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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

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ABSTRACT

The aim of the study was to evaluate milk fatty acid (FA) profile, animal performance, 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 d in milking were allotted into 3 experimental groups. Diets were characterized by chopped grass hay administered ad libitum and by 800 g/head and day of 3 experimental concentrates containing 84.5 g of soybean oil/kg of dry matter (DM) and 52.8 g/kg of DM of bentonite (control diet), chestnut tannin extract (CHT diet), or quebracho tannin extract (QUE diet). The trial lasted 4 wk. Milk yield was recorded daily, and milk composition and blood parameters were analyzed weekly. At the end of the experiment, samples of rumen fluid were collected to analyze pH, volatile fatty acid profile, and the relative proportions of *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* in the rumen microbial population. Hepatic functionality, milk yield, and gross composition were not affected by tannin extracts, whereas milk FA 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 + 8.71% 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 versus hydrolyzable tannins to interfere with rumen microbial metabolism, as indirectly confirmed by changes in the relative proportions of *B. fibrisolvens* and *B. proteoclasticus* populations and by changes in the molar proportions of volatile fatty acids. The effect of the CHT diet on the milk FA profile and microbial species considered in this trial was intermediate between that of QUE and the control diet, suggesting a differential effect of condensed and hydrolyzable tannins on rumen microbes. Compared with control animals, the presence of *B. fibrisolvens* increased about 3 times in ewes fed CHT and about 5 times in animals fed QUE. In contrast, the abundance of *B. proteoclasticus* decreased about 5- and 15-fold 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: tannin, milk fatty acid, sheep, microbial population

INTRODUCTION

During the last decade, several efforts have been done to enhance the level of healthy FA in milk and dairy products with the aim of improving the nutritional quality of foods deriving from ruminants (Chilliard et al., 2007; Mele, 2009). This objective may be achieved by applying feeding strategies based on dietary supplementation with polyunsaturated marine or vegetables oils or oilseeds (Shingfield et al., 2013), 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 PUFA. Previous studies demonstrated that adding vegetable oils rich in linoleic acid (*cis*-9,*cis*-12 18:2; **LA**) oil in the diet of small ruminants increased the content of *cis*-9,*trans*-11 CLA and VA in milk fat 2- to 3-fold (Mele et al., 2006, 2008; Gómez-Cortés et al., 2008). However, because the extent of rumen biohydrogenation (**BH**) of PUFA is usually more than 80 to 90%, the amount of supplemented lipid needed to achieve an effective enhancement of *cis*-9,*trans*-11 CLA and VA in milk fat from sheep and goats ranges from 60 to 100 g/head and

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day, leading to an increase in feeding costs. Moreover, although small ruminants are less sensitive than dairy cows to milk fat depression syndrome, high levels of lipids coupled with low forage diets may induce a decrease in milk fat content (Bauman and Griinari, 2003; Shingfield et al., 2013). As a consequence, in the last years, increasing interest has been devoted to feed ingredients able to slow the extent of rumen BH of dietary PUFA to obtain significant accumulations of VA in the rumen and, thus, an increase of the transfer of this FA from rumen to duodenum and then to the mammary gland (resulting in an increase of *cis*-9,*trans*-11 CLA, which originates by mammary desaturation of VA), while using lesser amounts of lipid supplementation.

Several in vitro studies have demonstrated that tannins are able to interfere with rumen BH or methane production, according to their polyphenolic nature (Bhatta et al., 2009; Khiaosa-Ard et al., 2009; Buccioni et al., 2011). Moreover, ewes and cows fed diets containing less than 4% tannins on a DM basis resulted in higher retention of nitrogen and lower plasma urea concentrations, because of the ability of tannin to protect feed protein from rumen microbial degradation (Frutos et al., 2004a). The effect on rumen microorganism activity has been related to the ability of tannins to interfere with the membranes of rumen bacteria, binding enzymes or by deprivation of metal ions, such as iron (Patra and Saxena, 2011). Among bacterial species involved in BH processes of PUFA, *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* seem to be the most sensitive (Vasta et al., 2010), but specific studies on the effects of different types of tannins on rumen microbial population are still scarce. Moreover, results from in vitro and in vivo experiments often show conflicting results on the effect of tannins on the accumulation of BH intermediates in the rumen and on the productive response of the animal (Vasta et al., 2009a; Toral et al., 2011, 2013). This is probably due to differences in tannin species and percentage inclusion in the diet and to associative effects between tannins and other diet ingredients such as lipids.

The aim of the present study was to evaluate the effect of moderate amount (<2%) of chestnut or quebracho tannin extracts (hydrolyzable and condensed tannins, respectively) in diets supplemented with soybean oil on the milk FA profile and on the relative abundance of *B. fibrisolvens* and *B. proteoclasticus* in the rumen microbial community. Moreover, because sheep milk is mainly used for cheese making, a further objective of the present study was to evaluate the effect of tannin addition on the gross composition and clotting characteristics of milk. Finally, because tannins may exert a toxic effect in ruminants, causing necrosis of the liver and lesions in the digestive tract (Reed, 1995; Hervás et al., 2003a), this experiment studied the effect of these feeding strategies on blood parameters, with a special focus on indicators of hepatic function.

MATERIALS AND METHODS

Experimental Design

Animals. Eighteen multiparous Comisana ewes at 122 ± 6 DIM kept at the Experimental Section of the Department of Applied Biology, University of Perugia Italy, were allotted into 3 experimental groups, homogeneous for BW (68.1 ± 7.83 kg), and each group was kept in multiple pens (6 ewes for each pen). The trial lasted 4 wk after 15 d of adaptation to the feeding regimen. The handling of the animals was according to Institutional Animal Care and Use Committee of the University of Perugia. The ewes were milked twice daily at 0730 and 1730 h using a milking machine (43 kPa; 150 pulsations/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 1 kg of milk at 6.5% fat (Cannas et al., 2004). Diets were composed of chopped grass hay (particle size >4 cm in length) administered ad libitum and 800 g/head and day of a concentrate that contained 84.5 g of soybean oil/kg of DM and 52.8 g/kg of DM of bentonite (control diet, **CON**), 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 to obtain a tannin concentration in the diet of almost 1.6% of expected DMI. On the basis of results from previous studies in literature, this dose was considered safe for the animal and practical for farmers (Hervás et al., 2003a,b; Frutos et al., 2004a,b). The experimental concentrates were offered after each milking, and 100 g/head of rolled barley was offered during milking. Chestnut hydrolyzable tannins (750 g/kg DM of tannic acid equivalent; by Gruppo Mauro Saviola srl Radicofani, Siena, Italy) and extract of quebracho tannins (456 g/kg DM of tannic acid equivalent; 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 using a Cyclotec 1093 mill (PBI International, Milan, Italy) using a mesh size of 1 mm.

Table 1. Ingredients, chemical composition, and FA profile of the experimental concentrates and the hay and rolled barley administered to ewes

Item	Grass hay	Rolled barley	Experimental concentrate[1]		
			Control	CHT	QUE
Ingredient, g/kg of DM					
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
Dicalcium phosphate			5.3	5.3	5.3
Chemical composition, g/kg of DM (unless otherwise noted)					
OM	847.0	859.9	816.9	858.1	869.6
CP	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 of DM	7.8	9.9	13.1	14.1	14.1
NE_L, Mcal/kg of DM	0.9	1.2	2.0	2.1	2.1
FA, g/100 g of total FA					
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
18:2n-6	28.5	45.0	51.4	51.7	51.8
18:3n-3	2.8	6.0	5.8	5.6	5.8
Others	18.1	4.9	1.9	2.0	2.1

[1]CHT = chestnut tannin extract; QUE = quebracho tannin extract.

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

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

[4]Condensed tannins extracted from quebracho (*Schinopsis lorentzii*) containing 456 g of tannic acid equivalent/kg of DM (provided by Guido Lapi spa, Castel Franco di Sotto, Pisa, Italy).

Crude protein, ether extract, and ash were determined according to the AOAC methods 976.06, 920.39, and 942.05, respectively (AOAC International, 1995). Neutral detergent fiber, ADF, and lignin were determined according to Van Soest et al. (1991) using heat-stable amylase and sodium sulfite, and results were expressed inclusive of residual ash. Metabolizable energy and NE_L were 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 3 aliquots for analysis: the first aliquot was processed to assess fat, lactose, protein, and casein contents by using Milkoscan 6000 FT (Foss Electric, Hillerød Denmark), and total SCC according to ISO 13366-2/IDF 148-2 (ISO-IDF, 2006), by using a Fossomatic 5000 (Foss Electric) 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 using a Maspress apparatus (Foss Italia, Padua, Italy), according to Zannoni and Annibaldi (1981). The following rennet parameters were determined: clotting time (**r**)—the time from rennet addition to the beginning of coagulation; firming time ($\mathbf{k_{20}}$)—the time needed for the amplitude to reach 20 mm on the recording chart; and curd firmness ($\mathbf{a_{30}}$)—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 FAME were quantified using valeric acid (5:0) and nonadecanoic acid (19:0) methyl esters (Sigma Chemical Co.) as internal standards and identified by comparison of the relative retention times of FAME peaks from samples, with those of the standard mixture 37 Component FAME Mix (Supelco, Bellefonte, PA; 4:0–24:0) and individual *trans*-9 18:1 and *trans*-11 18:1 (Sigma-Aldrich, St. Louis, MO), individual *cis*-9,*trans*-11 18:2 (Matreya Inc., Pleasant Gap, PA), CLA mix standard (Sigma-Aldrich, St. Louis, MO), and published isomeric profiles (Kramer et al., 1997, 2004; Cruz-Hernandez et al., 2006). The 18:1 isomer elution sequence was performed according to Kramer et al. (2008). Moreover, a standard mix of α-linolenic acid (**α-LNA**) isomers (47792, Supelco, Bellefonte, PA) and of LA isomers (47791, Supelco) and published isomeric profiles (Destaillats et al., 2005) were used to identify the isomers of interest. Two bacterial acid methyl ester mixes (Supelco; Matreya, Pleasant Gap, PA) and individual standard for methyl ester of *iso* 14:0, *anteiso* 14:0, *iso* 15:0, and *anteiso* 17:0 (Larodan, Malmo, Sweden) were used to identify the branched FA profile. Inter- and intraassay coefficients of variation were calculated by using a reference standard butter (CRM 164, Community Bureau of Reference, Brussels, Belgium), and the detection threshold of FA was 0.01 g/100 g of FA (Contarini et al., 2013). All FA composition results are expressed in grams per 100 grams of FA.

Blood Sampling and Analysis. Samples of blood were collected from each animal at the end of every experimental week from the jugular vein. Blood was stored in tubes without anticoagulant, and serum was separated by centrifugation (5,000 × *g* for 30 min at 25°C). Total protein (colorimetric biuret method), urea (kinetic enzymatic method), albumin (**ALB**; colorimetric BCG method), γ-glutamyl-transferase (**γ-GT**; kinetic SZASZ-Tris method), serum glutamic-oxaloacetic-transaminase (**SGOT**; kinetic UV IFCC method), and serum glutamic-pyruvic-transaminase (**SGPT**; kinetic UV IFCC method) were detected using diagnostic kits (ASR01120, ASR01143, ASR0128012, ASR01194, ASR01229, ASR01219, Assel s.r.l., Rome Italy) with an auto blood-analyzer for hematology (Vegavet AMS, Analyzer Medical System, Rome, Italy). Globulin (**GBL**) content was estimated by the difference between total protein and albumin contents.

Rumen Sampling and Analysis. On the last day of the experimental period, animals were milked and given free access to their ration for 1 h, according to Toral et al. (2013). Then, feeds were removed and 3 h later, rumen liquor samples were collected from each ewe using a stomach tube connected to a manual pump. Immediately after collection, each sample of rumen liquor was measured for pH, partitioned into 2 amounts and stored at −80°C until analysis for total VFA content (10 mL) and microbiological assay (5 mL). The analysis of VFA (2:0, acetic; 3:0, propionic; 4:0, butyric; *iso* 4:0, isobutyric; 5:0, valeric; *iso* 5:0, isovaleric) of rumen liquor samples was performed by HPLC: a volume of 10 mL of rumen liquor was diluted with 0.1 *N* H_2SO_4 (1:1) and centrifuged (5,000 × *g* for 15 min at 4°C) to separate the liquid phase from the feed residuals. After, the liquid phase was microfiltered (SLMV033RS, 0.45-μm Millex-HV, Merck-Millipore, Billerica, MA) and the sample was directly injected in the HPLC apparatus using an Aminex 85 HPX-87 H ion exclusion column (300 mm × 7.8 mm; 9-μm particle size; Bio-Rad, Milan, Italy) kept at 40°C; the detection wavelength was 220 nm. The analyses were carried out applying an isocratic elution (flux 0.6 mL/min) with a 0.008 *N* H_2SO_4 solution as mobile phase; the injection loop was 20 μL. Individual VFA were identified using a standard solution of 4.50 mg/mL of acetic acid, 5.76 mg/mL of propionic acid, 7.02 mg/mL of butyric acid and isobutyric acid, 8.28 mg/mL of valeric acid and isovaleric acid in 0.1 *N* H_2SO_4 (338826, 402907, B103500, 58360, 75054, 129542, respectively; Sigma-Aldrich). Quantification was done using an external calibration curve based on the standards described above. Data were expressed in millimoles per liter.

DNA Extraction and Quantitative Real-Time PCR Analysis. Total DNA was extracted from 1 mL of rumen liquor using the Fast DNA SPIN kit for soil (Qbiogene, Carlsbad, CA) with some modifications. Briefly, each sample was thawed on ice, transferred to a 15-mL tube containing 4.5 mL of a buffer solution (150 m*M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, and 4% SDS) and incubated for 15 min at 70°C. The liquid was centrifuged at 200 × *g* at 4°C for 5 min. One milliliter of the supernatant was centrifuged at 14,600 × *g* at 4°C for 5 min, and the pellet was processed according to the Fast DNA SPIN kit for soil. The extracted DNA was eluted in 50 μL of nuclease-free water and its concentration and quality were verified by agarose gel electrophoresis. Relative abundances of *B. fibrisolvens* and *B. proteoclasticus* in rumen liquor samples were measured by real-time quantitative (**q**) PCR, using total bacterial DNA as reference (Denman and McSweeny, 2005). The primers used in this study were identified from the literature to amplify partial 16S rRNA gene of total bacteria (Maeda et al. 2003), *B. fibrisolvens* (Stevenson and Weimer 2007), and *B. proteoclasticus* (Paillard et al. 2007). For each primer pair, reaction efficiencies were derived from a standard curve generated from a 5-fold serial dilution of pooled

DNA. Real-time qPCR analysis was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hertfordshire, UK) in a total volume of 20 μL. For *B. fibrisolvens* and total bacteria, 0.5 ng of DNA was added to 10 μL of SSo Advanced Universal SYBR Green Supermix (Bio-Rad) and 400 n*M* each primer. For *B. proteoclasticus*, 35 ng of DNA was added to 10 μL of SSo Advanced Universal Probes Supermix (Bio-Rad) containing 400 n*M* of each primer and 250 n*M* of molecular beacon. Amplification conditions were 95°C for 3 min, 40 cycles of 95°C for 15 s, and 60°C (*B. fibrisolvens* and total bacteria) or 55°C (*B. proteoclasticus*) for 30 s. To determine amplification specificity, following all non-probe-based qPCR reactions, a melting curve was constructed in the range of 60°C to 95°C. Cycle threshold values were converted into normalized relative quantities, corrected by PCR efficiency using Q-Gene software (Simon, 2003). The *B. fibrisolvens and B. proteoclasticus* 16S rRNA gene values were expressed as percentage of total bacteria.

Statistical Analysis of FA Data

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

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

where y_{ijkl} is the observation; μ is the overall mean; D_i is the fixed effect of diet ($i = 1$ to 3); T_j is the fixed effect of sampling time ($j = 1$ to 4); I_k is the random effect of the ewe nested within the diet ($k = 1$ to 6); $(D \times T)_{ij}$ is the interaction between diet and sampling time; and e_{ijkl} is the residual error. The covariance structure was compound symmetry, which was selected based on Akaike's information criterion of the mixed model of SAS. Statistical significance of the diet effect was tested against variance of sheep nested within diet according to repeated measures design theory (Littell et al., 1998).

Data of relative abundance of *B. fibrisolvens* and *B. proteoclasticus* were normalized by $\log_{10}$ transformation and checked for normal distribution by the Shapiro-Wilk test (SAS Institute, 1999). Data of VFA and normalized data of microbial abundance were processed using one-way ANOVA (SAS Institute, 1999) with a model that included diet and experimental error:

$$y_{ij} = \mu + D_i + e_{ij},$$

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

RESULTS

Animal Performance, Milk Composition, and Blood Parameters

During the experiment, the concentrate offered was completely consumed by the animals, irrespective of treatment (~760 g/head and day), allowing similar intakes of soybean oil (~63 g/head and day) and tannin extracts for sheep in the CHT and QUE groups (~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 groups CON, CHT, and QUE, respectively.

Dietary treatments did not affect milk yield, whereas several milk components (except total solids and linear score) and rheological parameters (except clotting time) varied significantly ($P < 0.01$) over time, as shown in Table 2.

Blood parameters did not change across dietary treatments but they did change during the experimental period except for the glutamic transaminases SGPT and SGOT (Table 3). The diet × time interaction was significant for total protein and GLB (Table 3).

Rumen pH and FA Profile

The average pH value of rumen liquor was not affected by dietary treatments and was 6.69 ± 0.07. Compared with the control diet, the CHT and QUE diets influenced rumen fermentation, as indirectly confirmed by the changes in VFA profile (Table 4). In particular, QUE induced decreases in concentrations of acetic, propionic, valeric, and isovaleric acids ($P < 0.05$), whereas CHT enhanced concentrations of acetic ($P < 0.05$) and butyric acids ($P < 0.01$; Table 4).

Effect of Tannins on Relative Abundance of B. fibrisolvens and B. proteoclasticus

Supplementation with chestnut and quebracho tannins significantly affected the relative abundances of *B. fibrisolvens* and *B. proteoclasticus* in rumen liquor. The proportion of *B. fibrisolvens* ranged from 0.008 to 0.057% of total bacteria, whereas that of *B. proteoclasticus* ranged from 0.018 to 0.380%. Compared with rumen liquor from ewes fed CON, the presence of *B. fibrisolvens* increased about 3-fold ($P < 0.001$) in ewes fed CHT and about 5-fold ($P < 0.001$) in animals fed the QUE diet (Table 4). In contrast, the abundance of *B. proteoclasticus* decreased about 5-fold ($P < 0.001$) and 15-fold ($P < 0.001$) in rumen liquor of ewes fed CHT and QUE diets, respectively (Table 4).

Table 2. Milk yield and composition(g/100 g, unless otherwise noted) from ewes fed 800 g/head per day of a concentrate containing 84 g of soybean oil/kg of DM plus 0 (control diet), 52.8 g/kg of DM of a chestnut tannin extract (CHT diet), or 52.8 g/kg of DM of quebracho tannin extract (QUE diet)

	Diet				*P*-value[1]		
Item	Control	CHT	QUE	SEM	D	T	D × T
Milk yield, g/d	710	837	800	80.0	0.291	0.092	0.351
Milk composition							
Fat	7.20	7.15	7.26	0.510	0.340	<0.001	0.981
Lactose	4.78	4.69	4.81	0.100	0.400	<0.001	0.981
Protein	6.15	6.41	6.22	0.161	0.571	<0.001	0.982
Casein	4.91	5.07	5.00	0.132	0.021	<0.001	0.790
Urea, mg/dL	31.39	34.18	33.26	2.790	0.961	<0.001	0.372
Total solids, g/d	129	150	145	15.0	0.751	0.770	0.370
Casein index[2]	79.83	79.03	80.54	0.563	0.021	<0.001	0.371
Linear score[3]	4.20	3.43	3.77	1.354	0.760	0.591	0.141
Clotting parameters[4]							
r, min:s	20:14	21:07	19:38	3:23	0.372	0.751	0.791
k_{20}, min:s	1:55	1:53	1:33	0:23	0.141	<0.001	0.231
a_{30}, mm	39.37	36.57	42.01	10.63	0.430	0.003	0.991

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

[2]Casein index = total casein/total protein × 100.

[3]Linear score = $\log_2$(SCC/12,500).

[4]Where r = clotting time—the time from rennet addition to the beginning of coagulation; k_{20} = firming time—the time needed for the amplitude to reach 20 mm on the recording chart; and a_{30} = curd firmness—the amplitude of the trace 30 min after rennet addition.

FA Composition of Milk

The FA profile of milk was modified by inclusion of tannin in the diets (Table 5). Milk PUFA content increased with CHT (+0.97%) and QUE (+15.24%) supplementation, but only the QUE diet resulted in an increase of MUFA content (QUE +3.96%) and in a decrease in SFA content (QUE −3.38%). Data are shown in Table 5. Tannins increased LNA, LA, and VA contents in milk fat ($P < 0.001$), and this effect was more evident with QUE supplementation, which also enhanced CLA content. Compared with both CON and CHT diets, the 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 was higher in milk fat from ewes fed QUE diet than in milk fat from the other 2 groups ($P < 0.01$). Feeding the QUE diets increased both *cis*-9 18:1 (oleic acid) and *cis*-12 18:1 contents, whereas *cis*-11 18:1 decreased ($P < 0.01$). The content of *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 latter being present only in trace amounts (data not shown). Both diets including tannins, moreover, decreased concentrations of 14:0 ($P < 0.01$), *iso* 14:0 ($P < 0.05$), 16:0 ($P < 0.01$), and *iso* 16:0 ($P < 0.05$) and increased concentrations of *cis*-9 16:1 ($P < 0.01$) and *cis*-9 17:1 ($P < 0.05$); only the CHT diet decreased *anteiso* 17:0 ($P < 0.01$). The amount of branched-chain FA significantly

Table 3. Blood parameters from ewes fed 800 g/head per day of a concentrate containing 84 g of soybean oil/kg of DM plus 0 (control diet), 52.8 g/kg of 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]		
Item[1]	Control	CHT	QUE	SEM	D	T	D × T
P tot, g/dL	7.83	7.44	7.61	0.133	0.169	<0.001	0.023
Urea, mg/dL	53.12	58.05	55.33	2.590	0.461	<0.001	0.127
ALB, g/dL	4.03	3.89	3.95	0.044	0.157	<0.001	0.069
GLB, g/dL	3.80	3.54	3.66	0.148	0.446	<0.001	0.009
γ-GT, U/L	56.99	61.40	63.87	4.200	0.520	<0.001	0.681
SGPT, U/L	20.33	16.99	19.37	2.520	0.678	0.441	0.490
SGOT, U/L	121.24	132.06	159.79	25.842	0.569	0.784	0.389

[1]P tot = total protein; ALB = albumin; GBL = globulin; γ-GT = γ-glutamyltransferase; SGPT = serum glutamic-pyruvic-transaminase; SGOT = serum glutamic-oxaloacetic-transaminase.

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

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

	Diet				
Item	Control	CHT	QUE	SEM	*P*-value[1]
VFA (m*M*)					
2:0	33.63[b]	43.50[a]	26.91[c]	2.967	0.014
3:0	8.36[a]	8.30[a]	5.49[b]	0.958	0.025
iso 4:0	0.53	0.34	0.38	0.115	0.491
4:0	6.71[b]	19.18[a]	6.06[b]	2.514	0.004
5:0	0.59[a]	0.46[a]	0.29[b]	0.094	0.029
iso 5:0	1.60[a]	1.63[a]	1.08[b]	0.205	0.038
Total VFA	50.89[b]	73.07[a]	39.82[c]	4.421	0.021
Population[2]					
B. fibrisolvens	−1.96[c] (0.011)	−1.48[b] (0.034)	−1.27[a] (0.054)	0.047	<0.001
B. proteoclasticus	−0.47[a] (0.339)	−1.12[b] (0.075)	−1.66[c] (0.022)	0.048	<0.001

[a–c]Means within a row with different letters differ ($P < 0.05$).

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

[2]Log_{10} of % 16S rRNA gene of total bacteria (observed values in parentheses).

decreased in milk fat from ewes fed either the QUE or CHT diet, particularly that of *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 almost 40 g/head and day of chestnut or quebracho tannin extract had no detrimental effects on blood parameters or productive performance. However, based on the diet × time interaction on total protein and GLB, further studies are needed to assess if long-term supplementation of dietary tannins may significantly affect protein utilization by dairy sheep. These data are in accordance with Liu et al. (2011) and Toral et al. (2011, 2013), who evaluated the effects of chestnut and quebracho tannins on growth and productive performances in ewes. The literature reports that condensed tannins are not absorbed in the intestine and, hence, they are not able to interfere with the metabolism of organs such as liver (McSweeney et al., 1988; Garg et al., 1992; Terrill et al., 1994). On the other hand, several researchers have demonstrated that rumen microorganisms are able to degrade hydrolyzable tannins, the toxicity of which seems to be due to absorption and accumulation of phenols in the bloodstream because of the liver's inability to completely detoxify them (Murdiati, 1992; Makkar, 2003). In this trial, the blood parameters of ewes showed that hepatic functionality was not perturbed by QUE and by CHT extract. Recently, Liu et al. (2013), in a feeding trial using dairy cows during the transition period, demonstrated that chestnut tannins were able to reduce the oxidative status of liver and to decrease the inflammatory status of mammary gland. Although the literature reports that ellagic acid and ellagitannins contained in fruits, nuts, seeds, and woods (e.g., chestnut tree) are metabolized in the stomach and small intestine, forming urolithins that are potent antiinflammatory agents targeting several tissues, including mammary gland (Cerdá et al., 2005; Espín et al., 2007; Landete, 2011), no effects were observed on milk SCC in the present study. However, SCC is only an indirect marker of the inflammatory status of the mammary gland.

In the current study, both CHT and QUE diets showed a significant effect on rumen microbial metabolism, as confirmed by the variation in several branched-chain FA in milk, that are an important diagnostic parameter for rumen microbial activity (Vlaeminck et al., 2006; Fievez et al., 2012). Interestingly, the ratio between *iso* and *anteiso* odd branched-chain FA, which is related to growth of cellulolytic bacteria (Vlaeminck et al., 2006), showed a similar trend to that observed for the acetic acid and total VFA concentrations, that increased with CHT and decreased with QUE (Table 4 and 5). Several differences were observed, in the molar proportion of rumen VFA among treatments, because the CHT diet increased the concentrations of acetic and butyric acids, whereas the QUE diet decreased all VFA with the exception of butyric acid. Interestingly, according to Waghorn (2008), hydrolyzable tannins contained in chestnut may be metabolized in the rumen to gallic and ellagic acids, which may be further metabolized to acetic and butyric acids. In the case of tannins from the QUE diet, the strong effect on VFA concentration was probably due to the depressive effect of condensed tannins on both carbohydrate and protein degradation, leading, in the last case, to reductions of VFA such as valeric and isovaleric acids, which origin from deamination of amino acids (Patra and Saxena, 2011). However,

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

	Diet				*P*-value[1]		
FA, g/100 g of FA	Control	CHT	QUE	SEM	D	T	D × T
4:0	3.10	3.27	3.33	0.061	0.194	0.160	0.126
6:0	2.52	2.56	2.52	0.041	0.227	0.371	0.259
8:0	2.60	2.58	2.60	0.024	0.364	0.291	0.200
10:0	4.91	4.75	4.90	0.070	0.195	0.195	0.187
cis-9 10:1	0.15^{b}	0.15^{b}	0.17^{a}	0.002	<0.001	0.237	0.134
11:0	0.03	0.03	0.03	0.001	0.279	0.346	0.236
12:0	2.79^{a}	2.63^{b}	2.81^{a}	0.034	0.002	0.249	0.639
cis-9 12:1	0.02	0.02	0.02	0.001	0.332	0.322	0.427
iso 13:0	0.02	0.02	0.02	0.001	0.137	0.428	0.217
anteiso 13:0	0.02	0.02	0.02	0.002	0.278	0.093	0.274
13:0	0.04	0.04	0.04	0.001	0.632	0.109	0.253
iso 14:0	0.07^{a}	0.06^{b}	0.06^{ab}	0.002	0.042	0.285	0.315
14:0	8.91^{a}	8.32^{c}	8.77^{b}	0.056	<0.001	0.043	0.638
iso 15:0	0.17^{a}	0.15^{b}	0.16^{a}	0.010	0.328	0.173	0.428
cis-9 14:1	0.15^{b}	0.14^{b}	0.17^{a}	0.003	<0.001	0.082	0.837
anteiso 15:0	0.32^{a}	0.30^{b}	0.31^{ab}	0.003	<0.001	0.040	0.543
15:0	0.80^{b}	0.80^{b}	0.82^{a}	0.005	0.027	0.173	0.842
iso 16:0	0.20^{a}	0.18^{b}	0.18^{b}	0.006	0.026	0.186	0.322
16:0	23.70^{a}	23.30^{b}	22.81^{c}	0.091	<0.001	0.036	0.260
cis-9 16:1	0.63^{b}	0.66^{b}	0.71^{a}	0.007	<0.001	0.328	0.162
iso 17:0	0.28	0.28	0.28	0.002	0.387	0.193	0.282
anteiso 17:0	0.30^{a}	0.29^{b}	0.31^{a}	0.004	0.007	0.204	0.424
17:0	0.56	0.57	0.55	0.005	0.095	0.198	0.736
cis-9 17:1	0.15^{b}	0.15^{ab}	0.16^{a}	0.004	0.040	0.202	0.317
18:0	10.58^{b}	11.51^{a}	9.37^{c}	0.118	<0.001	0.028	0.863
trans-6,8 18:1	0.60	0.63	0.57	0.018	0.098	0.293	0.322
trans-9 18:1	0.69	0.68	0.66	0.017	0.575	0.328	0.587
trans-10 18:1	0.99	0.90	0.89	0.034	0.126	0.284	0.487
trans-11 18:1	6.03^{c}	6.45^{b}	6.86^{a}	0.096	<0.001	0.382	0.294
trans-12 18:1	0.72^{b}	0.75^{a}	0.61^{c}	0.015	<0.001	0.287	0.164
cis-9 18:1	18.67^{b}	18.46^{c}	19.18^{a}	0.094	<0.001	0.094	0.328
cis-11 18:1	0.24^{b}	0.24^{a}	0.21^{c}	0.01	0.031	0.330	0.274
cis-12 18:1	0.46^{ab}	0.45^{b}	0.48^{a}	0.005	0.001	0.283	0.242
cis-9,*cis*-12 18:2	3.82^{c}	3.93^{b}	4.18^{a}	0.033	<0.001	0.193	0.143
cis-9,*cis*-12,*cis*-15 18:3	0.61^{c}	0.63^{b}	0.71^{a}	0.007	<0.001	0.432	0.264
20:0	0.29^{b}	0.31^{a}	0.28^{b}	0.004	0.005	0.329	0.265
cis-9,*trans*-11 18:2	2.81^{b}	2.76^{b}	3.49^{a}	0.046	<0.001	0.082	0.328
cis-11 20:1	0.12	0.05	0.06	0.034	0.328	0.182	0.583
cis-11,*cis*-14,*trans*-14 20:3	0.14	0.14	0.14	0.002	0.218	0.285	0.163
22:0	0.16	0.17	0.16	0.003	0.005	0.329	0.265
cis-9 22:1	Trace	Trace	Trace	—	—	—	—
24:0	0.05	0.05	0.04	0.005	0.483	0.284	0.275
SFA	61.46^{a}	61.17^{a}	59.38^{b}	0.096	0.031	0.024	0.073
MUFA	29.60^{b}	29.68^{b}	30.77^{a}	0.090	0.620	0.039	0.087
PUFA	7.39^{c}	7.46^{b}	8.51^{a}	0.049	0.020	0.017	0.097
OIAR[2]	0.72	0.74	0.71	0.015	0.043	0.193	0.152
DI[3]	0.016^{b}	0.016^{b}	0.019^{a}	0.001	0.001	0.294	0.213
<16:0[4]	37.68^{a}	36.76^{b}	37.44^{a}	0.092	0.043	0.091	0.233
>16:0[5]	61.76^{c}	62.60^{a}	62.23^{b}	0.077	0.038	0.128	0.396

[a–c]Means within a row with different letters differ ($P < 0.05$).

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

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

[3]Desaturation index (*cis*-9 14:1/14:0 + *cis*-9 14:1).

[4]De novo fatty acids calculated according to Fievez et al. (2012).

[5]Preformed FA calculated according to Chilliard et al. (2000) and Fievez et al. (2012).

previous studies reported controversial data concerning the effect of tannins on total VFA or on their molar proportion in rumen liquor. Hervás et al. (2003b), Liu et al. (2011), and Toral et al. (2011), in fact, found that tannins did not affect total VFA concentration or their molar proportions in rumen liquor from ewes, whereas Bhatta et al. (2009) found that condensed tannins from mimosa reduced total VFA and increased production of

propionate. These controversial results may arise from the use of different dosages or different kind of tannins and of associative effects between tannins and other ingredients of the basal diet.

Data about the microbiologic characterization of rumen liquor showed that the presence of tannins resulted in an increase in relative abundance of *B. fibrisolvens*, whereas the *B. proteoclasticus* population was strongly depressed, particularly with the QUE diet. These data are in accordance with previous in vivo and in vitro studies (Vasta et al., 2010; Buccioni et al., 2011) that reported a significant effect of CHT and QUE on rumen BH, favoring the accumulation of VA and negatively affecting the growth of *B. proteoclasticus*. Hence, the effect of tannin extracts on the milk FA profile observed in the present experiment could be due to the modulation of rumen BH because of changes in the microbial ecosystem. Nevertheless, some in vivo studies on rumen bacterial diversity in cows and ewes suggest that *B. fibrisolvens* and *B. proteoclasticus* do not play a dominant role in rumen lipid metabolism and that other as-yet-uncultured 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). The literature also provides evidence that alterations in rumen outflow 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). Because diet composition plays a fundamental role in the selection of rumen microorganisms, further studies are needed to better clarify the effect of tannins on specific bacterial strains involved in BH processes. Moreover, data in the present experiment suggested that the CHT and QUE extracts affected milk FA composition in different ways, not only by modulating BH of LA. The SA content in milk fat, in fact, was significantly lower in samples from ewes fed the QUE diet, whereas the CHT diet resulted in the highest content of SA. At the same time, compared with that in CON, OA content was higher in milk fat from ewes fed QUE diet and lower in the milk fat from ewes fed the 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, almost 50% of OA and *cis*-9,*trans*-11 CLA secreted in sheep milk originates from SCD activity (Frutos et al., 2014). In regard to this last point, it is worth noting that the ratio of 14:1 to 14:0, which is considered a proxy of Δ^9-desaturation in the mammary gland (Mele et al., 2007), was significantly higher in milk fat from ewes fed the QUE diet, suggesting a positive effect of this type of tannin extract on the activity of SCD, which in turn affected the ratio between substrate and products of the enzyme. Similar results were reported 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 investigation.

Saturated FA content was significantly decreased by both tannin treatments, but the effect was more evident for the 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 the QUE diet, whereas that from ewes fed the CHT diet was intermediate (Table 5). As regards FA involved in the rumen BH, the QUE diet significantly enhanced the content of LA in milk fat, which accounted for more than 4% of total milk FA and enhanced the content of intermediates of its BH (*cis*-9,*trans*-11 CLA and VA), which accounted for more than 3 and 6% of milk FA, respectively. In addition, *cis*-12 18:2, a putative intermediate of rumen BH, was significantly but marginally enhanced by the QUE diet (Table 5). Previous experiments 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 in the last step of rumen BH of LA (managed by *B. proteoclasticus*), which probably resulted in an increased flux of VA (and maybe of *cis*-9,*trans*-11 CLA) to the mammary gland. Because *cis*-9,*trans*-11 CLA is mainly produced in the mammary gland by Δ^9-desaturation of VA (Bauman and Griinari, 2003), the content of *cis*-9,*trans*-11 CLA also significantly increased. In the case of the CHT diet, the amount of LA and its rumen BH products in milk fat were intermediate between control and QUE treatments, suggesting a differential effect of hydrolyzable tannins compared with condensed tannins, as also shown by the relative quantification of *B. fibrisolvens* and *B. proteoclasticus* populations in rumen fluid (Table 4). These results did not agree with findings reported by Toral et al. (2011, 2013), who evaluated the effect of both quebracho and chestnut tannins on milk FA composition in dairy ewes fed a diet rich in LA. In both studies, those authors reported that tannins were not able to affect BH of LA, as suggested by the lack of significant changes in milk FA composition.

In those experiments, however, the amount of quebracho (58.98 g/head and day) or chestnut tannins (26.21 g/head and day) were, respectively, higher and lower than that adopted in the present trial (~40 g/head and day), and the diets consisted of a TMR instead of ad libitum administration of hay (such as adopted in this experiment), suggesting that the effect of tannins on rumen BH could depend on the dose included in the diet, the diet composition, and the physical form of the basal diet. The content of *trans*-10 18:1 in milk fat did not differ across treatments, and the content of *trans*-10,*cis*-12 18:2 was below the limit of detection. Previous in vitro and in vivo studies reported that tannins did not stimulate alternative rumen BH pathway of LA and, therefore, no accumulation of *trans*-10 18:1 and 18:2 isomers has been reported (Vasta et al., 2009a; Buccioni et al., 2011; Toral et al., 2011; Vasta and Luciano, 2011). However, Toral et al. (2013) reported that QUE tannins tended to increase *trans*-10 18:1 content in milk fat over time, suggesting that the effects of tannins should be evaluated in long-term experiments.

CONCLUSIONS

The use of soybean oil in the diet of dairy ewes coupled with practical doses of quebracho or chestnut tannin extracts resulted in significant changes of milk FA composition without affecting other milk components, milk yield, or hepatic functionality. In particular, the QUE diet seemed to be more efficient in disturbing rumen BH of PUFA, increasing contents of *cis*-9,*trans*-11 CLA and VA in milk fat. The perturbing effect of tannin extracts on rumen BH, and on microbe metabolism generally, was indirectly confirmed by the relative abundance of *B. fibrisolvens* and *B. proteoclasticus* populations and by the VFA molar proportion results. However, dose–response studies are needed to elucidate the minimum amount of tannin extracts needed to obtain a reliable and reproducible effect of LA on BH to maximize the enrichment of VA and *cis*-9,*trans*-11 CLA contents in milk fat using lower amounts of lipid supplementation. Finally, although neither type of tannin extract affected hepatic functionality or mammary gland health, studies involving long-term supplementation of hydrolyzable and condensed tannins are needed to confirm this result over a longer period.

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