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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 syntheses. 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; $p = .0326$), 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

(Vasta et al. 2010; Patra and Saxena 2011; Buccioni et al. 2015a, 2015b; Vasta et al. 2019). At the same time, both hydrolysable and condensed tannins have been associated with a reduction in methane emissions, although different mechanisms have been proposed to explain this effect (Vasta et al. 2019).

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 Mondovì, 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 Mondovì, 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 Mondovì, 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) (Licitra et al. 1996). Fatty

Table 1. Composition of the feeds studied in this *in vitro* trial (g/100 g DM[-1]).

	FEED[2]				
	CON	MF	GF	TF	CF
Ingredient					
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	0	4	0	0
Tara tannin extract	0	0	0	4	0
Chestnut tannin extract	0	0	0	0	4

[1]DM: dry matter.
[2]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.).

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

	Feed[2]				
	CON	MF	GF	TF	CF
Chemical composition					
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

[1]DM: Dry Metter
[2]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.).

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 CO_2, 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 CO_2. 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 $5000 \times g$ 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 H_2SO_4 0.1 N solution (1:1) and centrifuged ($5000 \times g$ 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 millex – HV) and the sample was directly injected into the HPLC apparatus using an ion exclusion column Aminex 85 HPX − 87 H (300 mm × 7.8 mm; Å-sized pores, 9 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 H_2SO_4 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 H_2SO_4 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 Shimadtzu 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 mixed. The individual data from each bottle were used. The model used the SAS 9.2 package.

$$Y_{ijk} = D_i + T_j + D * T_{ij} + \text{batch}(\text{random})_k + e_{ijk}$$

where y is the observation; D_i is the fixed effect of diet (i = 1 to 5); T_j is the fixed effect of sampling time (j = 1 to 3); $D*T_{ij}$ is the interaction between diet and sampling time; $\text{batch}(\text{random})_k$ 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

Table 3. Methane production (mmol/L) at each sampling time.

Time, hours	Feed[1]					SEM	p Value		
	CON	MF	GF	TF	CF		D[2]	T[3]	DxT[4]
6	0.13	0.11	0.14	0.14	0.12	0.122	.033	<.0001	.761
12	0.17	0.15	0.17	0.14	0.14				
24	0.18	0.17	0.18	0.16	0.16				

[1]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.).
[2]D: diet.
[3]T: sampling time effect.
[4]DxT: interaction between diet and sampling time.

Table 4. Ammonia production (mg/L) at each sampling time (6, 12, 24 h).

	Feed[1]						p Value		
Time	CON	MF	GF	TF	CF	SEM[2]	D[3]	T[4]	DxT[5]
6	105.75a	30.23c	33.01c	42.37bc	59.01b	14.178	$p < .0001$	$p < .0001$	$p < .0001$
12	54.52a	20.74b	33.83ab	58.92a	33.21ab				
24	248.60a	99.00d	129.19c	131.28c	179.93b				

[1]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.).
[2]Different letters (a, b, c) indicate significant differences for $p < .05$; SEM: standard error of mean.
[3]D: diet.
[4]T: sampling time effect.
[5]DxT: interaction between diet and sampling time.

Table 5. Volatile fatty acid (VFA) production (mmol/L) at each sampling time (0, 6, 12, 24 h).

Feed[1]							p Value			
Time, hours	VFA	CON	MF	GF	TF	CF	SEM[2]	D[3]	T[4]	DxT[5]
	Acetate									
0		34.96	34.07	25.56	39.59	26.44	11.23	.047	<.001	.274
6		53.88	72.36	68.32	58.52	46.72				
12		83.74	88.55	102.47	85.52	65.22				
24		69.43	67.93	104.48	99.92	84.43				
	Propionate									
0		7.46	7.37	6.03	9.23	9.08	4.98	.266	<.001	.081
6		16.48	24.85	23.31	17.51	14.09				
12		32.37	36.34	38.54	27.42	25.46				
24		26.84	23.62	38.60	42.24	34.45				
	Butyrate									
0		4.59	2.03	3.48	3.44	1.94	1.58	.414	<.001	.053
6		3.38	5.57	5.59	5.62	5.80				
12		7.46	6.67	7.47	7.39	5.28				
24		4.72	6.29	8.44	8.71	13.66				
	Total VFA									
0		47.71	44.80	36.03	56.44	38.61	17.23	.104	<.001	.106
6		74.66	107.05	98.88	81.71	68.88				
12		126.36	135.49	150.83	120.33	96.58				
24		103.98	98.99	153.96	150.85	136.88				
	C2/C3									
0		4.81	4.86	4.76	4.76	4.88	0.95	.643	.01	.329
6		3.46	3.20	2.93	3.64	3.62				
12		2.58	2.65	2.75	4.04	2.61				
24		2.86	3.24	2.59	2.47	2.34				

[1]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.).
[2]Different letters (a, b, c) indicate significant differences for $p < .05$; SEM: standard error of mean.
[3]D: diet.
[4]T: sampling time effect.
[5]DxT: 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

during fermentation, irrespective of the treatment ($p = .01$).

Fatty acid and DMA profile

Considering the effect of the interaction between Diet × 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 hydrolisable 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 × 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	Feed[1] CON	MF	GF	TF	CF	SEM[2]	D[3]	p Value T[4]	DxT[5]
6	C13iso	0.38	0.16	0.14	0.23	0.24	0.071	.345	.422	.522
12		0.15	0.15	0.16	0.14	0.24				
24		0.20	0.15	0.16	0.32	0.20				
6	C13	0.28	0.20	0.23	0.25	0.31	0.039	.045	.098	.962
12		0.26	0.23	0.25	0.23	0.30				
24		0.30	0.28	0.26	0.28	0.32				
6	C14iso	0.64	0.52	0.45	0.64	0.59	0.062	.001	.005	.116
12		0.54	0.48	0.43	0.45	0.64				
24		0.90	0.58	0.56	0.62	0.58				
6	C15iso	5.75ab	6.44a	6.39a	5.32ab	4.52b	0.591	.003	.136	.031
12		7.41 a	6.07b	7.59a	6.26b	4.80c				
24		5.55 b	4.25c	7.20a	6.49b	6.13b				
6	C15ante	3.47	3.11	2.92	4.07	3.84	0.381	.028	.137	.460
12		3.32	3.06	3.14	3.06	4.22				
24		4.68	3.35	3.43	3.78	4.03				
6	C15	1.84	1.75	1.59	2.19	2.21	0.165	.016	.013	.345
12		1.80	1.70	1.59	1.67	2.05				
24		2.32	2.02	1.98	1.81	2.24				
6	C16iso	1.01	0.85	0.88	1.02	1.09	0.124	.014	.056	.532
12		0.85	0.80	0.92	0.72	1.08				
24		1.31	0.94	0.91	0.88	1.24				
6	C16	22.81	23.09	21.06	25.52	26.77	1.264	<.001	.180	.278
12		23.14	22.67	20.24	22.36	26.12				
24		26.62	23.67	22.21	22.64	25.94				
6	C17iso	0.56	0.48	0.44	0.63	0.57	0.060	.005	.165	.680
12		0.49	0.45	0.36	0.50	0.54				
24		0.63	0.42	0.50	0.56	0.59				
6	C16:1c7	1.66	1.46	1.48	1.40	1.05	0.302	.381	<.001	.890
12		1.16	1.10	1.21	0.91	1.49				
24		1.26	1.22	0.76	0.92	0.92				
6	C16:1c9	0.44	0.47	0.38	0.45	0.42	0.067	.194	.694	.393
12		0.49	0.45	0.45	0.45	0.41				
24		0.60	0.36	0.45	0.45	0.44				
6	C17ante	1.20	1.10	1.09	1.33	1.31	0.150	.269	.185	.796
12		0.99	1.01	1.01	1.04	1.33				
24		1.45	1.14	1.14	1.15	1.28				
6	C17	0.96	0.95	0.85	0.95	0.93	0.038	.114	.040	.733
12		0.92	0.90	0.86	0.89	0.87				
24		0.93	0.97	0.94	0.94	0.90				
6	C18	29.95	30.05	31.22	24.37	20.01	2.955	<.001	.043	.509
12		31.30	32.51	35.58	29.78	23.55				
24		25.61	34.73	35.38	32.72	26.74				
6	C18:1t6 + t7 + t8	0.81	0.87	1.11	0.86	1.05	0.115	.760	<.001	.439
12		0.64	0.71	0.64	0.69	0.76				
24		0.45	0.64	0.47	0.59	0.38				
6	C18:1t9	0.40	0.47	0.58	0.45	0.59	0.051	.375	<.001	.341
12		0.33	0.38	0.37	0.40	0.43				
24		0.30	0.36	0.30	0.34	0.27				
6	C18:1t10	1.01	1.21	1.34	1.13	1.29	0.127	.277	<.001	.419
12		0.83	0.95	0.76	1.01	0.82				
24		0.52	0.76	0.58	0.83	0.42				
6	C18:1t11	2.07	2.20	2.92	2.48	3.12	0.321	.014	.141	.330
12		1.98	2.08	2.33	2.55	1.84				
24		1.55	2.09	1.88	2.63	1.86				
6	C18:1t12	0.77	0.86	1.09	0.85	1.03	0.070	.006	<.001	.448
12		0.58	0.67	0.68	0.64	0.79				
24		0.38	0.53	0.58	0.51	0.44				
6	C18:1c9	5.43	5.30	5.60	5.86	7.26	0.420	<.001	.002	.144
12		4.70	5.07	4.50	6.46	6.50				
24		5.15	4.99	4.35	4.93	5.55				
6	C18:1t15	0.44	0.37	0.49	0.29	0.41	0.054	.079	<.001	.100
12		0.23	0.27	0.32	0.27	0.40				
24		0.25	0.25	0.22	0.23	0.23				
6	C18:1c11	1.06	1.35	1.11	1.72	1.30	0.177	.324	.008	.065
12		1.99	1.46	1.48	1.50	1.55				
24		1.81	1.35	1.62	1.58	1.72				
6	C18:1c12	1.34	1.31	1.29	1.62	1.34	0.158	.370	.012	.422
12		1.24	1.10	1.22	0.99	1.36				
24		1.23	0.86	1.14	1.12	1.27				

(*continued*)

Table 6. Continued.

Time, hours	FA	CON	MF	GF	TF	CF	SEM[2]	D[3]	T[4]	DxT[5]
									Feed[1]					p Value		
6	C18:1c14 + t16	0.42	0.41	0.58	0.38	0.29	0.042	.006	.008	.122
12		0.31	0.35	0.38	0.32	0.28				
24		0.32	0.36	0.33	0.29	0.26				
6	C18:2 c9, c12	8.54	8.92	8.55	10.36	11.18	0.783	<.001	.129	.301
12		8.53	9.23	7.53	8.61	11.11				
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	.026	<.001	.350
12		0.99	1.20	0.92	1.09	1.40				
24		1.11	1.11	0.80	0.94	1.19				

[1]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.).
[2]Different letters (a, b, c) indicate significant differences for $p < .05$, within row; SEM: standard error of mean.
[3]D: diet.
[4]T: sampling time effect.
[5]DxT: interaction between diet and sampling time.
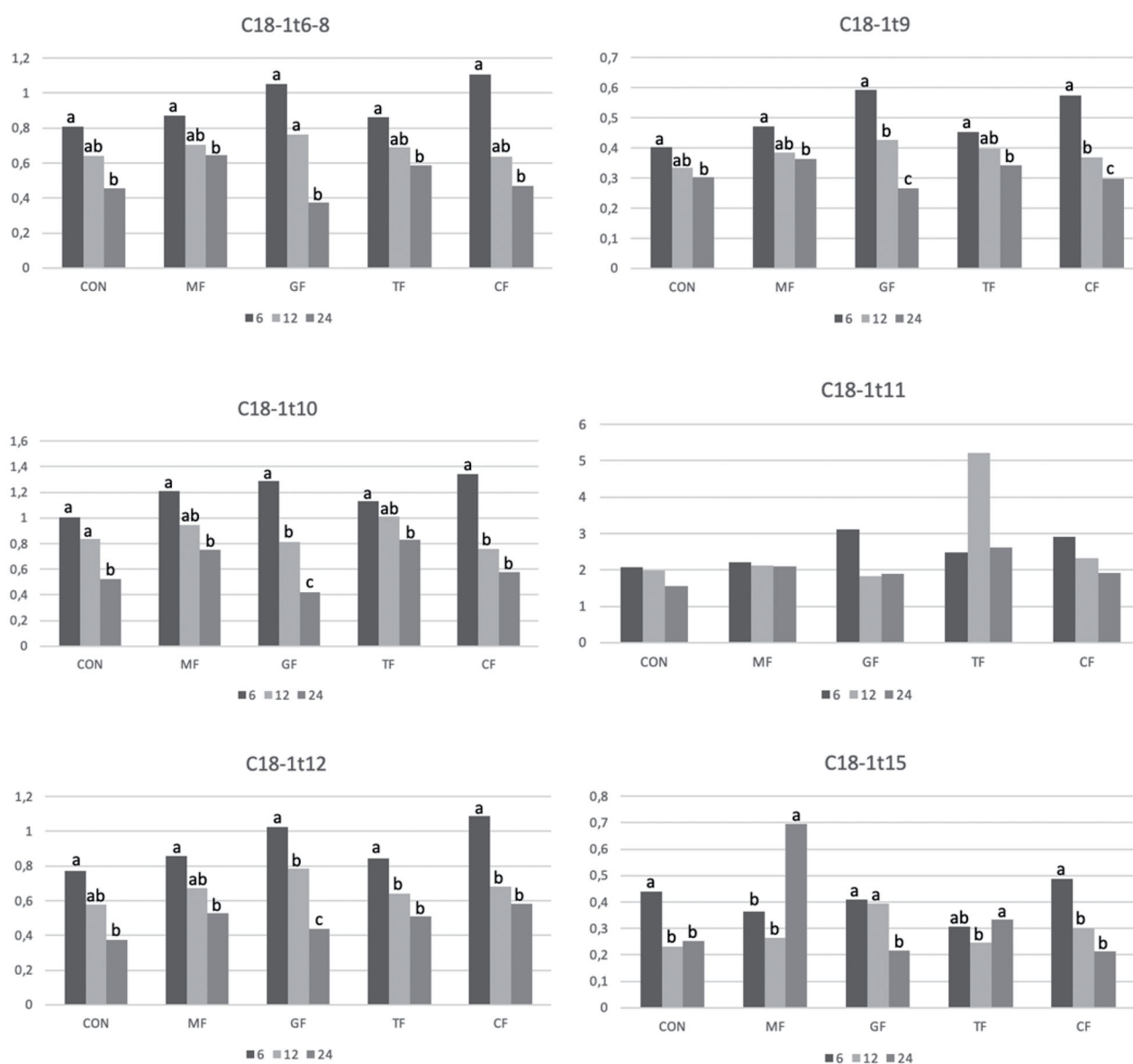

Figure 1. Trend of main C18:1 isomers during the *in vitro* fermentation. Control feed (CON); Mimosa feed (MF) with 4 g/100 g $_{DM}$ of Mimosa tannin extract (from *Acacia dealbata* Link); Gambier feed (GF) with 4 g/100 g $_{DM}$ of Gambier tannin extract (from *Uncaria gambir* W. Hunter); Tara feed (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 $p < .05$.

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	CON	MF	GF	TF	CF	SEM[2]	D[3]	T[4]	DxT[5]
	DMA 14:0									
6		0.43	0.40	.42	0.33	0.44	0.029	<0.001	0.012	.770
12		0.38	0.37	.37	0.27	0.35				
24		0.33	0.36	.41	0.29	0.42				
	DMA 15ante									
6		0.29	0.31	.31	0.33	0.44	0.038	0.049	0.071	.763
12		0.26	0.28	.30	0.26	0.30				
24		0.32	0.29	.28	0.30	0.38				
	DMA 15:0									
6		0.36	0.33	.38	0.29	0.36	0.037	0.006	0.039	.384
12		0.32	0.30	.33	0.26	0.37				
24		0.27	0.37	.311	0.23	0.29				
	DMA 16iso									
6		0.17	0.11	.14	0.16	0.11	0.030	0.015	0.003	.077
12		0.15	0.13	.19	0.11	0.14				
24		0.25	0.22	.21	0.18	0.11				
	DMA 16:0									
6		3.48	2.99	3.20	2.22	2.80	0.300	0.048	0.17	.670
12		3.03	3.11	3.27	2.97	2.53				
24		2.69	2.83	2.85	2.34	2.53				
	DMA 16:1									
6		0.31	0.29	.34	0.34	0.27	0.077	0.153	0.029	.801
12		0.28	0.19	.26	0.25	0.27				
24		0.33	0.20	.24	0.24	0.22				
	DMA 17ante									
6		0.30	0.27	.22	0.19	0.39	0.067	<0.001	0.255	.195
12		0.19	0.36	.22	0.19	0.33				
24		0.30	0.42	.22	0.23	0.32				
	DMA 18:0									
6		0.17	0.21	.16	0.13	0.25	0.034	<0.001	0.175	.098
12		0.14	0.14	.15	0.09	0.25				
24		0.17	0.21	.18	0.12	0.15				
	Total DMA									
6		5.67	4.91	5.09	3.99	5.38	0.394	0.002	0.121	.631
12		4.77	4.87	5.10	4.38	4.53				
24		4.64	4.94	4.67	3.93	4.43				

[1]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.).
[2]Different letters (a, b, c) indicate significant differences for $p < .05$, within row; SEM: standard error of mean.
[3]D: diet.
[4]T: sampling time effect.
[5]DxT: interaction between diet and sampling time.

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	CON	MF	CF	GF	TF	SEM[2]	p Value
DMA 14:0	0.38a	0.38a	0.40a	0.40a	0.30b	0.016	<.001
DMA 15:0	0.32a	0.34a	0.34a	0.34a	0.26b	0.027	.049
DMA 15ante	0.29b	0.29b	0.37a	0.30b	0.30b	0.023	.006
DMA 16:0	3.07ab	2.98ab	2.62b	3.11a	2.51b	0.185	.015
DMA 16:1	0.31	0.23	0.28	0.25	0.27	0.071	.048
DMA 16iso	0.19a	0.15ab	0.12b	0.18a	0.15ab	0.022	.153
DMA 17ante	0.31b	0.36ab	0.43a	0.22b	0.20b	0.053	<.001
DMA 18:0	0.16b	0.19ab	0.22a	0.17b	0.11c	0.027	<.001

[1]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.).
[2]Different letters (a, b, c) indicate significant differences for $p < .05$, within row; SEM: standard error of mean.

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

al. (2015) who found that methanogenesis was lower when feeds including chestnut tannin at three different concentrations (0.5, 0.75 and 1 mg/mL) were fermented *in vitro*.

Acetate is produced by bacteria during fibre degradation. The start point of the reaction is the conversion of pyruvate to acetilphosphate and formiate. Formiate is degraded into CO_2 and H_2 and is very toxic for microorganisms. Bacteria thus convert H_2 into methane to protect themselves. Consequently, the decrease in methane emissions is often linked to a decrease in fibre rumen degradability. Interestingly, in our experiment, the methane produced was significantly 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 H_2 sinks available in the microbial ecosystem of the rumen. This hypothesis is confirmed by the highest value of C17: 0iso concentration found in CF along with the fact that there was no negative effect on cellulolytic bacteria.

Acetate is a fundamental part of milk fat synthesis. Chestnut tannin extract thus represents an interesting opportunity in dairy ruminant feeding to improve the sustainability of productions. Although MF lowered methanogenesis, it decreased acetate and C17:0 iso productions, thus indicating a possible detrimental effect on cellulolytics. These data confirm that tannins in selectively modulating rumen processes.

Costa et al. (2018) compared mimosa tannins with chestnut tannins and observed a reduced growth of cellulolytic bacteria with condensed tannins compared to hydrolysable tannins, which was gentler. The authors hypothesised that the higher level of depolymerisation in hydrolysable tannins plays a fundamental role in the rumen ecosystem (McSweeney et al. 1999). Due to possible detrimental effects of MF on cellulolytic bacteria, a decrease in C14:0 iso, C15:0 iso and C16:0 iso concentrations was observed (Vlaeminck et al. 2006; Buccioni et al. 2011).

In our study, tannins decreased the concentration of ammonia in each fermenter and at each time of fermentation, thus highlighting how tannins interfere with microorganism syntheses. Our data are in agreement with Mannelli et al. (2018), who found a decrease in rumen protein degradation when tannins were added to feeds *in vitro*. In our trial, MF had the strongest effect, demonstrating once again that condensed tannins interfere the most with the protein metabolism of the rumen.

However, our data did not reveal whether the decrease in ammonia concentration was due to a direct detrimental effect on proteolytic bacteria or to the ability of tannins in complexing proteins, making them unavailable for rumen microorganism syntheses (Buccioni et al. 2017).

In our study, all the tannin extracts reduced biohydrogenation rate thus facilitating the accumulation of vaccenic acid and decreasing the stearic acid content. The same results were found by Carreño et al. (2015) who compared different doses (20–40–60–80 g/kg of diet DM) of condensed (quebracho and grape) and hydrolysable tannins (chestnut and oak) in an *in vitro* trial.

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 reported 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 *Ruminococcaceae* 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 bound 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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