Diets supplemented with condensed and hydrolysable tannins affected rumen fatty acid profile and plasmalogen lipids, ammonia and methane production in an in vitro study

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ABSTRACT
The livestock sector constitutes 14.5% of global green-house gas (GHG) emissions and soil and water pollution due to nitrogen excretion. Methane and nitrogen excretions in ruminants can be mitigated by specific feeding strategies, and tannins reduce methanogenesis and ammonia synthesis. In our study, two kinds of condensed tannins (Mimosa and Gambier) and two kinds of hydrolysable tannins (Chestnut and Tara) were added (4 g/100 g DM) to a basal feed (barley: 48 g/100 g DM, wheat bran: 23 g/100 g DM, dehydrated alfalfa hay: 15 g/100 g DM, soybean meal: 10 g/100 g DM and molasses: 2 g/100 g DM), inoculated with rumen fluid and fermented for 24 h. The methane, ammonia, fatty acid and plasmalogen lipid profile were determined. The results confirmed that tannins are an important family of heterogeneous compounds whose effect on rumen metabolism is strongly linked to their different characteristics. Chestnut tannin extract was shown to be a good compromise. It improved the sustainability of ruminant rearing by decreasing methanogenesis (control feed 0.159 vs chestnut feed 0.137 mmol/L rumen fluid; \( p = .0326 \)) and ammonia production (control feed 248 vs chestnut feed 179 mg/L rumen fluid; \( p < .0001 \)) and enhancing acetate synthesis (production rate: chestnut 68.68% vs 49.64% of control).

HIGHLIGHTS
- Tannin extracts from trees and shrubs can be used to modulate rumen fermentation.
- The positive effect of chestnut tannin extract was demonstrated on methane and ammonia production.
- Tannins showed no protective action on C18:2 trans 11.

Introduction
Livestock systems contribute approximately 40% of agricultural output and support the livelihoods of almost 1.3 billion people (FAO 2009). However, the livestock sector is growing rapidly and accounts for 14.5% of the global green-house gas (GHG) emissions and soil and water pollution due to nitrogen excretions. Sustainable strategies for livestock farming and animal nutrition are thus essential (Dehority 2004; Buccioni et al. 2014).

Methane and nitrogen excretions in ruminants can be mitigated by several approaches, including specific feeding strategies. Tannins reduce methane and ammonia emission by interfering with rumen microbial activities. Tannins belong to the family of phenolic compounds which is a large class of plant secondary metabolites. In the plant kingdom, they are particularly found in trees, shrubs and in some spontaneous herbaceous plants.

Depending on their molecular structure, tannins are classified as condensed or hydrolysable. These two groups are characterised by very different chemical and bioactive properties. Protein microbial degradation is usually reduced by dietary tannins, leading to a lower accumulation of ammonia in the rumen liquor.
Several kinds of condensed or hydrolysable tannins have been tested in animal nutrition and in many cases, changes in the microbial community profile of rumen have been reported (Vasta et al. 2010; Buccioni et al. 2011, 2015a, 2015b, 2017). Some types of tannins show also specific antimicrobial properties, such as ‘gambier’ or ‘catechu’ which are extracted from the leaf and twigs of the *Uncaria gambir* shrub (Achmadi 2019). Similarly, tannin extracts from leaves of Tara (*Caesalpinia spinosa* Molina Kuntze) have antimicrobial properties against coliform bacteria in monogastric animals (Huang et al. 2018).

The aim of this *in vitro* study was to evaluate the effects of the dietary addition of two kinds of condensed tannins (Mimosa and Gambier) and two kinds of hydrolysable tannins (Chestnut and Tara) on ammonia and methane production, fatty acids and plasmalogen lipid profile, and biohydrogenation process in rumen liquor fermented for 24 h.

### Materials and methods

#### Experimental design

**Feed**

A basal feed of barley (48 g/100 g dry matter – DM), wheat bran (23 g/100 g DM), dehydrated alfalfa hay (15 g/100 g DM), soybean meal (10 g/100 g DM) and molasses (2 g/100 g DM) was used as the control feed (CON). This basal feed was supplemented with 4 g/100 g DM of mimosa tannin extract (from *A. dealbata* Link) composed of 65% tannin (Silva Chimica srl, San Michele Mondovi, Cuneo, Italy), mimosa feed (MF); 4 g/100 g DM of gambier tannin extract (from *U. gambir* W. Hunter) composed of 48% tannin (Figli di Guido Lapi spa, Santa Croce sull’Arno, Pisa, Italy), gambier feed (GF); 4 g/100 g DM of Tara tannin extract (from *C. spinosa* Molina Kuntze) composed of 95% tannin (Silva Chimica srl, San Michele Mondovi, Cuneo, Italy), Tara feed (TF); 4 g/100 g DM of chestnut tannin extract (from *C. sativa* Mill.) composed of 75% tannin (Silva Chimica srl, San Michele Mondovi, Cuneo, Italy), chestnut feed (CF). The tannin content was determined according to Burns (1963).

The dose of tannins adopted was similar to that previously used in *in vivo* trials (Salami et al. 2018; Valenti et al. 2019). Experimental feed ingredients and their chemical composition are reported in Tables 1 and 2, respectively.

**Feed sampling and analysis**

Feeds were sampled before fermentation, dried at 40 °C, ground using a Cyclotec 1093 mill (PBI International, Milan, Italy) with a mesh size of 1 mm and stored at −80 °C until the analysis. Crude protein (CP) and ether extract (EE) were determined according to AOAC methods 976.06 and 920.39, respectively (AOAC International 1995). The contents of neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin (ADL) were determined using heat-stable amylase and sodium sulphite, according to Van Soest et al. (1991). The results were expressed as inclusive of residual ash. Neutral detergent insoluble protein was estimated according to the Cornell Net Carbohydrate and Protein System (CNCPS) (Licita et al. 1996).

#### Table 1. Composition of the diets studied in this *in vitro* trial (g/100 g DM⁻¹).

| Ingredient        | CON  | MF  | GF  | TF  | CF  |
|-------------------|------|-----|-----|-----|-----|
| Barley            | 480  | 479 | 479 | 479 | 479 |
| Wheat bran        | 230  | 229 | 229 | 229 | 229 |
| Dehydrated alfalfa hay | 150  | 149 | 149 | 149 | 149 |
| Soybean meal      | 100  | 99  | 99  | 99  | 99  |
| Molasses          | 20   | 20  | 20  | 20  | 20  |
| Supplement for Min-vit | 20   | 20  | 20  | 20  | 20  |
| Mimosa tannin extract | 0    | 4   | 0   | 0   | 0   |
| Gambier tannin extract | 0    | 4   | 0   | 0   | 0   |
| Tara tannin extract | 0    | 0   | 0   | 4   | 0   |
| Chestnut tannin extract | 0    | 0   | 0   | 0   | 4   |

1DM: dry matter.

2Control feed (CON); Mimosa feed (MF) with 4 g/100 g DM of Mimosa tannin extract (from *Acacia dealbata* Link); Gambier feed (GF) with 4 g/100 g DM of Gambier tannin extract (from *Uncaria gambir* W. Hunter); Tara feed (TF) with 4 g/100 g DM of Tara tannin extract (from *Caesalpinia spinosa* Molina Kuntze) and Chestnut feed (CF) with 4 g/100 g DM of Chestnut tannin extract (from *C. sativa* Mill.).

#### Table 2. Chemical composition of the diets studied in this *in vitro* trial (g/100 g DM⁻¹).

| Chemical composition | CON | MF | GF | TF | CF |
|----------------------|-----|----|----|----|----|
| CP                   | 14.18 | 13.49 | 14.43 | 14.08 | 14.50 |
| EE                   | 0.98  | 0.93 | 1.07 | 0.96 | 0.98 |
| NDF                  | 26.73 | 26.00 | 25.66 | 26.74 | 26.84 |
| NDFIP                | 2.65 | 2.19 | 2.42 | 2.26 | 2.17 |
| ADF                  | 12.96 | 12.86 | 12.04 | 12.41 | 12.94 |
| ADL                  | 1.34 | 0.86 | 0.98 | 0.78 | 0.95 |
| ASH                  | 7.30 | 7.00 | 7.78 | 6.76 | 6.96 |

1DM: Dry Matter.

2Control feed (CON); Mimosa feed (MF) with 4 g/100 g DM of Mimosa tannin extract (from *Acacia dealbata* Link); Gambier feed (GF) with 4 g/100 g DM of Gambier tannin extract (from *Uncaria gambir* W. Hunter); Tara feed (TF) with 4 g/100 g DM of Tara tannin extract (from *Caesalpinia spinosa* Molina Kuntze) and Chestnut feed (CF) with 4 g/100 g DM of Chestnut tannin extract (from *C. sativa* Mill.).
acids were extracted according to Folch et al. (1957), and methylated by sodium methylate in methanol (MeO−Na+/MeOH, 0.5 M) according to Christie (1982). The fatty acid profile was then determined by gas chromatography.

**Animals**

The experiment was conducted at the Centro di Ricerche Agro-Ambientali “Enrico Avanzi” of the University of Pisa, Italy. Animals were handled according to the guidelines of Italian law on animal welfare for experimental animals (Italian Ministry of Health 2014).

Five Massese ewes were conditioned with the basal diet used as the control feed in the in vitro trial. The animals had continuous access to water. On day 21, rumen liquor samples were collected from each ewe (about 0.5 L per animal) using a stomach tube connected to a manual pump according to Ramos-Morales et al. (2014). Rumen liquor samples were collected 3 h after the morning feed. Immediately after collection, rumen liquor samples were transferred by a temperature-controlled box (39°C) to the laboratory and prepared for the in vitro trial.

**In vitro batch fermentation**

Rumen liquor was filtered through four layers of cheese-cloth into a flask under a continuous flow of CO2, the volatile fatty acid profile was analysed (as described below) and buffered (1:3, v/v) with McDougal solution (McDougall 1948) under anaerobic conditions.

Feeds (2 g of DM) were incubated with 200 mL of inoculum in a 250 mL fermenter under a continuous flow of CO2. Three fermentation runs were carried out, and each treatment was fermented in triplicate per run. The apparatus consisted of a thermostatic chamber (39–40°C) equipped with eighteen 250 mL fermenters, continuously stirred, and provided with one inlet to release gas through a valve and connected to an electronic pressure transducer. The valve was connected to a gas chromatograph (micro-GC Agilent 3000) in order to measure and characterise the total gas production during the fermentation from each fermenter. The fermentation pressure was continuously measured by pressure piezo-resistive transducers, registered every 30 s over 24 h using a dedicated programmable logic controller connected to a PC.

**Gas sampling and analysis**

The cumulative volume of gas produced in each glass fermentation vessel was calculated according to Ragaglini et al. (2014) using the ideal gas law and the molar volume of ideal gases at standard temperature and pressure conditions (1 bar, 273.15 K). These data were used to calculate the percentage of methane produced. The headspace gas in the fermenters was collected at 6, 12 and 24 h and immediately analysed by a gas chromatograph (micro-GC Agilent 3000), to determine methane according to Angelidaki et al. (2009). Data were expressed as mmol/L of total gas.

**Rumen liquor sampling and analysis**

At the end of each fermentation time, the pH of the rumen liquor samples was measured (pH metre XS PH7) and the samples were quickly filtered through four layers of cheese-cloth into a flask and split into four aliquots for the analysis described below.

**Ammonia determination**

Five mL of rumen liquor were acidified with 5 mL of 0.2 M HCl solution. Samples were then centrifuged at 5000×g for 30 min at room temperature. The concentration of ammonia was determined according to Weatherburn (1967) using a UV spectrophotometer (Varian, Cary 50 SCAN). Data were expressed as mg/L.

**Volatile fatty acid (VFA) determination**

The rumen liquor volatile fatty acids (C2:0, acetic; C3:0, propionic; C4:0, butyric) were analysed by high performance liquid chromatography (HPLC): a volume of 10 mL of rumen liquor was diluted with H2SO4 0.1 N solution (1:1) and centrifuged (5000×g for 15 min at 4°C) to separate the liquid phase from the feed residuals. The liquid phase was then micro-filtered (0.45 μm sized particle; Biorad, Milan, Italy) and kept at 40°C. The detection wavelength was 220 nm. The analyses were carried out using an isocratic elution (flux 0.6 mL/min) with a H2SO4 0.008 N solution as the mobile phase. The injection loop was 20 μL. Individual volatile fatty acids 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 in H2SO4 0.1 N (cod. 338826, 402907, B103500, respectively; Sigma-Aldrich, St Louis, MO, USA). The quantification was obtained using an external calibration curve based on the standards described above. Data were expressed as mmol.
**Fatty acid and plasmalogen lipid characterisation**

Plasmalogen lipids are contained in the microbial membrane and are strictly related to the bacterial species (Alves et al. 2013). They are commonly identified and quantified by converting them into dimethylacetals (DMA), an artefact formed during the methylation of rumen fatty acids for chromatographic analysis (Alves et al. 2013). Two other aliquots of rumen liquor were thus freeze-dried and subsequently analysed in order to determine the fatty acid and DMA profile according to Jenkins (2010) as modified by Alves et al. (2013). Briefly: FA were trans-esterified using C9:0 and C19:0 (1 mg/mL) as internal standards (Kramer et al. 1997; Alves et al. 2013). Fatty acids and DMA fractions were then separated by thin-layer chromatography (TLC). The fatty acids and DMA were identified by a GC2010 Shimadzu gas chromatograph (Shimadzu, Columbia, MD, USA) equipped with a flame-ionisation detector and a high polar fused-silica capillary column (Chrompack CP-Sil 88 Varian, Middelburg, the Netherlands; 100 m, 0.25 mm i.d.; film thickness 0.20 μm). Hydrogen was used as the carrier gas at a flow of 1 mL/min. A split/splitless injector was used with a split ratio of 1:80. An aliquot of the sample was injected under the following GC conditions: the oven temperature started at 60 °C which was held for 1 min. It was then increased to 173 °C at a rate of 2 °C/min, and held for 30 min, before being once again increased to 185 °C at 1 °C/min and held for 5 min, and then to 220 °C at a rate of 3 °C/min, and held for 19 min. The injector temperature was set at 270 °C and the detector temperature was set at 300 °C. Data related to fatty acids and DMA were expressed as g/100 of fatty acids + DMA.

**Statistical analysis**

At each sampling time, data related to methane production, ammonia, volatile fatty acids, fatty acids and DMA profile were processed as a completely randomised design using the following linear model: 

\[
Y_{ijk} = D_i + T_j + D \times T_{ij} + \text{batch(random)}_k + e_{ijk}
\]

where \(y\) is the observation; \(D_i\) is the fixed effect of diet (1 = 1 to 5); \(T_j\) is the fixed effect of sampling time (j = 1 to 3); \(D \times T_{ij}\) is the interaction between diet and sampling time; batch(random)\(_k\) is the random effect of the fermentation run (k = 1 to 3) and \(e_{ijk}\) is the residual error.

Multiple comparisons among means were performed using the Tukey test.

**Results**

**Gas production and pH**

The overall amount of millimoles of methane produced during the fermentation time increased for all the treatments (time effect was significant, \(p < .0001\)). There was no interaction between the time and treatments (\(p = .7608\)), whereas the diet effect was significantly different (\(p = .03\)). The average amount of methane produced was lower when MF, CF and TF were fermented, compared with CON and GF (CON, 0.159 a; MF, 0.141 b; GF, 0.0.160 a; TF, 0.146 ab; CF, 0.137 b; \(SEM = 0.009; p = .0326\); means of diet effect expressed as mmol/L in rumen liquor; Table 3).

Data related to pH value after 24 h of fermentation did not show significant differences among treatments (CON 6.67; MF 6.67; GF 6.67; TF 6.69; CF 6.70).

**Ammonia production**

The pattern of ammonia production differed significantly across treatments, resulting in a significant effect of the interaction Diet × Time (Table 4). After 6 h, the ammonia content in rumen liquor from CON fermenters was two to three times higher, compared to the other treatments, irrespective of the type of tannin (Table 4). After 12 h, the ammonia nearly halved from 105.75 to 54.52 mg/L in the CON fermenter, whereas in the fermenters containing tannins, the ammonia content did not vary or decreased slightly. After 24 h, the content of ammonia increased

| Time, hours | Feed1 | p Value | SEM | \(D^2\) | \(T^3\) | DxT4 |
|-------------|-------|---------|-----|--------|-------|------|
| 6           | 0.13  | 0.11    | 0.14| 0.14   | 0.12  | 0.033|
| 12          | 0.17  | 0.15    | 0.14| 0.14   | 0.13  | <.0001|
| 24          | 0.18  | 0.17    | 0.18| 0.16   | 0.16  | .761 |

1Control feed (CON); Mimosa feed (MF) with 4 g/100 g DM of Mimosa tannin extract (from Acacia dealbata Link); Gambier feed (GF) with 4 g/100 g DM of Gambier tannin extract (from Uncaria gambir W. Hunter); Tara feed (TF) with 4 g/100 g DM of Tara tannin extract (from Casealpinia spinosa Molina Kuntze) and Chestnut feed (CF) with 4 g/100 g DM of Chestnut tannin extract (from Castanea sativa Mill.).

2\(D\): diet.

\(\times\): sampling time effect.

4DxT: interaction between diet and sampling time.
in all fermenters, however the final content was significantly higher in the rumen liquor from the CON fermenter, compared to the other treatments. At the end of fermentation, the average ammonia content was significantly lower in fermenters containing MF (Table 4).

**Volatile fatty acid production**

Both acetate and propionate production increased throughout the fermentation, irrespective of the treatment (Table 5).

The effect of the treatment was significant for acetate. Considering the concentration of each single volatile fatty acid at the end of fermentation, the mean acetate value from the GF treatment was significantly higher than those from the other treatments ($p < .05$). Interestingly, considering the percentage of production from 6 h to 24 h, acetate was highest in CF fermenters. The butyrate content also increased for all the treatments throughout the fermentation, with the exception of the CON treatment, for which the butyrate content in the rumen liquor remained constant ($DxT$ interaction $p = .053$, Table 5). The total amount of volatile fatty acids increased during fermentation and tended to be higher for the GF treatment ($p = .104$). Overall, the ratio $C2:0/C3:0$ did not change across treatments, and there was a general decrease

| Feed1  | Feed1  | Feed1  | Feed1  | Feed1  | SEM2 | D3 | T4 | DxT5 |
|--------|--------|--------|--------|--------|------|----|----|------|
| CON    | MF     | GF     | TF     | CF     |      |    |    |      |
| 6 68.39 | 53.89  | 64.13  | 62.64  | 56.92  |     |   |   |      |
| 12 66.54 | 57.08  | 68.04  | 65.27  | 59.00  |     |   |   |      |
| 24 69.43 | 63.93  | 72.94  | 69.77  | 63.31  |     |   |   |      |

1Control feed (CON); Mimosa feed (MF) with 4 g/100 g DM of Mimosa tannin extract (from *Acacia dealbata* Link); Gambier feed (GF) with 4 g/100 g DM of Gambier tannin extract (from *Uncaria gambir* W. Hunter); Tara feed (TF) with 4 g/100 g DM of Tara tannin extract (from *Casealpinia spinosa* Molina Kuntze) and Chestnut feed (CF) with 4 g/100 g DM of Chestnut tannin extract (from *Castanea sativa* Mill.).

2Different letters (a, b, c) indicate significant differences for $p < .05$; SEM: standard error of mean.

3D: diet.

4T: sampling time effect.

5DxT: interaction between diet and sampling time.
during fermentation, irrespective of the treatment \( (p = .01) \).

**Fatty acid and DMA profile**

Considering the effect of the interaction between Diet \( \times \) Time (DxT), the fatty acid profile did not show changes, apart from C15iso. In the rumen liquor fermenting CON, the C15iso content increased during the first 12 h, then decreased. Inversely, the C15iso content in the MF fermentation decreased from 6 to 24 h and, at the end of fermentation, the C15iso content was the lowest compared with CON and the other tannin treatments (Table 6). During fermentation, the C15iso content increased in both the hydrolysable tannin sources (TF and CF) and in GF treatment, but with a different temporal pattern: the TF and GF treatments reached the maximum content after 12 h, whereas the CF treatment reached it at the end of the fermentation time (24 h). Considering the diet effect, C17:0iso was decreased by both MF and GF but not by both TF and CF: CON, 0.559 a; MF, 0.452 b; GF, 0.432 b; TF, 0.561 a; CF, 0.564 a \((SEM = 0.039; p = .005)\) (means of diet effect expressed as g/100 g total FA in RL). In contrast, C14iso was affected by all the polyphenol extracts tested in this trial: CON 0.694 a; MF 0.525 c; GF 0.477 d; TF 0.569 b; CF 0.601 b; SEM 0.033; \( p = .014 \) while C15iso and C16iso showed a selective sensitivity to the different feeds (C15iso: CON 6.235 b; MF 5.586 c; GF 7.059 a; TF 6.021 b; CF 5.151 c; SEM 0.349; \( p = .003 \); C16iso: CON 1.054 a; MF 0.862 b; GF 0.902 ab; TF 0.873 b; CF 1.132 a; SEM 0.0829; \( p = .001 \); means of diet effect expressed as g/100 g FA in RL).

The biohydrogenation pathway of linoleic acid (C18:2 c9, c12) and linolenic acid (C18:3 c9, c12, c15) was affected differently by each kind of tannin. On average, the sources of hydrolysable tannins showed a higher content of the main dietary unsaturated fatty acids (linoleic and linolenic acid), particularly for the CF treatment (Table 6). For the condensed tannin sources (MF and GF), the average content of linoleic and linolenic acid did not differ significantly from the values obtained for the CON treatment: linoleic acid, CON 8.989 c; MF 8.430 cd; GF 7.705 d; TF 9.145 b; CF 10.813 a; SEM = 0.511; \( p < .001 \); linolenic acid, CON 1.065 bc; MF, 1.166 bc; GF 0.959 c; TF 1.133 bc; CF 1.372 a; SEM = 0.100; \( p = .026 \); vaccenic acid, CON 1.866 c; MF 2.138 bc; GF 2.388 bc; TF 3.435 a; CF 2.283 bc; SEM = 0.497; \( p = .014 \).

The end product of the rumen biohydrogenation of unsaturated fatty acid (C18:0, stearic acid) was contained to a higher extent in the rumen liquor from CON, MF and GF compared with the CF treatment: SA, CON 28.952 b; MF 32.429 a; GF 34.059 a; TF 28.958 b; CF 23.430 c; \( SEM = 2.064; p < .001 \) (means of diet effect expressed as g/100 g total fatty acids in rumen liquor).

Interestingly, oleic acid (C18:1 c9) was affected by all the tannin extracts but not by CF: CON, 5.092 c; MF, 5.119 c; GF, 4.810 c; TF, 5.740 b; CF, 6.430 a; SEM = 0.282; \( p < .001 \) (means of diet effect expressed as g/100 g total fatty acids in rumen liquor).

In the case of C18:1 t12 and C18:1 t16 (coeluted with C18:1 c14) the inclusion of tannins in the inoculum resulted in a higher content of these fatty acids compared with the CON treatment, irrespective to the kind of tannin adopted: C18:1 t12, CON, 0.575 c; MF, 0.6879 b; GF, 0.783 a; TF, 0.665 b; CF, 0.7490 a; SEM = 0.040; \( p < .001 \); C18:1 t16, CON, 0.351 bc; MF, 0.409 b; GF, 0.435 b; TF, 0.329 c; CF, 0.636 a; SEM = 0.082; \( p < .006 \); means of diet effect expressed as g/100 g total fatty acids in rumen liquor).

The content of the trans isomers of C18:1, which are considered as intermediates of the biohydrogenation of dietary unsaturated fatty acid (C18:1 t6-8; C18:1 t9; C18:1 t10; C18:1 t12; C18:1 t15), decreased during fermentation (Figure 1).

The main conjugated linoleic acid isomer (CLAc9, t11) and other minor linoleic acid isomers were not detected at any of the sampling times.

Eight different DMA were detected in the rumen liquor during the fermentation, irrespective of the treatment considered. The total amount of DMA ranged between 4% (TF treatment) and 5% (CON treatment) of the sum of fatty acids and DMA. DMA 16:0 was the most present in all treatments, representing from 55% (CF treatment) to 62% (GF treatment) of total DMA. Similarly to the fatty acids profile, DMA concentration did significantly vary considering the interaction effect Diet \( \times \) Time. Conversely, the diet effect was significant for both total and individual DMA detected, with the exception of DMA 16:1.

The dietary sources of condensed tannins and hydrolysable tannins showed a similar total DMA content and composition compared with CON. On the other hand, the content of DMA 14:0, DMA 15:0 and DMA 18:0 was significantly lower in rumen liquor from TF (Tables 7 and 8).

**Discussion**

Chestnut extract reduced methanogenesis without decreasing either the acetate syntheses or the C17:0 iso concentration, thus indicating that cellulolytic
Table 6. Fatty acid (FA) profile (g/100 g total fatty acid) at each sampling time (6, 12, 24 h).

| Time, hours | FA          | CON | MF  | GF  | TF  | CF  | SEM | D1  | T4  | Dxt5 |
|------------|-------------|-----|-----|-----|-----|-----|-----|-----|-----|------|
| 6          | C13iso      | 0.38| 0.16| 0.14| 0.23| 0.24| 0.071| 0.345| 0.422| 0.522|
| 12         | C13         | 0.28| 0.20| 0.23| 0.25| 0.31| 0.039| 0.045| 0.098| 0.962|
| 24         | C14iso      | 0.64| 0.52| 0.45| 0.64| 0.59| 0.062| 0.001| 0.005| 0.116|
| 12         | C15iso      | 5.75ab| 6.44a| 6.39a| 5.32ab| 4.52b| 0.591| 0.003| 0.136| 0.031|
| 24         | C15ante     | 3.47| 3.11| 2.92| 4.07| 3.84| 0.381| 0.028| 0.137| 0.460|
| 6          | C16iso      | 3.22| 3.06| 3.14| 3.06| 4.22| 0.321| 0.028| 0.137| 0.460|
| 12         | C16         | 1.84| 1.75| 1.59| 2.19| 2.21| 0.165| 0.016| 0.013| 0.345|
| 24         | C16:1c7     | 1.66| 1.46| 1.48| 1.40| 1.05| 0.302| 0.381| <.001| 0.890|
| 6          | C17iso      | 0.56| 0.48| 0.44| 0.46| 0.57| 0.060| 0.005| 0.165| 0.680|
| 12         | C17ante     | 0.49| 0.45| 0.36| 0.50| 0.54| 0.063| 0.50| 0.56| 0.59|
| 24         | C18         | 22.81| 23.09| 21.06| 25.52| 26.77| 1.264| <.001| 0.180| 0.278|
| 6          | C18:1c7     | 1.16| 1.10| 1.21| 0.91| 1.49| 0.126| 0.76| 0.92| 0.92|
| 12         | C18:1c9     | 0.44| 0.47| 0.33| 0.45| 0.42| 0.49| 0.45| 0.45| 0.41|
| 24         | C18:1c17    | 0.60| 0.36| 0.45| 0.45| 0.44| 1.31| 0.94| 0.88| 1.24|
| 6          | C18:1t6 + t7 + t8 | 0.81| 0.87| 1.11| 0.86| 1.05| 0.015| 0.76| <.001| 0.439|
| 12         | C18:1t9     | 0.40| 0.47| 0.58| 0.45| 0.59| 0.049| 0.375| <.001| 0.341|
| 24         | C18:1t10    | 0.33| 0.38| 0.37| 0.40| 0.43| 0.30| 0.36| 0.34| 0.27|
| 6          | C18:1t11    | 1.01| 1.21| 1.34| 1.13| 1.29| 0.127| 0.277| <.001| 0.199|
| 12         | C18:1c9     | 5.43| 5.30| 5.60| 5.86| 7.26| 0.420| <.001| 0.002| 0.144|
| 24         | C18:1c12    | 1.34| 1.31| 1.29| 1.62| 1.34| 0.158| 0.370| 0.012| 0.422|
| 6          | C18:1c12    | 1.23| 1.24| 1.12| 1.14| 1.27| 0.123| 0.86| 0.11| 0.27|

(continued)
Table 6. Continued.

| Time, hours | Feed1 | p Value |
|-------------|-------|---------|
|             | FA    | CON     | MF    | GF    | TF    | CF    | SEM2 | D3   | T4   | DxT5 |
| 6           | C18:1c14 + t16 | 0.42 | 0.41 | 0.58 | 0.38 | 0.29 | 0.042 | 0.006 | 0.008 | 0.122 |
| 12          |       | 0.31 | 0.35 | 0.38 | 0.32 | 0.28 |       |       |       |       |
| 24          |       | 0.32 | 0.36 | 0.33 | 0.29 | 0.26 |       |       |       |       |
| 12          | C18:2 c9, c12 | 8.54 | 8.92 | 8.55 | 10.36 | 11.18 | 0.783 | <.001 | 0.129 | 0.301 |
| 24          |       | 9.90 | 7.14 | 7.04 | 8.47 | 10.15 |       |       |       |       |
| 6           | C18:3 c9, c12, c15 | 1.09 | 1.19 | 1.16 | 1.37 | 1.52 | 0.127 | 0.026 | <.001 | 0.350 |
| 12          |       | 0.99 | 1.20 | 0.92 | 1.09 | 1.40 |       |       |       |       |
| 24          |       | 1.11 | 1.11 | 0.80 | 0.94 | 1.19 |       |       |       |       |

1Control feed (CON); Mimosa feed (MF) with 4 g/100 g DM of Mimosa tannin extract (from *Acacia dealbata* Link); Gambier feed (GF) with 4 g/100 g DM of Gambier tannin extract (from *Uncaria gambir* W. Hunter); Tara feed (TF) with 4 g/100 g DM of Tara tannin extract (from *Casealpinia spinosa* Molina Kuntze) and Chestnut feed (CF) with 4 g/100 g DM of Chestnut tannin extract (from *Castanea sativa* Mill.).

2Different letters (a, b, c) indicate significant differences for $p < .05$, within row; SEM: standard error of mean.

3D: diet.

4T: sampling time effect.

5DxT: interaction between diet and sampling time.

Figure 1. Trend of main C18:1 isomers during the *in vitro* fermentation. Control feed (CON); Mimosa feed (MF) with 4 g/100 g DM of Mimosa tannin extract (from *Acacia dealbata* Link); Gambier feed (GF) with 4 g/100 g DM of Gambier tannin extract (from *Uncaria gambir* W. Hunter); Tara feed (TF) with 4 g/100 g DM of Tara tannin extract (from *Casealpinia spinosa* Molina Kuntze) and Chestnut feed (CF) with 4 g/100 g DM of Chestnut tannin extract (from *Castanea sativa* Mill.). Different letters (a, b, c) indicate significant differences for $p < .05$. 

bacteria are probably not particularly affected by this kind of polyphenol (Dehority 2004; Vlaeminck et al. 2006). Similar results were found by Liu et al. (2011) who evaluated the productive performances and rumen fermentation of dairy ewes fed with a diet supplemented with chestnut tannin, and by Jayanegara et

### Table 7. Profile of identified dimethylacetals (DMA) (g/100 g of total fatty acid + DMA) in rumen liquor samples at each sampling time (6, 12, 24 h).

| Time, hours | DMA 14:0 | Feed¹ | SEM² | p Value |
|-------------|---------|-------|------|---------|
| 6           | 0.43    | MF    | 0.029| <0.001 |
| 12          | 0.38    | CF    | 0.012| 0.77    |
| 24          | 0.33    | TF    |      |         |

### Table 8. Profile of identified dimethylacetals (DMA) (g/100 g of total fatty acid + DMA) in rumen liquor samples as means of diet effect.

| DMA 14:0 | Feed¹ | SEM² | p Value |
|---------|-------|------|---------|
| 0.38a   | MF    | 0.016| <.001   |
| 0.32a   | CF    |      |         |
| 0.30b   | TF    |      |         |

¹Control feed (CON); Mimosa feed (MF) with 4 g/100 g DM of Mimosa tannin extract (from Acacia dealbata Link); Gambier feed (GF) with 4 g/100 g DM of Gambier tannin extract (from Uncaria gambir W. Hunter); Tara feed (TF) with 4 g/100 g DM of Tara tannin extract (from Casealpinia spinosa Molina Kuntze) and Chestnut feed (CF) with 4 g/100 g DM of Chestnut tannin extract (from Castanea sativa Mill.).
²Different letters (a, b, c) indicate significant differences for p < .05, within row; SEM: standard error of mean.
³D: diet.
⁴T: sampling time effect.
⁵DxT: interaction between diet and sampling time.

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### Table 7. Profile of identified dimethylacetals (DMA) (g/100 g of total fatty acid + DMA) in rumen liquor samples at each sampling time (6, 12, 24 h).

| Time, hours | DMA 14:0 | Feed¹ | SEM² | D¹ | T² | DxT³ |
|-------------|---------|-------|------|----|----|------|
| 6           | 0.43    | MF    | 0.029| <0.001| 0.012| 0.77 |
| 12          | 0.38    | CF    | 0.012| 0.77 |
| 24          | 0.33    | TF    |      |      |

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### Table 8. Profile of identified dimethylacetals (DMA) (g/100 g of total fatty acid + DMA) in rumen liquor samples as means of diet effect.

| DMA 14:0 | Feed¹ | SEM² | p Value |
|---------|-------|------|---------|
| 0.38a   | MF    | 0.016| <.001   |
| 0.32a   | CF    |      |         |
| 0.30b   | TF    |      |         |

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*bacteria are probably not particularly affected by this kind of polyphenol (Dehority 2004; Vlaeminck et al. 2006). Similar results were found by Liu et al. (2011) who evaluated the productive performances and rumen fermentation of dairy ewes fed with a diet supplemented with chestnut tannin, and by Jayanegara et
stimulating alternate H₂ sinks available in the micro-

thus preserving the efficiency of cellulolytic bacteria or

degradability was not decreased by chestnut tannin,
significantly lower when CF was fermented.

in our experiment, the methane produced was signifi-
cantly lower when CF was fermented.

Our findings suggest that in our trial the fibre degradability was not decreased by chestnut tannin, thus preserving the efficiency of cellulolytic bacteria or stimulating alternate H₂ sinks available in the microbial ecosystem of the rumen. This hypothesis is confirmed by the highest value of C17:0 iso concentration found in CF along with the fact that there was no negative effect on cellulolytic bacteria.

Our findings confirmed the selective action of tannins on microbial activities. In terms of the effect of diet, CF decreased linoleic and linolenic acid hydrogenation but not the last step of this process. In contrast, the other feeds affected the conversion of vaccenic acid to stearic acid only, which seems to be the most sensitive to tannins.

The accumulation of vaccenic acid could be explained by hypothesising either a direct detrimental effect on microorganisms involved in the last step of the biohydrogenation processes or an inhibition of the enzymes involved in the double bound saturation. Similar results were found in Buccioni et al. (2015a, 2015b, 2017), who found that chestnut tannin extract inhibits Butyrivibrio proteoclasticum which converts vaccenic acid into stearic acid.

Several authors have demonstrated that there is still no clear understanding of the role of rumen microbial species in the biohydrogenation pathway nor about the microorganisms involved in these processes. What is, however, clear is that B. proteoclasticus does 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 Ruminococaceae might be more relevant (Boeckaert et al. 2008; Kim et al. 2008; Belenguer et al. 2010; Huws et al. 2010, 2011; Halmemies-Beauchet-Filleau et al. 2013; Castro-Carrera et al. 2014).

In our study, tannins did not carry out a protective action on C18:2 cis9 trans11. In fact, no trace of this fatty acid was detected, indicating that there may have been a complete hydrogenation within 6 h.

Chestnut tannin may have induced the accumulation of oleic acid due to a decrease in the biohydrogenation rate of cis9 double bond or to a decrease in its isomerisation (Mosley et al. 2002).
Our results showed that the total amount of DMAs did not vary across the treatments, in accordance with Cappucci et al. (2018) who used different doses of olive crude phenolic concentrate in the diet of dairy ewes. We found that DMA 16:0 was the most present in all the treatments, in accordance with the results reported by Alves et al. (2013) and our previous study (Cappucci et al. 2018).

All DMAs, with the exception of DMA 16:1, were affected by diet, but the Diet × Time effect did not show any significant differences in the DMA profile among fermenters, suggesting that the tannins tested in our trial did not induce great perturbation in rumen microbiota.

Conclusions
In our trial, commercial doses of MF, GF, TF and CF were tested. All the extracts affected ammonia production, methanogenesis, and the production of fatty acids. We thus conclude that incorporating chestnut tannins into the CF diet improves the sustainability of ruminant rearing since it lowers methanogenesis and ammonia production without compromising the synthesis of acetate.

Disclosure statement
No potential conflict of interest was reported by the author(s).

Ethical approval
Animals were handled according to the guidelines of Italian law on animal welfare for experimental animals (Italian Ministry of Health 2014).

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