Running title: **Sheep milk and tannins**

Milk fatty acid composition, rumen microbial population and animal performances in response to diets rich in linoleic acid supplemented with chestnut or quebracho tannins in dairy ewes.

A. Buccioni[1*], M. Pauselli[2], C. Viti[1], S. Minieri[1], G. Pallara[1], V. Roscini[2], S. Rapaccini[1], M. Trabalza Marinucci[3], P. Lupi[1] , G. Conte[4], M. Mele[4].

[1] Dipartimento di Scienze delle Produzioni Agro-alimentari e dell'Ambiente, University of Florence, Piazzale delle Cascine 18, 50144 Firenze, Italy

[2] Dipartimento di Scienze Agrarie Alimentari ed Ambientali, University of Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy

[3] Dipartimento di Medicina Veterinaria, University of Perugia, Via S. Costanzo 4, 06126 Perugia, Italy.

[4] Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy

[*] Corresponding author: arianna.buccioni@unifi.it

INTERPRETATIVE SUMMARY

Since tannins are able to interfere with lipid rumen metabolism, they can represent a dietary ingredient to modulate biohydrogenation of polyunsaturated fatty acids. This study showed that the use of soybean oil in the diet of dairy ewes coupled with practical doses of quebracho or chestnut tannin extracts resulted in an increase of bioactive fatty acid content in milk. Nevertheless, the kind of tannin (condensed or hydrolysable) affected milk fatty acid profile in different way suggesting that a differential response of rumen microbioma to the nature of tannin extract could be occurred.

ABSTRACT

The aim of the study was to evaluate milk fatty acid profile, animal performances and rumen microbial population in response to diets containing soybean oil supplemented or not with chestnut and quebracho tannins in dairy ewes. Eighteen Comisana ewes at 122 ± 6 days in milking were allotted into 3 experimental groups. Diets were characterized by chopped grass hay *ad libitum* administered and by 800 g / head and day of three experimental concentrates containing 84.5 g of soybean oil / kg of DM and 52.8 g / kg DM of bentonite (Control diet) or chestnut tannin extract (CHT diet) or quebracho tannin extract (QUE diet). The trial lasted 4 weeks. Milk yield was recorded daily, while milk composition and blood parameters were analysed weekly. At the end of the experiment, samples of rumen fluid were collected in order to analyse pH, volatile fatty acid profile and the relative proportion of *Butyrivibrio fibrisolvens* and *B. proteoclasticus* in the rumen microbial population.

Hepatic functionality, milk yield and gross composition were not affected by tannin extracts, while milk fatty acid composition was characterized by significant changes in the concentration

of linoleic acid (CHT + 2.77 % and QUE + 9.23 %), vaccenic acid (CHT + 7.07 % and QUE + 13.88 %), rumenic acid (CHT - 1.88 % and QUE + 24.24 %), stearic acid (CHT + 8.711 % and QUE - 11.45 %) and saturated fatty acids (CHT – 0.47 % and QUE -3.38 %). These differences were probably due to the ability of condensed *vs* hydrolizable tannins to interfere with rumen microbial metabolism, as indirectly confirmed by changes in the relative proportion of *B. fibrisolvens* and *B. proteoclasticus* populations and by changes in the volatile fatty acid molar proportion. The effect of CHT diet on milk fatty acids profile and on the microbial species considered in this trial was intermediate between QUE and control diet, suggesting a differential effect of condensed and hydrolysable tannins on rumen microbes. In comparison to control animals, the presence of *B. fibrisolvens* increased about three times in ewes fed CHT and about five times in animals fed QUE diet. On the contrary, the abundance of *B. proteoclasticus* decreased about five and fifteen times in rumen liquor of ewes fed CHT and QUE diets, respectively. The use of soybean oil and a practical dose of QUE or CHT extract in the diet of dairy ewes can be an efficient strategy to improve the nutritional quality of milk.

Key words: tannins, milk fatty acids, sheep, microbial population.

INTRODUCTION

During the last decade several efforts have been done to enhance the level of healthy fatty acids (FA) in milk and dairy products with the aim to improve the nutritional quality of foods deriving from ruminants (Chilliard et al. 2007; Mele et. al, 2009). This objective may be achieved applying feeding strategies based on dietary supplementation with polyunsaturated marine or vegetables oils or oilseeds (Shingfield et al., 2013), in order to accumulate in the rumen conjugated linoleic acid (*trans*-11, *cis*-9 CLA) precursors, such as *trans*-11 18:1 (vaccenic acid, VA) and to increase the duodenal passage of polyunsaturated fatty acids (PUFA). Previous studies demonstrated that adding vegetable oils rich in linoleic acid (*cis*-9, *cis*-12 18:2; LA) oil

68 in the diet of small ruminants, the content of *cis*-9, *trans*-11 CLA and VA in milk fat increased
69 by two-three times (Mele et al., 2006; Gomez-Cortes et al., 2008; Mele et al. 2008). However,
70 since the extent of rumen biohydrogenation (BH) of PUFA is usually more than 80 – 90 %, the
71 amount of supplemented lipid needed to achieve an effective enhancement of *cis*-9, *trans*-11
72 CLA and VA in milk fat from sheep and goats ranges from 60 to 100 g / head and day, leading
73 to an increase of feeding costs. Moreover, although small ruminants are less sensitive than dairy
74 cow to the milk fat depression syndrome, high levels of lipids coupled with low forage diets
75 may induce a decrease in the milk fat content (Bauman and Griinari, 2003; Shingfield et al.,
76 2013). As a consequence, in the last years, increasing interested has been devoted to feed
77 ingredients able to slow rumen BH extent of dietary PUFA, in order to obtain significant
78 accumulation of VA in the rumen and, thus, an increase of the transfer of this fatty acid from
79 rumen to duodenum and lastly to the mammary gland (allowing to obtain also an increase of
80 *cis*-9, *trans*-11 CLA, which originates by the mammary desaturation of vaccenic acid), by using
81 less amounts of lipid supplementation.
82 Several in vitro studies demonstrated that tannins are able to interfere with rumen BH or
83 methane production, according to their polyphenolic nature (Bhatta et al., 2009; Khiahosa-Ard
84 et al., 2009; Buccioni et al., 2011). Moreover, fed ewes and cows with diets containing less than
85 4 % of tannins on dry matter basis, resulted in a higher retention of nitrogen and in a lower
86 plasma urea concentration, as a consequence of the tannin ability to preserve feed protein from
87 rumen microbial degradation (Frutos et al., 2004a). The effect on rumen microorganism activity
88 has been related to the ability of tannins to interfere with the membranes of rumen bacteria,
89 binding enzymes or by metal ions deprivation (Patra and Saxena, 2011). Among bacterial
90 species involved in BH processes of PUFA, *B. fibrisolvens* and *B. proteoclasticus* seem to be
91 the more sensitive (Vasta et al. 2010), but specific studies about the effect of different kind of
92 tannins on rumen microbial population are still scarce. Moreover, results from *in vitro* and *in*

93 *vivo* experiments were often contrasting as regard the effect of tannins on the accumulation of
94 BH intermediates in the rumen and on the productive response of the animal (Vasta et al.,
95 2009a; Toral et al., 2011, Toral et al., 2013). This aspect is probably due to differences in tannin
96 species and in percentage of inclusion in the diet and to associative effects between tannins and
97 other diet ingredients such as lipids.
98 The aim of the present study was to evaluate the effect of moderate amount (less than 2 %) of
99 chestnut or quebracho tannin extracts (hydrolysable and condensed tannins, respectively) in
100 diets supplemented with soybean oil on the milk fatty acid (FA) profile and on the relative
101 abundance of *B. fibrisolvens* and *B. proteoclasticus* in the rumen microbial community.
102 Moreover, since sheep milk is mainly used for cheese-making, a further objective of the present
103 study was to evaluate the effect of tannin addiction on the gross composition and clotting
104 characteristics of milk. Finally, since in ruminants tannins may exert a toxic effect, causing
105 necrosis of liver and lesion in the digestive tract (Reed, 1995; Hervas et al., 2003a), this
106 experiment was carried out also to study the effect of these feeding strategies on blood
107 parameters with special focus to the indicators of hepatic functions.

108 **MATERIALS AND METHODS**

109 ***Experimental design.***

110 ***Animals***. Eighteen multiparous Comisana ewes at 122 ± 6 days in milking (DIM) kept at the
111 Experimental Section of the Department of Applied Biology - University of Perugia Italy, were
112 allotted into 3 experimental groups, homogeneous for body weight (68.1 ± 7.83 kg; BW), and
113 each group was kept in multiple pens (6 ewes for each pen). The trial lasted 4 weeks after 15
114 days of adaptation to the feeding regimen. The handling of the animals was according to
115 Institutional Animal Care and Use Committee (IACUC, 2014) of University of Perugia. The

ewes were milked twice daily at 07:30 and 17:30 h using a milking machine (43 kPa; 150 pulsation / min) and daily individual milk yield was recorded.

Diets. The experimental diets were formulated according to the nutrient requirements of a ewe weighing 68 kg and producing one kg of milk at 6.5 % of fat (Cannas et al., 2004). Diets were composed by chopped grass hay (particle size > 4 cm of length) *ad libitum* administered and 800 g / head and day of a concentrate, which contained 84.5 g of soybean oil / kg DM and 52.8 g / kg DM of bentonite (control diet), or 52.8 g / kg DM of chestnut tannins (CHT diet) or 52.8 g / kg DM of quebracho tannins (QUE diet). The chemical composition of feeds and the ingredients of concentrates are presented in Table 1. The dose of tannins was chosen in order to obtain a tannin concentration in the diet of nearly 1.6 % of expected DM intake. On the basis of results from previous studies in literature, this dose was considered as safe for the animal and practical for the farmers (Hervas et al., 2003a; Hervas et al., 2003b; Frutos et al., 2004a; Frutos et al., 2004b). The experimental concentrates were offered after each milking, whereas 100 g/head of rolled barley were offered during milking. Chestnut hydrolysable tannins (750 g / kg DM of equivalent tannic acid; by Gruppo Mauro Saviola srl Radicofani, Siena, Italy), and extract of quebracho tannins (456 g / kg DM of equivalent tannic acid; by Guido Lapi spa Castel Franco di Sotto, Pisa, Italy) were titrated according to Burns (1963).

Sampling and analysis.

Feed sampling and analysis. Samples of feeds and orts were weekly collected and stored at -80 °C until analysis. Samples were freeze dried and then ground for chemical analysis by mill Cyclotec 1093 (PBI International, Milan, Italy) using a mesh size of 1 mm. Crude protein (CP), ether extract (EE) and ash were determined according to the AOAC methods 976.06, 920.39 and 942.05, respectively (AOAC, 1995). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin were determined according to Van Soest et al. (1991) using heat stable amylase and sodium sulphite, and expressed inclusive of residual ash. Metabolizable energy

(ME) and Net energy for lactation (NEl) was calculated according to Cannas et al. (2004). Feed FA were extracted according to Folch et al., (1957), esterified according to Christie (1982) with 19:0 (Sigma Chemical Co., St Louis, MO) as the internal standard, and identified using the same procedure described below for FA of milk samples.

Milk sampling and analysis. Individual milk samples from morning and evening milking were collected weekly and allotted into three aliquots for analysis: the first aliquot was processed in order to assess fat, lactose, protein and casein content by using Milkoscan 6000 FT (Foss Electric, Hillerød Denmark), and total somatic cell count (SCC) according to ISO 13366-2|IDF 148-2 (2006), by using a Fossomatic 5000 (Foss Electric, Hillerød Denmark) and expressed as linear score (linear score = $\log_2$ [SCC / 12,500]; Shook, 1993). The second aliquot was processed to determine the milk rennet characteristics at 35°C by a Maspress apparatus (Foss Italia, Padua, Italy), according to Zannoni and Annibaldi (1981). The following rennet parameters were determined: clotting time (r) that is the time from rennet addition to the beginning of coagulation, firming time (k_{20}) that is the time needed for the amplitude to reach 20 mm on the recording chart, and curd firmness (a_{30}) that is the amplitude of the trace 30 min after rennet addition. The third aliquot of milk samples was stored at -80°C until analysis for FA extraction and composition by gas-chromatography according to Buccioni et al. (2010). Individual fatty acid methyl esters (FAMEs) were quantified using valeric acid (5:0) and nonadecanoic acid (19:0) methyl esters (cod W275204 and cod N5377, respectively; Sigma Chemical Co., St. Louis, MO) as internal standards and identified by comparison of the relative retention times of FAMEs peaks from samples, with those of the standard mixture 37 Component FAMEs Mix (Supelco, Bellefonte, PA, USA 4:0 - 24:0 (cod 18919 - 1AMP, Supelco, Bellefonte, PA, USA), individual *trans*-9 18:1 and *trans*-11 18:1 (cod 46903 and v1381 respectively, Sigma-Aldrich, St. Louis, Missouri, USA), individual *cis*-9, *trans*-11 18:2

(cod 1255, Matreya Inc Pleasant GAP, PA, USA.), CLA mix standard (cod 05632; Sigma-Aldrich, St. Louis, Missouri, USA) and published isomeric profile (Kramer et al., 1997; Kramer et al., 2004; Cruz-Hernandez et al., 2006). The 18:1 isomers elution sequence was performed according to Kramer et al. (2008). Moreover, standard mix of α-linolenic acid (α-LNA) isomers (47792, Supelco, Chemical Co., St. Louis, MO) and of LA isomers (47791, Supelco, Chemical Co., St. Louis, MO) and published isomeric profiles (Destaillats et al., 2005) were used to identify the isomers of interest. Two bacterial acid methyl ester mix (47080-U Supelco, Chemical Co., St. Louis, MO; GLC110, Matreya, Pleasant Gap, PA) and individual standard for methyl ester of *iso* 14:0, *anteiso* 14:0, *iso* 15:0 and *anteiso* 17:0 (21-1211-11, 21-1210-11, 21-1312-11 and 21-1415-11, Larodan Malmo, Sweden) were used to identify branched FA profile. Inter and intra-assay coefficients of variation were calculated by using a reference standard butter (CRM 164, Community Boureau of Reference, Bruxelles, Belgium) and detection threshold of FA was 0.01g / 100g of FA (Contarini et al., 2013). All FA composition results are expressed as g / 100g of FA.

Blood sampling and analysis. Samples of blood were collected from each animal at the end of every experimental week by punching the jugular vein. Blood was stored into tubes without anticoagulant and serum was separated by centrifugation (5000 x g for 30 min at 25°C). Total protein (Colorimetric method BIURET), urea (kinetic enzymatic metod), albumine (ALB; colorimetric BCG method), γ-glutamil-transferase (γ-GT; kinetic SZASZ-tris method), serum glutamic-oxaloacetic-transaminase (SGOT; kinetic UV IFCC method), serum glutamic-pyruvic-transaminase (SGPT; kinetic UV IFCC method) were detected using diagnostic kits (cods ASR01120; ASR01143; ASR0128012; ASR01194; ASR01229; ASR01219, Assel s.r.l., Rome Italy) with an auto blood-analyzer for hematology (Vegavet AMS, Analyser Medical

188 System, Rome, Italy). Globuline (GBL) content was estimated by the difference between total
189 protein and albumin contents.

190 ***Rumen sampling and analysis.*** The last day of the experimental period, animals were milked
191 and given free access to their ration for 1 h according to Toral et al (2013). Then feeds were
192 removed and 3 h later, rumen liquor samples were collected from each ewe using a stomach
193 tube connected to a manual pump. Immediately after collection, each sample of rumen liquor
194 was measured for pH, partitioned into two amounts and stored at -80°C until analysis for total
195 volatile fatty acid (VFA) content (10 mL) and microbiological assay (5 ml). The analysis of
196 VFA (2:0, acetic; 3:0, propionic; 4:0, butyric; *iso* 4:0, *iso* butyric; 5:0, valeric; *iso* 5:0, *iso*
197 valeric) of rumen liquor samples was performed by high performance liquid chromatography
198 (HPLC): a volume of 10 mL of rumen liquor was diluted with H_2SO_4 0.1N (1:1) and centrifuged
199 (5000 x g for 15 min at 4°C) to separate the liquid phase from the feed residuals. After, the
200 liquid phase was micro-filtered (0.45 μm millex –HV) and the sample was directly injected in
201 the HPLC apparatus using a ion exclusion column Aminex 85 HPX – 87 H (300 mm x 7.8 mm;
202 Å-sized pores, 9 μm sized particle; Biorad, Milan, Italy) kept at 40°C; the detection wavelength
203 was 220 nm. The analyses were carried out applying an isocratic elution (flux 0.6 mL/min) with
204 a H_2SO_4 0.008N solution as mobile phase. The injection loop was 20 μL. Individual VFA were
205 identified using a standard solution of 4.50 mg / mL of acetic acid, 5.76 mg / mL of propionic
206 acid, 7.02 mg / mL of butyric acid and *iso* butyric acid, 8.28 mg / mL of valeric acid and of *iso*
207 valeric acid in H_2SO_4 0.1N (cods 338826, 402907, B103500, 58360, 75054, 129542
208 respectively; Sigma-aldrich, St Louis, Missouri, USA). The quantification was obtained using
209 an external calibration curve based on the standards above described. Data were expressed as
210 mM.

211 ***DNA extraction and quantitative real-time PCR analysis.*** Total DNA was extracted from 1
212 mL of rumen liquor using the Fast DNA SPIN kit for soil (Qbiogene, Carlsbad, CA, USA) with
213 some modifications. Briefly, each sample was thawed on ice, transferred to a 15 mL tube
214 containing 4.5 mL of a buffer solution (150 mM-NaCl, 10 mM-Tris-HCl, pH 8.0, 10 mM-
215 EDTA and 4% SDS) and incubated for 15 min at 70°C. The liquid was centrifuged at 200 × *g*
216 at 4°C for 5 min. One mL of the supernatant was centrifuged at 14,600 × *g* at 4°C for 5 min and
217 the pellet was processed according to the Fast DNA SPIN kit for soil. The extracted DNA was
218 eluted in 50 µL of nuclease-free water and its concentration and quality were verified by agarose
219 gel electrophoresis. Relative abundances of *B. fibrisolvens* and *B. proteoclasticus* in rumen
220 liquor samples were measured by real-time qPCR, using total bacterial as reference (Denman
221 and McSweeny, 2005). The primers used in this study were obtained from the literature in order
222 to amplify partial 16S rRNA gene of total bacteria (Maeda et al. 2003), *B. fibrisolvens*
223 (Stevenson and Weimer 2007) and *B. proteoclasticus* (Paillard et al. 2007). For each primer
224 pair, reaction efficiencies were derived from a standard curve generated from a 5-fold serial
225 dilution of pooled DNA. RT-qPCR analysis was performed using a CFX96 Real-Time PCR
226 Detection System (Bio-Rad Laboratories, Hertfordshire, UK) in 20 µL total volume. For *B.*
227 *fibrisolvens* and total bacteria 0.5 ng of DNA was added to 10 µL of SSo Advanced Universal
228 SYBR Green Supermix (Bio-Rad) and 400 nM each primer. For *B. proteoclasticus* 35 ng of
229 DNA was added to 10 µL of SSo Advanced Universal Probes Supermix (Bio-Rad) containing
230 400 nM and 250 nM of molecular beacon. Amplification conditions were 95°C for 3 min, 40
231 cycles of 95°C for 15 s, 60°C (*B. fibrisolvens* and total bacteria) or 55°C (*B. proteoclasticus*)
232 for 30 s..To determine amplification specificity, following all non-probe based qPCR reactions,
233 a melting curve was constructed in the range of 60°C to 95°C. Cycle threshold values were
234 converted into normalized relative quantities (Q), corrected by PCR efficiency using Q-Gene

235 software (Simon, 2003). *B. fibrisolvens and B. proteoclasticus* 16S rRNA gene values were
236 expressed as percent of total eubacteria.

237 ***Statistical analysis of fatty acids data.***

238 All data (e.g., animal performance, milk composition and blood parameters) recorded over the
239 course of the experiment were processed as completely randomized design with repeated
240 measures using the MIXED procedure of SAS (SAS, 1999):

241 $y_{ijkl} = \mu + D_i + T_j + I_k(D) + (D \times T)_{ij} + e_{ijkl}$

242 where y_{ijkl} is the observation; μ is the overall mean; D_i the fixed effect of diet (i = 1 to 3); T_j the
243 fixed effect of sampling time (j = 1 to 4); I_k is the random effect of the ewe nested within the
244 diet (k = 1 to 6); $(D_i \times T)_{ij}$ the interaction between diet and sampling time and e_{ijkl} the residual
245 error. The covariance structure was compound symmetry, which was selected on the basis of
246 Akaike's information criterion of the mixed model of SAS. Statistical significance of the diet
247 effect was tested against variance of sheep nested within diet according to repeated measures
248 design theory (Littell et al. 1998).
249 Data of relative abundance of *B. fibrisolvens* and *B. proteoclasticus* were normalized by log10
250 transformation and checked for normal distribution by Shapiro-Wilk test (SAS, 1999). Data of
251 VFA and normalized data of microbial abundance were processed using one way analysis of
252 variance (SAS, 1999) with a model that included diet and experimental error.
253 $y_{ij} = \mu + D_i + e_{ij}$

254 where y_{ij} is the observation; μ is the overall mean; D_i the diet (i = 1 to 3) and e_{ij} the residual
255 error. Multiple comparisons among means were performed using the Tukey's test.

256 **RESULTS**

257 ***Animal performance, milk composition and blood parameters.***

During the experiment, the concentrate offered was quite completely consumed by the animals, irrespective to the treatments (nearly 760 g / head and day), allowing to obtain similar intake of soybean oil (nearly 63 g / head and day) and of tannin extracts for sheep from CHT and QUE groups (nearly 40 g / head and day). The average DMI of diet was 2.53 ± 0.07, 2.29 ± 0.19 and 2.25 ± 0.11 kg / head and day for group C, CHT and QUE respectively.

Dietary treatments did not affect milk yield, while several milk components (with the exception of total solid and the linear score) and rheological parameters (with the exception of the clotting time) varied significantly ($P < 0.01$) over the time as shown in Table 2.

Also blood parameters did not change across dietary treatments, but they did during the experimental period with the exception of glutamic transaminase SGPT and SGOT (Table 3). The interaction DxT was significant for total protein and GLB (table 3).

Rumen pH and fatty acid profile.

The average pH value of rumen liquor was not affected by dietary treatments and it was 6.69 ± 0.07. Compared to the control diet, CHT and QUE diets influenced rumen fermentation, as indirectly confirmed by the changes in VFA profile (Table 4). In particular, QUE induced a decrease of 2:0, 3:0, 5:0 and *iso* 5:0 ($P < 0.05$), whereas CHT enhanced acetic ($P < 0.05$) and butyric acid ($P < 0.01$; table 4) concentration.

Effect of tannins on B. fibrisolvens and B. proteoclasticus relative abundance.

Chestnut and quebracho tannin supplementation significantly affected the relative abundances of *B. fibrisolvens* and *B. proteoclasticus* in rumen liquor. The proportion of *B. fibrisolvens* ranged from 0.008 % and 0.057 % of total bacteria, whereas that of *B. proteoclasticus* ranged from 0.018 % and 0.380 %. In comparison to rumen liquor from ewes fed control diet, the presence of *B. fibrisolvens* increased about three times ($P < 0.001$) in ewes fed CHT and about five times ($P < 0.001$) in animals fed QUE diet (Table 4). On the contrary, the abundance of *B.*

proteoclasticus decreased about five ($P < 0.001$) and fifteen ($P < 0.001$) times in rumen liquor of ewes fed CHT and QUE diets, respectively (Table 4).

Fatty acid composition of milk.

The FA profile of milk has been modified by tannin inclusion in diets (Table 5). Milk PUFA content increased with CHT (+ 0.97 %) and QUE (+ 15.24 %) supplementation, but only QUE diet resulted in an increase of MUFA content (QUE + 3.96 %) and in a decrease of SFA content (QUE - 3.38 %). Data are shown in Table 5. Tannins increased LNA, LA, VA content in milk fat ($P < 0.001$), and this effect was more evident in the case of QUE supplementation that enhanced also CLA content. In comparison to both control and CHT diets, QUE diet resulted in a significant decrease ($P < 0.01$) of 18:0. On the other hand, the content of 18:0 was highest in milk from ewes fed CHT diet. Interestingly, the desaturation index (DI) was higher in milk fat from ewes fed QUE diet than in milk fat from the other two groups ($P < 0.01$). QUE diets increased both *cis*-9 18:1 (oleic acid, OA) and *cis*-12 18:1 content while *cis*-11 18:1 decreased ($P < 0.01$). *Trans-12* 18:1, in contrast, increased with CHT ($P < 0.01$). No differences were found among diets for *trans*-10 18:1 and *trans*-10, *cis*-12 18:2, the last one being present only in trace (data not showed). Both diets including tannins, moreover, lowered 14:0 ($P < 0.01$), *iso* 14:0 ($P < 0.05$), 16:0 ($P < 0.01$), *iso* 16:0 ($P < 0.05$) and increased *cis*-9 16:1 ($P < 0.01$), *cis*-9 17:1 ($P < 0.05$) concentration, but only CHT decreased *ante* 17:0 ($P < 0.01$). The amount of branched chain fatty acids significantly decreased in milk fat from ewes fed both QUE and CHT diets, in particular as regard *iso* 14:0, *iso* 15:0, *anteiso* 15:0, *iso* 16:0 and *anteiso* 17:0 (Table 5).

DISCUSSION

In the present trial the intake of nearly 40 g / head and day of chestnut or quebracho tannin extract had no detrimental effects on blood parameters neither on productive performances.

306 However, on the basis of the interaction D X T on total protein and GLB, further studies are
307 needed in order to assess if long-term supplementation of dietary tannins may significantly
308 affect the protein utilization by dairy sheep. These data are in accordance with Liu et al. (2011)
309 and Toral et al. (2011; 2013) that evaluated the effect of chestnut and quebracho tannins on
310 growth and productive performances in ewes. Literature reports that the condensed tannins are
311 not absorbed in the intestine and, hence, they are not able to interfere with the metabolism of
312 internal organs such as liver (McSweeney et al., 1988; Garg et al., 1992; Terrill et al., 1994).
313 On the other hand, several authors demonstrated that rumen microorganisms are able to degrade
314 hydrolysable tannins, whose toxicity seemed to be due to the absorption and to the accumulation
315 of phenols in blood stream, as a consequence of liver inability to completely detoxify them
316 (Murdiati, 1992; Makkar, 2003). In this trial, the hematic parameters of ewes showed that
317 hepatic functionality was not perturbed not only by QUE but also by CHT extract. Recently Liu
318 et al. (2013), in a feeding trial using dairy cows during the transition period, demonstrated that
319 chestnut tannins were able to reduce the oxidative status of liver and to decrease the
320 inflammatory status of mammary gland. Although literature reported that ellagic acid and
321 ellagitannins contained in fruits, nuts, seeds and woods (as chestnut tree) are metabolized in the
322 stomach and in the small intestine forming urolithins that are potent anti-inflammatory targeting
323 several tissues, including mammary gland (Cerda et al., 2005; Espin et al., 2007; Landete et al.,
324 2011), in the present study no effects were observed on milk somatic cell count. On the other
325 hand, somatic cell count is only an indirect marker of the inflammatory status of the mammary
326 gland.
327 In the current study both CHT and QUE diets showed a significant effect on rumen microbial
328 metabolism, as confirmed by the variation of several branched-chain fatty acids in milk, that
329 are an important diagnostic parameter for rumen microbial activity (Vlaemink et al., 2006;
330 Fiviez et al., 2012). Interestingly, the ratio between *iso* and *anteiso* odd branched chain FA,

331 which has been related to the cellulolytic bacteria growth (Vlaemink et al., 2006), showed a
332 similar trend to that observed for the acetic acid and total VFA concentrations, that increased
333 with CHT and decreased with QUE (table 4 and table 6). Several differences were observed,
334 also, in the molar proportion of rumen VFA among treatments, because CHT increased the
335 concentration of 2:0 and 4:0 while QUE diet decreased all VFA with the exception of 4:0.
336 Interestingly, according to Waghorn (2008), hydrolysable tannins contained in chestnut may be
337 metabolized in the rumen to gallic and ellagic acids, which may be further metabolized to acetic
338 and butyric acid. In the case of tannins from QUE diet, the strong effect on the VFA
339 concentration was probably due to the depressive effect of condensed tannins on both
340 carbohydrate and protein degradation, leading, in the last case, also to a reduction of VFA such
341 as valeric and *iso*-valeric acid, which origin from deamination of aminoacids (Patra and Saxena,
342 2011). However, previous studies reported controversial data concerning the effect of tannins
343 on total VFA or on their molar proportion in rumen liquor. Hervás et al. (2003b), Liu et al.
344 (2011), and Toral et al. (2011), in fact, found that tannins did not affect total VFA concentration
345 neither their molar proportion in rumen liquor from ewes, while Bhatta et al. (2009) found that
346 condensed tannins from mimosa reduced total VFA and increased the propionate production.
347 Probably these controversial results may be a consequence of the use of different dosages or
348 different kind of tannins and of associative effects between tannins and other ingredients of the
349 basal diet.
350 Data about the microbiologic characterization of rumen liquor showed that the presence of
351 tannins resulted in an increase of *B. fibrisolvens* relative abundance, whereas the *B.*
352 *proteoclasticus* population was strongly depressed, particularly in the case of QUE diet. These
353 data are in accordance with previous *in vivo* and *in vitro* studies (Vasta et al., 2010; Buccioni et
354 al., 2011) that reported a significant effect of CHT and QUE on rumen BH, favoring the
355 accumulation of VA and negatively affecting the *B. proteoclasticus* growth. Hence, the effect

of tannin extracts on milk FA profile, observed in the present experiment, could be due to the modulation of rumen BH as consequence of changes in microbial ecosystem. Nevertheless, some *in vivo* studies on rumen bacterial diversity in cows and ewes suggested that *B. fibrisolvens* and *B. proteoclasticus* do not play a dominant role in the rumen lipid metabolism and that other as-yet-uncultivated bacteria phylogenetically classified as *Prevotella*, *Lachnospinaceae* incertae sedis and unclassified *Bacteroidales*, *Clostridiales* and *Ruminococcaceae* might be more relevant (Boeckaert et al., 2008; Belenguer et al., 2010; Huws et al., 2011; Castro-Carrera et al. 2014). Literature also provides evidence that alterations in rumen out-flow of 18-carbon BH intermediates and 18:0, induced by diets based on different types of forages or supplemented with fish oil, are not accompanied by significant changes in *B. proteoclasticus* group (Kim et al., 2008; Huws et al., 2010; Halmemies-Beauchet-Filleau et al., 2013). Since diet composition plays a fundamental role in the selection of rumen microorganisms, further studies are needed in order to better clarify the effect of tannins on specific bacteria strains involved in BH processes. Moreover, data of the present experiment suggested also that CHT and QUE extracts differentially affected milk fatty acid composition not only by modulating BH process of LA. The SA content in milk fat, in fact, was significantly lower in samples from ewes fed QUE diet, whereas CHT diet resulted in the highest content of SA. At the same time, in comparison with control diet, OA content was higher in milk fat from ewes fed QUE diet and lower in the milk fat from ewes fed CHT diet. The content of SA (as well as that of OA, VA and *cis*-9, *trans*-11 CLA) in milk fat is strictly regulated by the uptake of mammary tissue and by Stearoyl Co-A desaturase enzyme (SCD) activity, which converts SA to OA and VA to *cis*-9, *trans*-11 CLA. In particular, nearly 50 % of OA and *cis*-9, *trans*-11 CLA secreted in sheep milk originates from the SCD activity (Frutos et al., 2014). As regard this last point, it is worth of attention that the 14:1 / 14:0 ratio, which is considered a proxy of Δ^9 desaturation in mammary gland (Mele et al., 2007), was significantly higher in milk fat from

ewes fed QUE diet, suggesting a positive effect of this kind of tannin extract on the activity of SCD, which in turn affected the ratio between substrate and products of the enzyme. Similar results have been reported also by Vasta et al. (2009b) in intramuscular fat of lambs fed green herbage with QUE tannin. However, whether condensed tannins affect directly or indirectly (for instance by modulating the substrate availability to the mammary gland) the activity of SCD enzyme needs further investigations.

Saturated FA content was significantly decreased by both tannin treatments, but the effect was more evident for QUE diet, probably due to the depressive effect of PUFA on milk fat synthesis in mammary gland (Shingfield et al., 2013). The PUFA content, in fact, was significantly higher in milk fat from ewes fed QUE diet, whereas that from ewes fed CHT diet was intermediate (Table 5). As regard FA involved in the rumen BH, QUE diet significantly enhanced the content of LA in milk fat, which accounted for more than 4% of total milk FA and also the intermediates of its BH (*cis*-9, *trans*-11 CLA and VA), which accounted for more than 3 % and 6 % of milk FA, respectively. Also *cis*-12 18:2, a putative intermediate of the rumen BH, was significantly, but marginally enhanced by QUE diet (table 5). Previous experiment based on the use of similar or higher amounts of soybean oil in the diet of dairy ewes (Mele et al., 2006; Gómez-Cortés et al., 2008) reported lower levels of VA and *cis*-9, *trans*-11 CLA in milk fat (similar to that found for the control diet in the present study), suggesting a significant effect of QUE tannins on the last step of rumen BH of LA (managed by *B. proteoclasticus*), which probably resulted in an increasing flux of VA (and maybe of *cis*-9, *trans*-11 CLA) to the mammary gland. Since *cis*-9, *trans*-11 CLA is mainly produced in mammary gland by delta-9 desaturation of VA (Bauman and Griinari, 2003), also *cis*-9, *trans*-11 CLA content significantly increased. In the case of CHT diet, the amount of LA and its rumen BH products in milk fat was intermediate between control and QUE treatments, suggesting a differential effect of hydrolysable tannins in comparison to condensed tannins, as showed also by the results of the relative quantification of

406 *B. fibrisolvens* and *B. proteoclasticus* populations in rumen fluid (Table 4). These results did
407 not agree with findings reported by Toral et al. (2011; 2013) who evaluated the effect of both
408 quebracho and chestnut tannins on milk FA composition in dairy ewes fed a diet rich in LA. In
409 both cases, authors reported that tannins were not able to affect BH of LA, as suggested by the
410 lack of significant changes in milk FA composition. In these experiments, however, the amount
411 of quebracho (58.98 g / head and day) or chestnut tannins (26.21 g / head and day) were
412 respectively higher and lower than that adopted in the present trial (nearly 40 g / head and day)
413 and the diets consisted of a TMR instead of *ad libitum* administration of hay as that adopted in
414 this experiment, suggesting that the effect of tannins on rumen BH could be dependent either
415 to the dose included in the diet and to the diet composition and to the physical form of the basal
416 diet. The content of *trans*-10 18:1 in milk fat did not differ across treatments and the content of
417 *trans*-10, *cis*-12 18:2 was under the limit of detection. Previous *in vitro* and *in vivo* studies
418 reported that tannins did not stimulate alternative rumen BH pathway of LA and, therefore, no
419 accumulation of *trans*-10 18:1 and 18:2 isomers has been reported (Vasta et al., 2009a; Buccioni
420 et al., 2011; Toral et al., 2011; Vasta et al., 2011). However, Toral et al. (2013) reported that
421 QUE tannins tended to increase *trans*-10 18:1 content in milk fat over time, suggesting that the
422 tannins effects should be evaluated also in long-term experiments.
423
424 **CONCLUSION**
425 The use of soybean oil in the diet of dairy ewes coupled with practical doses of quebracho or
426 chestnut tannin extracts resulted in significant changes of milk FA composition, without
427 affecting the other milk components, milk yield and hepatic functionality. In particular, QUE
428 diet seemed to be more efficient to disturb rumen BH of PUFA, increasing *cis*-9, *trans*-11 CLA
429 and VA content in milk fat. The perturbing effect of tannin extracts on rumen BH and, more in

general, on microbe metabolism was indirectly confirmed also by the results about the relative abundance of *B. fibrisolvens* and *B. proteoclasticus* populations and by the results about VFA molar proportion. However, dose-response studies are needed in order to elucidate the minimum amount of tannin extracts needed to obtain a reliable and reproducible effect on BH of LA, in order to maximize the enrichment of VA and *cis*-9, *trans*-11 CLA content in milk fat, using lower amounts of lipid supplementation. Finally, although both types of tannin extracts did not affect hepatic functionality or the mammary gland health, further studies considering long-term supplementation of hydrolysable and condensed tannins are needed, in order to confirm this result also over a longer time period.

AKNOWLEDGENTS

The authors thanks Dr Roberta Pastorelli of "Centro di Ricerca per l'Agrobiologia e la Pedologia, Consiglio per la Ricerca e la sperimentazione in Agricoltura - Firenze, Italy", Mr Antonio Pezzati and Mrs Doria Benvenuti of "Dipartimento di Scienze delle Produzioni Agro-alimentari e dell'Ambiente, Firenze, Italy" for their precious technical assistance in lab, and Mr Giuseppe Ponti, Mr Attilio Mazzocanti and Mr Giuseppe Covarelli of "Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Perugia, Italy for their technical assistance in animal management during the trial. This research has been financed by Universities of Florence, Pisa, Perugia and by Gruppo Mauro Saviola srl.

448 **REFERENCES**

449 AOAC International. 1995. Official Methods of Analysis, International. Gaitersburg, MD.

450 Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. Annual
451 Review of Nutrition. 23: 203-227.

452 Belenguer, A., P. G. Toral, P. Frutos, and G. Hervás. 2010. Changes in the rumen bacterial
453 community in response to sunflower oil and fish oil supplements in the diet of dairy sheep. J.
454 Dairy Sci. 93 : 3275–3286.

455 Bhatta, R., Y. Uyeno, K. Tajima, A. Takenaka, Y. Yabumoto, I. Nonaka, O. Enishi, and M.
456 Kurihara. 2009. Difference in the nature of tannins on in vitro ruminal methane and volatile
457 fatty acid production and on methanogenic archea and protozoal populations. J. Dairy Sci. 92:
458 5512-5522.

459 Boeckaert, C., B. Vlaeminck, V. Fievez, L. Maignien, J. Dijkstra, and N. Boon. 2008.
460 Accumulation of trans C18:1 fatty acids in the rumen after dietary algal supplementation is
461 associated with changes in the Butyrivibrio community. Appl. Env. Microbiol 74(22): 6923–
462 6930.

463 Buccioni, A., S. Rapaccini, M. Antongiovanni, S. Minieri, G. Conte, and M. Mele. 2010.
464 Conjugated linoleic acid (CLA) and C18:1 isomers content in milk fat of sheep and their
465 transfer to Pecorino Toscano D.O.P. cheese. Int. Dairy J. 20: 190–194.

466 Buccioni, A., S. Minieri, S. Rapaccini, M. Antongiovanni, and M. Mele. 2011. Effect of
467 chestnut and quebracho tannins on fatty acid profile in rumen liquid- and solid-associated
468 bacteria: an in vitro study. Anim. 5: 1521-1530.

469 Burns, R. E. 1963. Methods of tannin analysis for forage crop evaluation. Technical Bullettin
470 number 32. Georgia Agricultural Experiment Station, Athens, GA, USA.

471 Cannas, A., L. O. Tedeschi, D. G. Fox, A. N. Pell, and P. J. Van Soest. 2004. A mechanistic
472 model for predicting the nutrients requirements and feed biological values for sheep. J. Anim.
473 Sci. 82: 149–169.
474 Castro-Carrera, T., P. G. Toral, P. Frutos, N. R. McEwan, G. Hervás, L. Abecia, E. Pinloche,
475 S. E. Girdwood, and A. Belenguer. 2014. Rumen bacterial community evaluated by 454
476 pyrosequencing and terminal restriction fragment length polymorphism analyses in dairy sheep
477 fed marine algae. J. Dairy Sci. 97: 1661–16690.
478 Cerda B., F. A. Tomas-Barberan, and J. C. Espin. 2005. Metabolism of antioxidant and
479 chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in
480 humans:identification of biomarkers and individual variability. J. Agric. Food Chem. 53: 227-
481 235.
482 Chilliard, Y., A. Ferlay, R. M. Mansbridge, and M. Doreau. 2000. Ruminant milk fat plasticity:
483 nutritional control of saturated, polyunsaturated, trans and conjugated fatty acids. Ann.
484 Zootech. 49: 181–205.
485 Chilliard, Y., F. Glasser, A. Ferlay, L. Bernard, J. Rouel, and M. Doreau. 2007. Diet, rumen
486 biohydrogenation and nutritional quality of cow and goat milk fat. Eur. J. Lipid Sci. Technol.
487 109: 828-855.
488 Christie, W.W. 1982. A simple procedure for rapid *trans*-methylation of glycerolipids and
489 cholesterol esters. J. Lipid Res. 23: 1072-1075.
490 Contarini, G., M. Povolo, V. Pelizzola, L. Monti, and G. Lercker. 2013. Interlaboratory
491 evaluation of milk fatty acid composition by using different GC operating conditions. J. Food
492 Compos Anal. 32: 131-140.
493 Cruz-Hernandez, C., J. K. G. Kramer, J. Kraft, V. Santercole, M. Or-Rashid, Z. Deng, M. E. R.
494 Dugan, P. Delmonte, and M. P. Yurawecz. 2006. Systematic analysis of trans and conjugated

495 linoleic acids in the milk and meat of ruminants. Pages 45–93 in Advances in Conjugated
496 Linoleic Acid Research. Vol. 3. M. P. Yurawecz, J. K. G. Kramer, O. Gudmundsen, M. W.
497 Pariza, and S. Banni, ed. AOCS Press, Champaign, IL.
498 Denman, S. E., and C. S. McSweeny. 2005. Quantitative (real-time) PCR. Pages 105-118. In
499 Methods in gut microbial ecology for ruminants. Makkar, H. P. S., and C. S. McSweeney ed.
500 Springer, Dordrecht, Netherlands.
501 Destaillats, F., J. P. Trottier, J. M. G. Galvez, and P. Angers. 2005. Analysis of alpha-linolenic
502 acid biohydrogenation intermediates in milk fat with emphasis on conjugated linolenic acids. J.
503 Dairy Sci. 88: 3231-3239.
504 Espin, J. C., R. Gonzalez-Barrio, B. Cerda, C. Lopez-Bote, A. I. Rey, and F. A. Tomas-
505 Barberan. 2007. Iberian pig as model to clarify obscure points in the bioavailability and
506 metabolism of ellagitannins in humans. J. Agric. Food Chem. 55: 10476-10485.
507 Fievez, V., E. Colman, J. M. Castro-Montoya, I. Stefanov, and B. Vlaeminck. 2012. Milk odd-
508 and branched-chain fatty acids as biomarkers of rumen function – an update. Anim. Feed Sci.
509 Technol. 172: 51–65.
510 Folch, J., M. Lees, and G. H. Sloane Stanley.1957. A simple method for the isolation and
511 purification of total lipids from animal tissue. J. Biol. Chem. 226: 497-509.
512 Frutos, P., G. Hervás, F. J. Giráldez, and A. R. Mantecón. 2004a. Review. Tannins and ruminant
513 nutrition. Span. J. Agr. Res. 2: 191-202.
514 Frutos P., M. Raso, G. Hervás, A. R. Mantecón, V. Pérez, and F. J. Giráldez. 2004b. Is there
515 any detrimental effect when a chestnut hydrolysable tannin extract is included in the diet of
516 finishing lambs? Anim. Res. 53: 127-136.
517 Frutos P., P. G. Toral, E. Ramos-Morales, K. J. Shingfield, A. Belenguer, and G. Hervás. 2014.
518 Oral administration of cobalt acetate alters milk fatty acid composition, consistent with an
519 inhibition of stearoyl-coenzyme A desaturase in lactating ewes. J. Dairy Sci. 97: 1036-1046.

520 Garg, S. K., H. P. S. Makkar, K. B. Nagal, S. K. Sharma, D. R. Wadhwa, and B. Singh. 1992.
521 Toxicological investigations into oak (*Quercus incana*) leaf poisoning in cattle. Vet. Human
522 Toxicol. 34: 161–164.
523 Gómez-Cortés, P., P. Frutos, A. R. Mantecón, M. Juárez, M. A. De La Fuente, and G. Hervás.
524 2008. Milk production, conjugated linoleic acid content, and in vitro ruminal fermentation in
525 response to high levels of soybean oil in dairy ewe diet. J. Dairy Sci. 91: 1560-1569.
526 Halmemies-Beauchet-Filleau, A., P. Kairenius, S. Ahvenjärvi, L. K. Crosley, S. Muetzel, P.
527 Huhtanen, A. Vanhatalo, V. Toivonen, R. J. Wallace, and K. J. Shingfield. 2013. Effect of
528 forage conservation method on ruminal lipid metabolism and microbial ecology in lactating
529 cows fed diets containing a 60:40 forage-to-concentrate ratio. J. Dairy Sci. 96 : 2428–2447.
530 Hervás, G., V. Pérez, F. J. Giráldez, A. R. Mantecón, M. M. Almar, and P. Frutos. 2003a.
531 Intoxication of sheep with quebracho tannin extract. J. Compar. Pathol. 129: 44-54.
532 Hervás, G., P. Frutos, F. J. Giráldez, A. R. Mantecón, and M. C. Álvarez Del Pino. 2003b.
533 Effect of different doses of quebracho tannins extract on rumen fermentation in ewes. Anim.
534 Feed Sci. and Technol. 109: 65-78.
535 Huws, S. A., E. J. Kim, M. R. F. Lee, M. B. Scott, J. K. S. Tweed, E. Pinloche, R. J. Wallace,
536 and N. D. Scollan. 2011. As yet uncultured bacteria phylogenetically classified as Prevotella,
537 Lachnospiraceae incertae sedis and unclassified Bacteroidales, Clostridiales and
538 Ruminococcaceae may play a predominant role in ruminal biohydrogenation. Envir. Microbiol.
539 13(6): 1500–1512.
540 Huws, S. A., M. R. F. Lee, S. M. Muetzel, M. B. Scott, R. J. Wallace, and N. D. Scollan. 2010.
541 Forage type and fish oil cause shifts in rumen bacterial diversity. FEMS Microbiol Ecol. 73:
542 396–407.
543 IACUC. 2014. Istitutional Animal Care and Use Committee. University of Perugia.
544 www.unipg.it/files/pagine/115/regolamentoOPBApageweb.pdf.

ISO13366-2|IDF148-2. 2006. Milk-Enumeration of somatic cells – Part 2: Guidance on the operation of fluoro – opto- electronic counters.

Khiaosa-Ard, R., S.F. Bryner, M. R. L. Scheeder, H. R. Wettstein, F. Leiber, and M. Kreuzer. 2009. Evidence for the inhibition of the terminal step of ruminal α-linolenic acid biohydrogenation by condensed tannins. J. Dairy. Sci. 92: 177–188.

Kim, E. J., S. A. Huws, M. R. F. Lee, J. D. Wood, S. M. Muetzel, R. J. Wallace, and N. D. Scollan. 2008. Fish oil increases the duodenal flow of long chain polyunsaturated fatty acids and trans-11 18:1 and decreases 18:0 in steers via changes in the rumen bacterial community. J. Nutr. 138: 889-896.

Kramer, J. K. G., V. Fellner, M. E. R. Dugan, F.D. Sauer, M. M. Mossoba, and M. P. Yurawecz. 1997. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. Lipids. 32: 1219-1228.

Kramer, J. K. G., C. Cruz-Hernandez, Z. Y. Deng, J. Q. Zhou, G. Jahreis, and M. E. R. Dugan. 2004. Analysis of conjugated linoleic acid and trans 18:1 isomers in synthetic and animal products. Am. J. Clin. Nutr. 79: 1137S-1145S.

Kramer, K. G., M. Hernandez, C. Cruz-Hernandez, J. Kraft, and M. E. R. Dugan. 2008. combining results of two GC separations partly achieves determination of all cis and trans 16:1, 18:1, 18:2 and 18:3 except CLA isomers of milk fat as demonstrated using Ag-Ion SPE fractionation. Lipids. 43: 259–273.

Landete, J. M. 2011. Ellagitannins, ellagic acid and their derived metabolites: a review about source, metabolism, functions and health. Food Res. Int. 44: 1150-1160.

Littell, R. C., P. R. Henry and C. B. Ammerman. 1998. Statistical analysis of repeated measures data using SAS procedures. J. Anim. Sci. 76: 1216-1231.

568 Liu, H., V. Vaddella, and D. Zhou. 2011. Effect of chestnut tannins and coconut oil on growth
569 performance, methane emission, ruminal fermentation, and microbial populations in sheep. J.
570 Dairy Sci. 94: 6069-6077.
571 Liu, H. W., D. W. Zhou, and K. Li. 2013. Effects of chestnut tannins on performance and
572 antioxidative status of transition dairy cows. J. Dairy Sci. 96: 5901–5907.
573 Maeda, H., C. Fujimoto, Y. Haruki, T. Maeda, S. Kokeguchi, M. Petelin, H. Arai, I. Tanimoto,
574 F. Nishimura, and S. Takashiba. 2003. Quantitative real-time PCR using TaqMan and SYBR
575 Green for Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella
576 intermedia, tetQ gene and total bacteria. FEMS Immunol. Med. Microbiol. 39: 81-86.
577 Makkar, H. P. S. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins,
578 and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rum. Res.
579 49: 241–256.
580 McSweeney, C. S., P. M. Kennedy, and A. John. 1988. Effect of ingestion of hydrolysable
581 tannins in Terminalia oblongata on digestion in sheep fed Stylosanthes hamata. Aust. J. Agric.
582 Res. 39: 235–244.
583 Mele, M., A. Buccioni, F. Petacchi, A. Serra, S. Banni, M. Antongiovanni, and P. Secchiari.
584 2006. Effect of forage/concentrate ratio and soy bean oil supplementation on milk yield and
585 composition from Sarda ewes. Anim. Res. 55: 273-285.
586 Mele, M., G. Conte, B. Castiglioni, S. Chessa, N. P. P. Macciotta, A. Serra, A. Buccioni, G.
587 Pagnacco, and P. Secchiari. 2007. Stearoyl-coenzyme a desaturase gene polymorphism and
588 milk fatty acid composition in Italian Holsteins. J. Dairy Sci. 90: 4458-4465.
589 Mele, M., A. Serra, A. Buccioni, G. Conte, A. Pollicardo, and P. Secchiari. 2008. Effect of
590 soybean oil supplementation on milk fatty acid composition from Saanen goats fed diets with
591 different forage:concentrate ratios. Ital. J. Anim Sci. 7: 297-311.

592 Mele, M. 2009. Designing milk fat to improve healthfulness and functional properties of dairy
593 products: From feeding strategies to a genetic approach. Ital. J. Anim. Sci. 8(2): 365-373.
594 Murdiati, T. B. 1992. Metabolism in sheep of garlic acid, tannic acid and hydrolysable tannin
595 from Terminalia oblongata. Aust. J. Agric. Res. 43: 1307-1319.
596 Paillard, D., N. McKain, M. T. Rincon, K. J. Shingfield, D. I. Givens, and R. J. Wallace. 2007.
597 Quantification of ruminal Clostridium proteoclasticum by real-time PCR using a molecular
598 beacon approach. J. Appl. Microbiol. 103: 1251-1261.
599 Patra, A. K., and J. Saxena. 2011. Exploitation of dietary tannins to improve rumen metabolism
600 and ruminant nutrition. J. Sci. Food Agric. 91: 24-37.
601 Reed, J. D. 1995. Nutritional toxicology of tannins and related polyphenols in forages legumes.
602 J. Anim. Sci. 73: 1516-1528.
603 SAS Institute, 1999. SAS User's Guide: Statistics. Version 8.0. SAS Institute Inc (Ed.), Cary,
604 NC.
605 Shingfield, K. J., M. Bonnet, and N. D. Scollan. 2013. Recent developments in altering the fatty
606 acid composition of ruminant-derived foods. Animal. 7: 132–162.
607 Shook, G. E. 1993. Genetic improvement of mastitis through selection on somatic cell count.
608 Vet. Clin. North Am. Food Anim. Pract. 9: 563–58.
609 Simon, P. 2003 Q-Gene: processing quantitative real-time RT-PCR data. *Bioinformatics*. 19:
610 1439-40.
611 Stevenson, D. M., and P. J. Weimer. 2007. Dominance of Prevotella and low abundance of
612 classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-
613 time PCR. Appl Microbiol Biotechnol 75, 165–174.
614 Terrill, T. H., G. C. Waghorn, D. J. Woolley, W. C. McNabb, and T. N. Barry 1994. Assay and
615 digestion of 14C-labelled condensed tannins in the gastrointestinal tract of sheep. Br. J. Nutr.
616 72: 467–477.

617 Toral, P., G. Hervàs, E. Bichi, A. Belenguer, and P. Fruitos. 2011. Tannins as feed additives to
618 modulate ruminal biohydrogenation: Effects on animal performance, milk fatty acid
619 composition and ruminal fermentation in dairy ewes fed a diet containing sunflower oil. Anim.
620 Feed Sci. Technol. 164: 199-206.
621 Toral, P., G. Hervàs, E. Bichi, A. Belenguer, and P. Fruitos. 2013. Effect of the inclusion of
622 quebracho tannins in diet rich in linoleic acid on milk fatty acid composition in dairy ewes. J.
623 Dairy Sci. 96: 431-439.
624 Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fibre, neutral
625 detergent fibre, and no starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:
626 3583–3597.
627 Vasta, V., M. Mele, A. Serra, M. Scerra, G. Luciano, M. Lanza, and A. Priolo. 2009a. Metabolic
628 fate of fatty acids involved in ruminal biohydrogenation in sheep fed concentrate or herbage
629 with or without tannins. J. Anim. Sci. 87: 2674-2684.
630 Vasta, V., A. Priolo, M. Scerra, K. G. Hallett, J. D. Wood, and O. Doran. 2009b. Δ9 desaturase
631 protein expression and fatty acid composition of longissimus dorsi muscle in lambs fed green
632 herbage or concentrate with or without added tannins. Meat Sci. 82: 357–364.
633 Vasta, V., D. R. Yanez-Ruiz, M. Mele, A. Serra, G. Luciano, M. Lanza, L. Biondi, and A.
634 Priolo. 2010. Bacterial and protozoal communities and fatty acid profile in the rumen of sheep
635 fed a diet containing added tannins. Appl. Envir. Microbiol. 76 (8): 2549-2555.
636 Vasta, V., and G. Luciano. 2011. The effect of dietary consumption of plants secondary
637 compounds on small ruminants' product quality. Small rumin. Res. 101: 150-159.
638 Vlaemink, B., V. Fievez, S. Tamminga, R. J. Dewhurst, A. van Vuuren, D. De Brabander, and
639 D. Demeyer. 2006. Milk odd and branched chain fatty acids in relation to the rumen
640 fermentation pattern. J. Dairy Sci. 89: 3954-3964.

641 Waghorn, G. 2008. Beneficial and detrimental effects of dietary condensed tannins for
642 sustainable sheep and goat production-Progress and challenges. Anim. Feed Sci. Technol. 147:
643 116-139.
644 Zannoni, M., and S. Annibaldi. 1981. Standardization of the rennet ability of milk by
645 Formagraph-I. Sci. Tecn. Latt. Cas. 32: 79–94.
646

647 Table 1. Ingredients, chemical composition and fatty acids profile of the experimental
648 concentrates and of the hay and rolled barley administered to the ewes.

			Experimental concentrates [1]		
	Grass hay	Rolled barley	Control	CHT	QUE
Ingredients (g / kg of dry matter)					
Barley			213.8	213.8	213.8
Corn			211.3	211.3	211.3
Wheat bran			158.5	158.5	158.5
Soybean meal (44 CP)			126.8	126.8	126.8
Beet pulp			89.8	89.8	89.8
Soybean oil[2]			84.5	84.5	84.5
Bentonite			52.8	-	-
Chestnut tannin extract[3]			-	52.8	-
Quebracho tannin extract[4]			-	-	52.8
Molasses			41.3	41.3	41.3
$CaCO_3$			10.6	10.6	10.6
Sodium bicarbonate			5.3	5.3	5.3
Di-calcium phosphate			5.3	5.3	5.3
Chemical composition (g / kg of DM)					
Organic matter	847.0	859.9	816.9	858.1	869.6
Crude Protein	111.2	121.0	165.6	173.7	170.3
Ether extract	12.0	16.1	109.4	105.4	102.4
NDF	636.4	134.1	174.7	181.4	172.1
ADF	501.3	54.2	77.6	72.4	74.3
ADL	105.7	14.9	10.6	13.3	8.7
Ash	69.6	21.0	84.6	39.9	39.4
ME (MJ / kg DM)	7.8	9.9	13.1	14.1	14.1
NEl (Mcal / kg DM)	0.9	1.2	2.0	2.1	2.1
Fatty acids (g / 100g of total fatty acids)					
16:0	35.5	18.2	14.0	14.4	14.9
18:0	5.8	4.6	3.6	3.4	3.4
18:1 cis-9	9.3	21.2	23.3	22.9	22.0
18:2 n-6	28.5	45.0	51.4	51.7	51.8
18:3 n-3	2.8	6.0	5.8	5.6	5.8
others	18.1	4.9	1.9	2.0	2.1

649 [1]CHT: chestnut tannin extract; QUE: quebracho tannin extract.

650 [2]Fatty acid profile of soybean oil (g / 100g of total fatty acids): 16:0, 11.01; 18:0, 3.6; *cis*9 18:1,
651 22.09; *cis*9 *cis*12 18:2, 53.7; *cis*9 *cis*12 *cis*15 18:3, 7.2.

652 [3]Hydrolysable tannins extracted from Chestnut wood (*Castanea sativa)* containing 750 g of
653 equivalent tannic acid/kg DM (provided by Gruppo Mauro Saviola srl Radicofani, Siena, Italy).

654 [4]Condensed tannins extracted from quebracho (*Schinopsis lorentzii*) containing 456 g of
655 equivalent tannic acid/kg DM (provided by Guido Lapi spa, Castel Franco di Sotto, Pisa, Italy).
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676 Table 2. Milk yield and composition from ewes fed 800 g / head / d of a concentrate containing
677 84 g of soybean oil / kg DM plus 0 (control diet) or 52.8 g / kg DM of a chestnut tannin extract
678 (CHT diet) or 52.8 g / kg of DM of quebracho tannin extract (QUE diet).

Item		Diet				P value[1]		
		Control	CHT	QUE	SEM[4]	D	T	D x T
Milk yield	g / d	710	837	800	80.0	0.2912	0.0923	0.3510
Milk composition g / 100 g								
Fat		7.20	7.15	7.26	0.510	0.3403	<0.0010	0.9810
Lactose		4.78	4.69	4.81	0.100	0.4002	<0.0010	0.9815
Protein		6.15	6.41	6.22	0.161	0.5710	<0.0010	0.9823
Casein		4.91	5.07	5.00	0.132	0.0210	<0.0010	0.7903
Urea	mg / dl	31.39	34.18	33.26	2.790	0.9612	<0.0010	0.3721
Total solids	g / d	129	150	145	15.0	0.7512	0.7700	0.3699
Casein Index[2]		79.83	79.03	80.54	0.563	0.0211	<0.0010	0.3711
Linear Score[3]		4.20	3.43	3.77	1.354	0.7600	0.5913	0.1415
Clotting parameters								
r	min	20'14"	21'07"	19'38"	3'23"	0.3722	0.7510	0.7913
k_{20}	"	1'55"	1'53"	1'33"	0'23"	0.1411	<0.0010	0.2312
a_{30}	mm	39.37	36.57	42.01	10.631	0.4300	0.0030	0.9910

[1] Probability of significant effect due to experimental factors: diet (D), time (T), and their interaction (D X T);

[2] Casein index: total casein / total protein *100.

[3] Linear Score = $\log_2$(SCC / 12,500) where SCC is Somatic Cell Count.

[4] Standard Error Mean

Table 3. Blood parameters from ewes fed 800 g / head / d of a concentrate containing 84 g of soybean oil / kg DM plus 0 (control diet) or 52.8 g / kg DM of a chestnut tannin extract (CHT diet) or 52.8 g / kg of DM of quebracho tannin extract (QUE diet).

					P value [3]		
Item[1]	**Control**	**CHT**	**QUE**	**SEM[2]**	**D**	**T**	**D X T**
P tot g / dl	7.83	7.44	7.61	0.133	0.1686	< 0.0001	0.0295
Urea mg / dl	53.12	58.05	55.33	2.590	0.4609	< 0.0001	0.1268
ALB g / dl	4.03	3.89	3.95	0.044	0.1571	< 0.0001	0.0694
GLB g / dl	3.80	3.54	3.66	0.148	0.4460	< 0.0001	0.0091
γ-GT U / l	56.99	61.40	63.87	4.200	0.5196	< 0.0001	0.6808
SGPT U / l	20.33	16.99	19.37	2.520	0.6781	0.4411	0.4904
SGOT U l	121.24	132.06	159.79	25.842	0.5691	0.7841	0.3892

[1] P tot: total protein; ALB: albumine; GBL: globuline; γ-GT: γ-glutamil-transferase; SGPT: serum glutamic-pyruvic-transaminase; SGOT: serum glutamic-oxaloacetic-transaminase.

[2] Standard Error Mean

[3] Probability of significant effect due to experimental factors: diet (D), time (T), and their interaction (D X T).

Table 4. Effect of tannins on volatile fatty acid (VFA) composition and relatives abundances of *Butyrivibrio* species in rumen liquor at the end of the experimental period from ewes fed 800 g / head / d of a concentrate containing 84 g of soybean oil / kg DM plus 0 (control diet) or 52.8 g / kg DM of a chestnut tannin extract (CHT diet) or 52.8 g / kg of DM of quebracho tannin extract (QUE diet).

VFA (mM)	C	CHT	QUE	SEM[1]	P value[2]
2:0	33.63 b	43.50 a	26.91 c	2.967	0.0138
3:0	8.36 a	8.30 a	5.49 b	0.958	0.0246
iso 4:0	0.53	0.34	0.38	0.115	0.4910
4:0	6.71 b	19.18 a	6.06 b	2.514	0.0039
5:0	0.59 a	0.46 a	0.29 b	0.094	0.0290
iso 5:0	1.60 a	1.63 a	1.08 b	0.205	0.0385
total VFA	50.89 b	73.07 a	39.82 c	4.421	0.0206
[3] Population, (log10 of % 16S rRNA gene of total eubactéria)					
B. fibrisolvens	-1.96 c (0.011)	-1.48 b (0.034)	-1.27 a (0.054)	0.047	< 0.0001
B. proteoclasticus	-0.47 a (0.339)	-1.12 b (0.075)	-1.66 c (0.022)	0.048	< 0.0001

[1] Standard Error Mean.

[2] Probability of significant effect due to experimental factors: diet (D); means within a row with different letters differ ($P < 0.05$).

[3] In brackets the observed values

Table 5. Fatty acid composition of milk from sheep fed 800 g / head / d of a concentrate containing 84 g of soybean oil / kg DM plus 0 (control diet) or 52.8 g / kg DM of a chestnut tannin extract (CHT diet) or 52.8 g / kg of DM of quebracho tannin extract (QUE diet).

	Diet				P value[2]		
Fatty acid g/100g FA	Control	CHT	QUE	SEM[1]	D	T	D x T
4:0	3.10	3.27	3.33	0.061	0.1936	0.1597	0.1261

6:0	2.52	2.56	2.52	0.041	0.2267	0.3713	0.2589
8:0	2.60	2.58	2.60	0.024	0.3642	0.2912	0.1997
10:0	4.91	4.75	4.90	0.070	0.1952	0.1953	0.1874
cis-9 10:1	0.15 b	0.15 b	0.17 a	0.002	0.0001	0.2374	0.1345
11:0	0.03	0.03	0.03	0.001	0.2787	0.3462	0.2359
12:0	2.79 a	2.63 b	2.81 a	0.034	0.0017	0.2489	0.6389
cis-9 12:1	0.02	0.02	0.02	0.001	0.3318	0.3218	0.4273
iso 13:0	0.02	0.02	0.02	0.001	0.1367	0.4281	0.2174
anteiso 13:0	0.02	0.02	0.02	0.002	0.2784	0.0927	0.2738
13:0	0.04	0.04	0.04	0.001	0.6324	0.1092	0.2531
iso 14:0	0.07 a	0.06 b	0.06 ab	0.002	0.0417	0.2849	0.3148
14:0	8.91 a	8.32 c	8.77 b	0.056	0.0001	0.0432	0.6382
iso 15:0	0.17 a	0.15 b	0.16 a	0.010	0.3281	0.1728	0.4281
cis-9 14:1	0.15 b	0.14 b	0.17 a	0.003	0.0001	0.0821	0.8372
anteiso 15:0	0.32 a	0.30 b	0.31 ab	0.003	0.0001	0.0398	0.5428
15:0	0.80 b	0.80 b	0.82 a	0.005	0.0269	0.1729	0.8425
iso 16:0	0.20 a	0.18 b	0.18 b	0.006	0.0258	0.1856	0.3217
16:0	23.70 a	23.30 b	22.81 c	0.091	0.0001	0.0362	0.2602
cis-9 16:1	0.63 b	0.66 b	0.71 a	0.007	0.0001	0.3278	0.1621
iso 17:0	0.28	0.28	0.28	0.002	0.3866	0.1930	0.2817
anteiso 17:0	0.30 a	0.29 b	0.31 a	0.004	0.0067	0.2036	0.4238
17:0	0.56	0.57	0.55	0.005	0.0948	0.1967	0.7362
cis-9 17:1	0.15 b	0.15 ab	0.16 a	0.004	0.0405	0.2018	0.3172
18:0	10.58 b	11.51 a	9.37 c	0.118	0.0001	0.0237	0.8632
trans-6,8 18:1	0.60	0.63	0.57	0.018	0.0984	0.2934	0.3218
trans-9 18:1	0.69	0.68	0.66	0.017	0.5754	0.3281	0.5872
trans-10 18:1	0.99	0.90	0.89	0.034	0.1265	0.2837	0.4875
trans-11 18:1	6.03 c	6.45 b	6.86 a	0.096	0.0001	0.3822	0.2943
trans-12 18:1	0.72 b	0.75 a	0.61 c	0.015	0.0001	0.2873	0.1642
cis-9 18:1	18.67 b	18.46 c	19.18 a	0.094	0.0001	0.0942	0.3284
cis-11 18:1	0.24 b	0.24 a	0.21 c	0.01	0.0307	0.3298	0.2741
cis-12 18:1	0.46 ab	0.45 b	0.48 a	0.005	0.0008	0.2832	0.2418
cis-9, *cis*-12 18:2	3.82 c	3.93 b	4.18 a	0.033	0.0001	0.1927	0.1426
cis-9, *cis*-12, *cis*-15 18:3	0.61 c	0.63 b	0.71 a	0.007	0.0001	0.4321	0.2641
20:0	0.29 b	0.31 a	0.28 b	0.004	0.0055	0.3291	0.2648
cis-9, *trans*-11 18:2	2.81 b	2.76 b	3.49 a	0.046	0.0001	0.0823	0.3281
cis-11 20:1	0.12	0.05	0.06	0.034	0.3282	0.1825	0.5829
cis-11, cis-14, trans-14 20:3	0.14	0.14	0.14	0.002	0.2176	0.2853	0.1628
22:0	0.16	0.17	0.16	0.003	0.0055	0.3291	0.2648
cis-9 22:1	trace	trace	trace	-	-	-	-
24:0	0.05	0.05	0.04	0.005	0.4826	0.2836	0.2749
SFA[3]	61.46 a	61.17 a	59.38 b	0.096	0.0312	0.0237	0.0731
MUFA[4]	29.60 b	29.68 b	30.77 a	0.090	0.6201	0.0390	0.0866

PUFA[5]	7.39 c	7.46 b	8.51 a	0.049	0.0199	0.0173	0.0974
OIAR[6]	0.72	0.74	0.71	0.015	0.0431	0.1932	0.1516
DI [7]	0.016 b	0.016 b	0.019 a	0.001	0.0009	0.2937	0.2130
< 16:0[8]	37.68 a	36.76 b	37.44 a	0.092	0.0431	0.0913	0.2331
> 16:0[9]	61.76 c	62.60 a	62.23 b	0.077	0.0379	0.1279	0.3960

721 [1] Standard Error Mean.

722 [2] Probability of significant effect due to experimental factors: diet (D), time (T), and their

723 interaction (D X T); means within a row with different letters differ ($P < 0.05$).

724 [3] SFA: saturated fatty acids

725 [4] MUFA: monounsaturated fatty acids

726 [5] PUFA: polyunsaturated fatty acids

727 [6] Ratio odd-*iso* to odd-*anteiso* FA: (*iso* 15:0 + *iso* 17:0) / (*anteiso* 15:0 + *anteiso* 17:0)

728 [7] Desaturation index, DI = (*cis*-9 14:1 / 14:0 + *cis*-9 14:1).

729 [8] < 16:0 de novo fatty acids calculated according to Fiviez et al 2012.

730 [9] > 16:0 preformed fatty acids calculated according to Chilliard et al., 2000 and Fiviez et al.,

731 2012