLA and α-LNA in milk fat than those reported
67 for grazing ewes or for non-gazing ewes fed diet supplemented with extruded linseed. However,
68 previous studies reported that extruded linseed supplementation in the diet of grazing ewes or cows
69 did not result in an increase of α-LNA content in milk fat, suggesting that the α-LNA contained in
70 linseed was almost completely biohydrogenated in the rumen (Addis et al., 2009; Lerch et al., 2012).
71 Tannins are bioactive phenolic compounds widely distributed in plant kingdom, which interfere with
72 protein and lipid metabolism in the rumen, by forming undegradable complexes with feed proteins
73 and by modulating the biohydrogenation (BH) of polyunsaturated fatty acids (PUFA; Buccioni et al.,
74 2012; Minieri et al., 2014). Tannins may differ in their solubility and other chemical and physical
75 characteristics, and also differ in their capacity to bind feed proteins or to influence the activity of
76 rumen microorganisms (Carreño et al., 2015; Frutos et al., 2004a, 2004b). In ruminants, tannins can
77 have beneficial effects if they are present in the diet at moderate concentrations (Patra and Saxena,
78 2011). In fact, when ewes and cows were fed on diets containing less than 4% of tannins on dry matter

basis, they had higher retention of nitrogen and lower plasma urea concentrations, as a consequence of the ability of tannin to preserve feed protein from rumen microbial degradation (Frutos et al., 2004a, 2004b; Min et al., 2003). A recent study on dairy cows reported that hydrolysable tannins extracted from chestnut wood positively affected animal health, by inhibiting lipid peroxidation and by increasing antioxidant enzymes activities in plasma and liver, without worsening their milk production (Liu et al., 2013). Moreover, the efficacy and selectivity of chestnut tannins (CHT) in limiting BH extent of dietary unsaturated fatty acids, without detrimental effects on rumen microbiome, have been proven by *in vitro* trials (Buccioni et al., 2011). However, *in vivo* effects of hydrolysable and condensed tannins on rumen BH of both linoleic acid (LA) and α-LNA are still controversial (Buccioni et al., 2015; Minieri et al., 2014; Toral et al., 2011, Toral et al., 2013).

The aim of the present study was to evaluate the effects on milk yield and composition of moderate concentrations of CHT in the diet of grazing dairy ewes fed a concentrate containing extruded linseed. In particular, this study aimed to evaluate the effect of dietary CHT supplementation on concentrations of the urea, casein, and α-LNA and CLA in milk.

2. Material and methods

2.1 Animals

Ninety-six multiparous Sarda ewes, average body weight 44.2 ± 3.4 kg, at mid lactation (fourth month) were allotted to two groups each of 48 animals (control group, C group; experimental group, CHE group), which were balanced for age and parity. The handling of the animals was according to Institutional Animal Care and Use Committee (IACUC, 2004) of University of Florence. The ewes were milked daily at 07:00 and 18:00 h using a milking machine (43kPa; 150 pulsation/min) and the daily milk yield was recorded.

2.2 Diets

Diets were formulated to meet requirements of a dairy ewe producing 1kg of milk at 6.5% of fat according to Cannas et al. (2004). Animals of both groups grazed 8 hours per day on the same pasture

105 composed a mixture (1:1:1) of ryegrass (*Lolium multiflorum*), oat (*Avena sativa*) and white clover
106 (*Trifolium repens*). The chemical and nutritional profile of pasture was: Dry matter (DM) 171.8 g/kg
107 of fresh matter, crude protein (CP) 233.0 g/kg of DM, ether extract (EE) 37.1 g/kg of DM, neutral
108 detergent fibre (assayed with heat stable amylase and expressed inclusive of residual ash, NDF) 371.2
109 g/kg of DM, acid detergent fibre (ADF) 255.3 g/kg of DM, acid detergent lignin (ADL) 38.1g/kg of
110 DM, soluble protein (PS) 10 g/ kg of DM, non protein nitrogen (NPN) 51.0 g/ kg of DM, Ash
111 140.3g/kg of DM, non fibre carbohydrates (NFC) 218.4 g/kg of DM. The feeding regimen of the two
112 groups differed only in the type of concentrate offered (data are expressed on DM basis): C group
113 received 450 g DM/head per day of the control concentrate (C) containing 90 g of extruded linseed,
114 whereas the other group (CHE group) received 500 g DM/head per day of the experimental
115 concentrate (CHE) containing 90 g of extruded linseed and 40 g of a commercial CHT extract
116 (Gruppo Mauro Saviola s.r.l., Radicofani, Siena, Italy). Chestnut tannin extract was previously
117 characterized by Romani et al. (2013) and contained 750 g of equivalent tannic acid/ kg DM,
118 determined according to Burns (1963). Extruded linseed contained 350 g/kg DM of oil and 330 g/kg
119 DM of crude protein. The ingredients and chemical composition of the concentrates are reported in
120 Table 1. The concentrates were pelleted and molasses was added to both the concentrates to avoid
121 the choice of dietary components by animals and to improve the palatability of the feeds. The amount
122 of concentrates offered was established on the basis of the expected intake of pasture estimated by
123 Cornell Net Carbohydrates and Protein System for sheep (Cannas et al., 2004) and taking into
124 consideration the smaller concentration of crude protein and net energy of CHE compared to C, due
125 to the inclusion of CHT.

126 **2.3 Experimental design**

127 Concentrates were offered during the morning (7:00) and the afternoon (18:00) milking and the daily
128 intake of DM (DMI) was individually registered on the basis of residuals. The trial lasted 7 weeks,
129 after a 3 weeks adaptation period. All the animals were weighed at the beginning and at the end of

130 the experiment. Once a week, milk samples from each individual ewe were collected during both
131 milkings and then combined in a single sample according to the morning and afternoon yield.

132 **2.4 Proximate analysis of diets**

133 Samples of pasture and concentrates were analysed for proximate composition according to AOAC
134 (1995) procedures (DM, 930.15; CP, 976.06; EE, 920.39; ash, 942.05) while the fibre fractions (NDF,
135 ADF, ADL) were analysed after Goering and van Soest (1970). NPN and PS were determined
136 according to Licitra et al. (1996). NFC content was calculated according to NRC (2001). Net Energy
137 lactation (NEl) was estimated according to Cannas et al. (2004).

138 **2.5 Milk analysis**

139 Milk samples were analysed for fat according to Gerber and Gerber-Van Gulik (ISO, 1975); milk
140 proteins, urea, total solids and lactose contents were determined by infrared analysis (Milkoscan 133
141 B, Italian Foss Electric, Padova, Italy). Moreover, pH was measured for each fresh milk sample
142 (Hanna Instruments, HI110, Villafranca Padovana, Italy) . Rennet clotting time (r), rate of curd
143 firming (K20), and curd firmness after 30 min (A30) were also measured on a Formagraph apparatus
144 (Delacroix-Buchet et al., 1994).

145 Milk production was standardised as Fat-Corrected Milk (FCM) at 6.5% fat, according to the
146 following formula: FCM = M (0.37+0.097F), where M is milk yield (kg) and F is milk fat percentage
147 (Pulina et al., 2002).

148 **2.6 Fatty acid analysis**

149 Milk fat was extracted according to Buccioni et al. (2010) and methyl esters of fatty acids (FAMEs)
150 were prepared with a base-catalyzed transesterification according to Christie (2001). The FAMEs
151 were separated and identified by gas-chromatography according to Buccioni et al. (2015).

152 The Desaturation index was calculated according to the following formula:

153 $$DI = (cis\text{-}9\ 14{:}1\ /\ 14{:}0 + cis\text{-}9\ 14{:}1)$$

154 Geometrical and positional isomers of CLA were separated and identified by silver ion HPLC
155 analysis (Sehat et al., 1998). The stationary phase was a silver ion column (ChromSpher5lipidcolumn,

156 250 x 4,6 mm i.d. Stainless steel, 5μ particle size; Varian Inc., Middelburg, The Netherland). The
157 mobile phase was a fresh mixture of acetonitrile in hexane (0.1%, v/v). The injection loop was 50 μL.
158 The solvent flow rate was standardized at1 mL/ min and UV was set at 233 nm. Pure single *cis*9,
159 *trans*11 and *trans*10, *cis*12 C18:2 (Matreya Inc., Pleasant Gap, PA, USA), CLA mix standard (cod
160 O5632; Sigma-Aldrich, St. Louis, MO, USA) and published isomeric profile (Kramer et al., 2008)
161 were used to identify the CLA isomers of interest. Since a reliable internal standard for CLA is not
162 yet available, the quantitative measurements were performed through a calibration curve using high
163 purity single isomer C18:2 *cis*9, *trans*11 (Matreya Inc., Pleasant Gap, PA, USA) and data were
164 referred to gas-chromatographic results. All results concerning the fatty acid composition are
165 expressed as g/ 100g of total lipids, with the exception of CLA isomers that were expressed as g/100g
166 of CLA.

167 **2.7 Identification and quantification of Casein fractions**

168 The identification and quantification of the milk casein fractions were performed according to Bonizzi
169 et al. (2008): milk samples were centrifuged (5000 x *g* for 10 min at 4°C) and the fat was removed.
170 A volume of 200 μL of skimmed milk was diluted with 0.8 mL of a denaturing solution containing 8
171 M urea, 165 mM Tris, 44 mM sodium citrate and 0.3% (v/v) β-mercaptoethanol solution. After
172 filtration through a 0.45-μm pore cellulose membrane (Phenomenex, Torrance, CA, USA), the
173 samples were directly analysed in a reverse-phase mode (RP- HPLC) using a Jupiter C4 column (250
174 mm × 4.6 mm, 300 Å-sized pores, 5-μm sized particles; Phenomenex, Torrance, CA, USA) kept at
175 room temperature; the detection wavelength was 220 nm. The analyses were carried out applying a
176 binary gradient profile to the mobile phase composition: eluent A was HPLC-grade water containing
177 0.1% (v/v) trifluoroacetic acid (TFA) and eluent B was HPLC-grade acetonitrile containing 0.1%
178 (v/v) TFA. The injection loop was 20 μL. The gradient elution programme (constant flow rate of 0.8
179 ml min^{-1}) was: 0–40 min linear gradient from 30% B to 50% B; 40–42 min linear gradient from 50%
180 B to 100% B; 42–43 min isocratic elution 100% B; 43–46 min linear gradient from 100% B to 30%

181 B, followed by a 5 min isocratic elution at the initial conditions. Data were expressed as g/100g of
182 total casein.
183 Casein index has been calculated according the following formula (Buccioni et al., 2015):
184 CI= (Total Casein Content/Total Crude Protein Content) x 100
185
186 **2.8 Statistical analysis**
187 All data (e.g., animal performance and milk composition) recorded over the course of the experiment
188 were processed as completely randomized design with repeated measures using the MIXED
189 procedure of SAS (SAS, 1999):
190 $$y_{ijkl} = \mu + D_i + T_j + I_k(D) + (D\times T)_{ij} + e_{ijkl}$$
191 where y_{ijkl} is the observation; μ is the overall mean; D_i the fixed effect of diet (i = 1 to 2); T_j the fixed
192 effect of sampling time (j = 1 to 7); I_k is the random effect of the ewe nested within the diet (k= 1 to
193 48); $(D_i \times T)_{ij}$ the interaction between diet and sampling time and e_{ijkl} the residual error. The
194 covariance structure was compound symmetry, which was selected on the basis of Akaike's
195 information criterion of the mixed model of SAS. Statistical significance of the diet effect was tested
196 against variance of sheep nested within diet according to repeated measures design theory (Littell et
197 al. 1998). Multiple comparisons among means were performed using the Tukey's test and differences
198 between treatment means were considered to be significant at $P \leq 0.05$.
199
200 **3 Results and Discussion**
201 **3.1 Animal performances**
202 At the end of the trial, the body weight of each animal did not vary with respect to initial status and
203 no differences between the two experimental groups were found (C group, 44.5 ± 3.9 kg *vs* CHE
204 group, 45.2 ± 4.5 kg; P=0.936). Hence, the dietary intake met the ewes' energy requirement. The
205 amount of concentrate offered was completely consumed by the animals of both groups (C group,
206 450 g/head per day; CHE group, 500 g/head per day) and, after the adaptation period, the CHE group

207 did not show any palatability problem due to CHT extract inclusion in the concentrate. It allowed a
208 similar intake of linseed oil (31.5 g/ head per day) for the two groups and a consumption of 40 g/
209 head per day of CHT for the CHE group. This result was in agreement with the literature, which
210 reported no detrimental effects of CHT extract on the DM intake (Buccioni et al., 2015; Sliwisnki et
211 al., 2002; Toral et al., 2011).
212 The effect of the sampling time and of the interaction diet x sampling time was not significant for all
213 the variables considered, so only the results about the effects of dietary treatments were reported.
214 **3.2 Milk yield and composition**
215 In the present experiment, CHE treatment resulted in a greater production of milk and of FCM (Table
216 2). Previous studies based on the use of *Lotus corniculatus* as fresh forage, reported a beneficial effect
217 of condensed tannins on milk production (Wang et al., 1996; Woodward et al., 1999), whereas
218 experiments based on the use of hydrolysable tannins from chestnut or condensed tannins from
219 quebracho in the diet of non-grazing ewes did not find any effect on milk yield (Buccioni et al., 2015;
220 Toral et al., 2013, 2011). Similarly, Liu et al. (2013) did not observe a positive effect of dietary
221 supplementation with CHT extract (at 10 g/ kg of DM intake; DMI) on milk yield and milk
222 composition of dairy cows during the transition period. Also fat and protein yield was greater in milk
223 from CHE group (P=0.039 and P=0.025, respectively) while the protein and urea contents were
224 significantly smaller (P<0.001), as a consequence of the dilution effect due to the greater milk
225 production (P<0.001) from ewes fed CHE (Table 2). A previous study on dairy cows reported that
226 the addition of quebracho and chestnut tannin over 18 g/ kg of DMI decreased milk true protein
227 concentration (Aguerre et al., 2010). In the present experiment, casein concentration, casein fraction
228 profile and rheological parameters were not significantly affected by CHE concentrate, whereas the
229 value of the casein index was greater (P=0.034), suggesting that the smaller content of total protein
230 in milk from sheep fed CHE concentrate was not due to the casein fraction, but, probably, to a smaller
231 content of the whey protein component of milk (Tables 2 and 3). As reported by Waghorn et al.
232 (1987), tannins may selectively affect the intestinal absorption of amino acids, in particular that of

233 non essential aminoacids (NEAA), which are less digested by nearly 10%, when tannins are added to
234 the diet. According to Min et al. (2001), tannins induce a selective apparent absorption of specific
235 aminoacids such as threonine (57%), valine (89%), isoleucine (94%), leucine (30%), tyrosine (41%),
236 phenylalanine (93%), histidine (90%) and lysine (59%). However, on the basis of the data collected
237 in this trial, it is not possible to assess if the observed differences in the Casein index values were due
238 to an effect of CHE tannins on the intestinal absorption of NEAA or to the interaction of tannins with
239 other digestive factors.
240 The urea content in milk from ewes fed CHE was significantly smaller than that in milk from ewes
241 fed C diet ($P<0.001$; Table 2) and this result was expected as tannins are able to decrease the rumen
242 degradability of dietary protein, increasing the efficiency in nitrogen recycling. Tannin-protein
243 complexes may be dissociated in the abomasum tract, increasing the amount of by-pass dietary
244 aminoacids available for the intestinal absorption (Patra and Saxena, 2011). The decrease in milk urea
245 and the increase in aminoacids availability could have favoured energy partitioning towards milk
246 production, as a consequence of the smaller energy cost of the urea recycling and of the greater
247 availability of aminoacids, which may increase the synthesis of glucose (Frutos et al., 2004a, 2004b).
248 Thus, when the nitrogen content of the diet is very high, as in the grazing period during the early
249 spring, the addition of hydrolysable tannins to the diet may improve the efficiency of nitrogen
250 recycling with beneficial effects on milk production. However, this effect is probably related also to
251 the nature of dietary protein (especially as regard the amount of soluble protein) and also to the
252 equilibrium between the energy and protein fraction of the diet (Pulina et al., 2002).

253 **3.3 Milk fatty acid profile**

254 The chestnut tannin extract inclusion in the concentrate had a marked effect on the FA profile of milk.
255 The concentration of LA and α-LNA in milk from ewes fed CHE concentrate was significantly greater
256 than that in C milk, probably as a consequence of a decrease in the BH rate of the rumen (Table 4).
257 However, the content of α-LNA in milk from C group was above 2% of the total lipids, a value similar
258 to that reported in a previous study adopting greater amounts of extruded linseed in the diet (210 g/

259 head per day, Mele et al., 2011) and nearly double that reported in a previous study on grazing dairy
260 ewes fed a dietary supplement of 200 g/head per day of extruded linseed (Addis et al., 2009). When
261 CHT was added to the concentrate, the content of α-LNA in milk fat was greater than that found in
262 milk from ewes fed C diet (+18%; P=0.001), suggesting CHT may protect unsaturated fatty acids
263 from BH in the rumen. This aspect is also suggested by the pattern of BH intermediates. In fact, the
264 milk fat concentration of all the BH intermediates, including *cis*9, *trans*11 CLA, was smaller than
265 that observed for milk samples from ewes fed C concentrate (P=0.001). It is well known that
266 polyphenols interfere with the rumen microbiome (by inhibiting enzymes, affecting bacterial
267 membranes or depriving metal ions), with effects on fibre digestibility, methane emissions and protein
268 degradation (Cieslak et al., 2014; Frutos et al., 2004a, 2004b; Goel and Makkar, 2012). However,
269 contrasting results are reported about the *in vivo* effect of hydrolysable and condensed tannins on the
270 rumen BH of PUFA. Studies on fattening lambs indicate a significant effect of condensed tannin on
271 rumen BH (Vasta et al., 2010, 2009a, 2009b), as a result of a shift in the composition of the rumen
272 microbial population. In particular, *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticum* are
273 strongly influenced by the presence of hydrolysed and condensed tannins in the diet (Buccioni et al.,
274 2015; Vasta et al., 2010). However, poor information on the effect of tannins on other microbial
275 species, as-yet-uncultivated bacteria phylogenetically classified as *Prevotella*, *Lachnospinaceae*
276 *incertae sedis* and unclassified *Bacteroidales*, *Clostridiales* and *Ruminococcaceae* playing a
277 dominant role in the rumen lipid metabolism, are not available in literature (Belenguer et al., 2010;
278 Boeckaert et al., 2008; Castro-Carrera et al. 2014; Huws et al., 2011). *In vivo* studies on lactating
279 dairy ewes fed diets with chestnut tannin or quebracho tannins in some cases failed to demonstrate
280 the efficacy of tannins on rumen BH (Toral et al., 2013, 2011), whereas more recently a significant
281 effect was reported by Buccioni et al. (2015). In the present study the greater concentration of LA, α-
282 LNA in milk fat from ewes fed CHE, along with the smaller concentration of the BH intermediates
283 and of stearic acid, suggested a reduction of the BH rate starting from the first step of the process.
284 Differences with previous researches may be due to the greater amount of PUFA offered to the ewes

285 (from pasture and from extruded linseed), and to the greater amount of CHT included in the diet. A
286 previous study, in fact, adopted a dose of 20 g/head per day for both vegetable oil and tannin extracts
287 (Toral et al., 2013). In the present study, taking into consideration the amount of concentrate offered
288 to the ewes in the CHE group, the intake of linseed oil accounted for nearly 30 g/head per day (500 g
289 of concentrate containing 90 g of extruded linseed, at 35% oil content) and the intake of CHT extract
290 was nearly 40 g/head per day (500 g of concentrate containing 8% of CHT extract). Both doses
291 accounted for less than 2% of the expected DMI of a lactating Sarda ewe (nearly 2200 g DMI per
292 day, considering the body weight and the level of production obtained in the present study; Cannas
293 et al., 2004). Therefore they may be considered as practical doses. Interestingly, the dose of CHT
294 extract used in the present study was the same as that used in a previous *in vivo* study with non-
295 grazing dairy ewes (Buccioni et al., 2015), in which similar results about the effect of CHT on rumen
296 BH of PUFA were obtained.
297 The contents of short chain FA (SCFA), medium chain FA (MCFA) and < C16:0FA were greater in
298 milk from ewes fed CHE than in milk from ewes fed C (P=0.001, P=0.001 and P=0.041 respectively;
299 Table 4). This result and the smaller content of > C16:0 FA (P=0.034), suggest an increase of *de novo*
300 FA production in mammary gland of ewes fed the CHE concentrate. The inclusion of unsaturated
301 vegetable oils in the ruminant diet usually decreases the neo-synthesis of SCFA and MCFA due to
302 the inhibitory effect of large amounts of circulating long chain FA (including some trans, mono and
303 polyenoic FA) on the expression of genes involved in FA synthesis (Shingfield et al., 2013). Since
304 the volatile fatty acid (VFA) production in the rumen was not evaluated in the present study, it is not
305 possible to establish if the effect of CHE concentrate on the content of SCFA and MCFA in milk was
306 due to an increase of acetate availability to the mammary gland or to a decrease of the inhibition
307 effect of the FA deriving from rumen BH. However, several authors reported that hydrolysable
308 tannins are able to increase the production of VFA especially of acetate (Buccioni et al., 2015;
309 Waghorn et al., 2008). Moreover, the relative milk concentration of *trans*9 *trans*11 and of *trans*10
310 *cis*12 CLA, which may exert a specific inhibitory activity on the lipid synthesis in mammary gland

311 (Shingfield et al., 2010), was greater in milk fat from ewes fed C concentrate compared to those fed
312 CHT (P=0.042 and P=0.032, respectively; Table 5). This results is consistent with literature that
313 reported the ability of tannins to decrease the production of C18:1 *trans*10 and C18:2 *trans*10-isomers
314 in the rumen (Buccioni et al., 2015, 2011; Minieri et al., 2014; Toral et al., 2011; Vasta and Luciano,
315 2011).
316 Also, the contents of *cis*9, *trans*11 CLA and of *cis*8, *trans*10 CLA were greater in milk from ewes
317 fed C concentrate (P=0.010 and P=0.012, respectively), whereas that of *cis*11, *cis*13CLA and *cis*10,
318 *cis*12 CLA was smaller (P=0.012 and P=0.010, respectively). In both cases *cis*9, *trans*11 CLA was
319 the main isomer, accounting for more than 60% of total CLA content.
320 The content of several branched chain fatty acids was smaller in milk from ewes fed the CHE
321 concentrate (Table 4), confirming that hydrolysable tannins may influence microbial activities
322 (Fieviez et al., 2012; Vlaemink et al., 2006).
323 Interestingly, the ratio C14:1*cis*9/(C14:0+C14:1 *cis*9), which is considered the best proxy of the
324 desaturation activity of mammary Stearoyl Co-A desaturase enzyme, was significantly smaller in
325 milk from ewes fed CHE concentrate (P=0.042). Similarly, the content of other products of the SCD
326 enzyme was smaller in milk from CHE group (C14:1 *cis*9, C17:1 *cis*9, C18:1 *cis*9). Hence, a lower
327 activity of SCD in ewes fed CHE could be hypothesised even if in the literature controversial data
328 are reported (Buccioni et al., 2015; Toral et al., 2013, 2011). It is well known that rumen bacteria may
329 metabolize hydrolysable tannins to phenolic substances with a lower molecular weight, which may
330 be adsorbed in the intestine (Makkar, 2003). To our knowledge no information are available about a
331 putative effect of these substances on the gene expression or activity of SCD enzyme.
332
333 **4 Conclusion**
334 The inclusion of CHT extracts in the concentrate for grazing ewes improved milk production and
335 resulted in a smaller amount of milk urea. Moreover, the concentration of LA and α-LNA in milk was
336 greater. At the same time significant changes in the concentration of rumen BH intermediates suggest

337 that the diet interfered with the rumen microbiome involved in BH processes. This effect was
338 probably amplified by the great content of α-LNA included in the diet, as a consequence of the
339 additive effect of pasture and linseed. The use of practical doses of CHT in the diet of grazing ewes
340 may improve the response to dietary linseed supplementation, resulting in milk with a greater
341 concentration of α-LNA.
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Table 1. Ingredients and chemical composition of the concentrates.

item	C[1]	CHE[2]
Ingredient (g/kg of DM)		
Barley meal	220.1	169.9
Extruded linseed	204.3	181.9
Wheat bran	175.9	131.2
Sorghum	105.2	159.9
Sunflower meal	99.6	90.3
Soybean meal (44% CP)	64.9	59.7
Molasses	50.0	50.0
Alfalfa hay meal	40.0	37.1
Chestnut tannin extract[3]	-	80.0
Mineral vitamin mix	40.0	40.0

Chemical composition[4]			
DM	kg	878.0	882.1
CP	g/kg DM	188.4	168.7
EE	”	102.1	91.3
NDF	”	219.3	193.7
ADF	”	112.8	107.2
ADL	”	31.2	29.2
NPN	”	1.6	1.4
PS	”	18.1	16.2
Ash	”	89.2	93.7
NFC	”	270.5	245.9
NEL	kcal/kg DM	1883	1750
g/100 g of fatty acids			
C14:0		0.1	0.1
C16:0		7.3	7.1
C16:1 *cis*9		0.1	0.2
C18:0		5.2	5.3
C18:1 *cis*9		17.0	17.1
C18:2 *cis*9 *cis*12		18.2	18.0
C18:3 *cis*9 *cis*12 *cis*15		52.1	52.3

[1]C, Control concentrate.

[2]CHE, Concentrate containing hydrolizable tannin extract.

[3]Hydrolizable tannin extracted from chestnut wood (*Castaneasativa*) containing 750 g of tannic acid equivalent/kg of DM (provided by Gruppo Mauro saviola s.r.l., Radicofani, Siena, Italy).

[4]Legenda: DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; NPN, non-protein nitrogen; PS, soluble protein; NFC, non fiber carbohydrates; NE, Net energy for lactation.

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562 Table 2. Milk yield and milk composition from ewes fed a diet containing 0 (C) or 80 g of chestnut

563 tannin/kg of DM (CHE).

Item		Diet		SEM[1]	P value[2]
		Control	CHE		
Milk yield	g/day	825 b	978 a	0.011	<0.001
6.5% FCM[3]		769 b	910 a	0.010	<0.001
Milk composition					
Fat percentage	g/100 g	5.80	5.78	0.020	0.425
Milk fat yield	g/day	47.8 b	56.5 a	0.921	0.039
Lactose	g/100 g	4.93	4.93	0.022	0.541
Protein percentage	g/100 g	5.56 a	5.12 b	0.010	<0.001
Milk protein yield	g/day	45.8 b	50.1 a	0.561	0.025

Casein percentage	g/100 g	4.27	4.19	0.051	0.356
Milk casein yield	g/day	35.2 b	40.9 b	0.253	0.031
Urea	mg/dl	44.7 a	37.9 b	0.521	<0.001
Milk pH		6.56	6.61	0.021	0.527
Total solids	g/day	137	149	12.000	0.943
Casein Index[4]	g/100 g of total crude protein	76.8 a	81.8 b	0.540	0.034
Clotting parameters					
r	min	20.0	20.7	0.012	0.891
k_{20}	min	1.56	1.51	0.012	0.879
a_{30}	mm	38.9	37.0	0.023	0.786

564 [1] Standard error of the mean.

565 [2] Probability of significant effect due to experimental diet; ns, not significant and a, b for P<0.05

566 [3] Fat Corrected Milk, FCM = M (0.37+0.097 F), where M is milk yield (kg) and F is milk fat

567 percentage

568 [4] Casein Index, CI= (Total Casein Content/Total Crude Protein Content) x 100

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574 Table 3. Casein fractions profile (g/100g of total Casein).

Item	Diet		SEM[1]	P value[2]
	C[3]	CHE[4]		
κ-casein	12.8	11.9	0.57	0.24
αS2-casein	16.3	15.9	0.43	0.56
αS1-casein	26.8	26.1	0.65	0.43
β-casein	43.8	45.9	0.74	0.06

575 [1] Standard error of the mean.

576 [2] Probability of significant effect due to experimental diet; a, b for P<0.05

577 [3]C, Control concentrate.

578 [4]CHE, Concentrate containing hydrolysable tannin extract.

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580 Table 4. Fatty acid profile (g/100g of total lipids) of milk.

Fatty acid	Diet		SEM[1]	P value[2]
	C[3]	CHE[4]		
C4:0	4.54	4.58	0.057	0.564
C6:0	2.93 b	3.21 a	0.067	0.001
C7:0	0.03	0.03	0.002	0.768
C8:0	2.81 b	3.12 a	0.049	0.001
C10:0	7.75 b	8.78 b	0.134	0.001
C10:1*cis*9	0.21	0.20	0.005	0.734
C11:0	0.06	0.07	0.003	0.744
C12:0	3.94 a	4.23 b	0.056	0.002
C12:1 *cis*9	0.04	0.04	0.002	0.112
C13:0	0.08 a	0.06 b	0.002	0.001
C13-*iso*	0.03 a	0.02 b	0.001	0.001
C14:0	9.17 a	9.68 b	0.067	0.001
C14-*iso*	0.09 a	0.06 b	0.003	0.001
C14:1 *cis*9	0.13 a	0.11 b	0.002	0.003
C15:0	1.02 a	0.91 b	0.008	0.001
C15-*anteiso*	0.53 a	0.44 b	0.007	0.001
C15-*iso*	0.26 a	0.17 b	0.005	0.001
C16:0	18.77 b	19.45 a	0.181	0.014
C16-*iso*	0.23	0.19	0.004	0.001
C16:1 *cis*9	0.58	0.53	0.017	0.069
C16:1*trans*9	0.50 a	0.42 b	0.009	0.001
C16-1 *cis*7	0.39	0.37	0.013	0.253
C17:0	0.59	0.55	0.016	0.068
C17-*anteiso*	0.36 a	0.33 b	0.004	0.001
C17-*iso*	0.48 a	0.38 b	0.030	0.017
C17:1 *cis*9	0.15 a	0.10 b	0.005	0.001
C18:0	10.39 a	9.13 b	0.257	0.002
C18-*iso*	0.04	0.03	0.003	0.374
C18:1 *cis*9	15.61 a	14.27b	0.166	0.001
C18:1 *cis*11	0.98 b	1.03 a	0.013	0.048
C18:1 *cis*12	0.42 a	0.57 b	0.022	0.002
C18:1 *cis*13	0.10	0.09	0.007	0.273
C18:1 *cis*14	0.09	0.09	0.015	0.813
C18:1 *cis*15	0.36 b	0.38 a	0.009	0.042
C18:1 *trans*6-8	0.46	0.48	0.011	0.399
C18:1 *trans*9	0.41	0.41	0.007	0.783
C18:1 *trans*10	0.72	0.75	0.022	0.386
C18:1 *trans*11	3.89 a	3.57 b	0.061	0.001
C18:1 *trans*12	0.87	0.88	0.074	0.374
C18:1 *trans*16	0.70	0.69	0.016	0.791

C18:2 *cis*9 *trans*11	2.20 a	1.85 b	0.036	0.001
C18:2 *cis*9 *cis*12	1.89 b	2.42 a	0.049	0.001
C18:2 *trans*11 *cis*15	1.14	1.16	0.028	0.112
C18:2 *cis*9 *trans*12	0.09	0.09	0.018	0.998
C18:2 *trans*9 *cis*12	0.07	0.06	0.013	0.678
C18:3 *cis*9 *cis*12 *cis*15	2.18 a	2.57 b	0.056	0.001
C18-3 *cis*9 *trans*11 *cis*15	0.36 a	0.29 b	0.009	0.001
C20:0	0.17	0.17	0.007	0.825
C20:4 *cis*5 *cis*8 *cis*11 *cis*14	0.10	0.10	0.003	0.338
C20:5 *cis*5 *cis*8 *cis*11 *cis*14 *cis*17	0.14	0.13	0.004	0.653
C21:0	0.16	0.15	0.014	0.625
C22:0	0.10	0.09	0.006	0.124
C22:5 *cis*7,*cis*10, *cis*13*cis*16 *cis*19	0.15	0.15	0.003	0.802
C22:6 *cis*4, *cis*7, *cis*10 *cis*13*cis* 16 *cis*19	0.10	0.09	0.009	0.107
C24:0	0.03	0.03	0.005	0.774
SCFA[5]	18.27b	19.92a	0.042	0.001
MCFA[6]	34.12b	35.24a	0.051	0.001
SFA[7]	63.31b	64.90a	0.079	0.048
MUFA[8]	26.61a	24.98b	0.095	0,039
PUFA tot[9]	8.42b	8.91a	0.059	0.049
PUFA n-3[10]	3.68b	4.02a	0.062	0.001
PUFA n-6[11]	2.05b	2.57a	0.066	0.001
OIAR[12]	0.83	0.71	0.062	0.176
DI[13]	0.014a	0.011b	0.001	0.042
<C16:0[14]	43.18b	45.22a	0.054	0.041
>C16:0[15]	66.47a	64.35b	0.048	0.034

581 [1] Standard error of the mean.

582 [2] Probability of significant effect due to experimental diet; a, b for P<0.05.

583 [3]C, Control concentrate.

584 [4]CHE, Concentrate containing hydrolizable tannin extract.

585 [5]SCFA, Short chain fatty acids.

586 [6]MCFA, Medium chain fatty acids.

587 [7]SFA, Saturated fatty acids.

588 [8]MUFA, Monounsaturated fatty acids.

589 [9]PUFA tot, polyunsaturated fatty acids.

590 [10]PUFA n-3, polyunsaturated fatty acids belonging to the n-3 series.

591 [11]PUFA n-6, polyunsaturated fatty acids belonging to the n-6 series.

592 [12]Ratio odd-*iso* to odd-*anteiso* FA: (*iso* 15:0 + *iso* 17:0) / (*anteiso* 15:0 + *anteiso* 17:0).

593 [13] Desaturation index, DI = (*cis*-9 14:1 / 14:0 + *cis*-9 14:1).

594 [14]<C16:0 de novo fatty acids calculated according to Fiviez et al 2012.

595 [15]>C16:0 preformed fatty acids calculated according to Chilliard et al., 2000 and Fiviez et al., 2012.

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618 Table 5. Conjugated Linoleic acid isomers profile (g/100g of total CLA) of milk.

Isomer	Diet		SEM[1]	P value[2]
	C[3]	CHE[4]		
*trans*13 *trans*15	1.10	1.20	0.111	0.622
*trans*12 *trans*14	2.06	2.00	0.132	0.061
*trans*11 *trans*13	8.76	8.83	0.033	0.080
*trans*10 *trans*12	5.35	5.65	0.021	0.065
*trans*9 *trans*11	2.63a	2.11 b	0.052	0.042
*trans*8 *trans*10	3.22	3.54	0.080	0.126
*trans*7 *trans*9	1.52	1.46	0.040	0.144
*trans*6 *trans*8	0.24	0.22	0.042	0.259
*cis*11 *trans*13	2.03	1.96	0.051	0.183
*trans*10 *cis*12	6.62 a	6.38 b	0.023	0.032
*cis*9 *trans*11	61.80 a	61.08 b	0.031	0.010
*cis*8 *trans*10	0.41 a	0.25 b	0.030	0.012
*trans*7 *cis*9	4.26	4.17	0.062	0.065
*cis*11 *cis*13	0.01 b	0.54 a	0.051	0.012
*cis*10 *cis*12	0.01	0.59 b	0.050	0.010
*cis*9 *cis*12	0.01	0.03	0.121	0.679

619 [1] Standard error of the mean.

620 [2] Probability of significant effect due to experimental diet; a, b for $P<0.05$.

621 [3]C, Control concentrate.

622 [4]CHE, Concentrate containing hydrolizable tannin extract.

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