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Effects of two tannin extracts at different doses in interaction with a green or dry forage substrate on *in vitro* rumen fermentation and biohydrogenation

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ABSTRACT

Extensive ruminant farming systems often face fluctuating pasture availability, resulting in two periods with very different forage quality. Tannins are natural bioactive compounds able to modify ruminants' digestive metabolism thanks to their bioactive properties. To evaluate a differential effect of tannins in different feeding situations, according to the availability of pasture along the year, an *in vitro* rumen incubation trial was performed. Buffered sheep rumen content was incubated *in vitro* with two different substrates, vetch pasture (VP) and vetch hay (VH), in the presence of two different tannin extracts, from quebracho (QUE) and a mixture of quebracho and chestnut tannins (MIX). Each of the tannin extract was tested at 0, 15 and 30 g of tannin/kg DM. The gas production was determined at 3.5 and 24 h of fermentation. After 24 h in anaerobic conditions, volatile fatty acids (VFA), ammonia and fatty acids (FA) were analysed. Interesting significative two-way interactions with different tannin extracts were observed in some fermentation parameters. Adding MIX to VH treatment resulted in a higher depression ($P < 0.050$) of ammonia production and iso-VFA proportions, compared to QUE supplementation, while no differences were observed in VP treatment. QUE was more effective than MIX ($P < 0.050$) in reducing acetate/propionate ratio when supplemented to VH treatment. However, the MIX treatment showed a higher increase ($P < 0.050$) of CO_2/CH_4 ratio after 24 h of incubation when supplemented at 30 g/kg, compared to QUE treatment. MIX and QUE exerted similar effects on rumen biohydrogenation. Tannin extracts decreased the rumenic/linoleic acid ratio and *iso*-FA proportions ($P < 0.050$) only when added to the VH treatment. These results demonstrate that tannin extracts are more effective in modulating rumen metabolism when associated with a hay-

Abbreviations: ADF, acid detergent fibre expressed inclusive of residual ash; ALA, C18:3 *c*9*c*12*c*15; aNDF, neutral detergent fibre assayed with a heat stable amylase and expressed inclusive of residual ash; BCFA, branched-chain fatty acids; BH, biohydrogenation; CP, crude protein; CT, condensed tannins; DDM, degraded dry matter; DM, dry matter; EE, ether extract; FA, fatty acids; FAME, fatty acid methyl esters; HT, hydrolysable tannins; IVDMD, *in vitro* dry matter degradability; LA, C18:2 *c*9*c*12; Lignin (sa), lignin determined by solubilization of cellulose with sulphuric acid; MIX, mixture of quebracho and chestnut tannin extracts; MUFA, monounsaturated fatty acids; OA, C18:1 *c*9; OBCFA, odd- and branched-chain fatty acids; OCFA, odd-chain fatty acids; OM, organic matter; PUFA, polyunsaturated fatty acids; QUE, quebracho tannin extract; RA, C18:2 *c*9*t*11; SA, C18:0; SFA, saturated fatty acids; VA, C18:1 *t*11; VFA, volatile fatty acids; VH, vetch hay; VP, vetch pasture.

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based diet. Also, the MIX treatment was more effective in protecting proteins from ruminal degradation, a key point in grazing periods. Therefore, extensive husbandry could benefit from different strategies for using dietary tannin extracts according to season and pasture availability.

1. Introduction

In certain environments, such as the Mediterranean area, extensive ruminant farming systems face a fluctuating pasture availability, resulting in two periods with very different forage type and quality. The favourable season presents a high amount of fresh herbage, leading to the ingestion of highly degradable protein with consequential nitrogen losses and excessive release of ammonia in the soil (Kingston-Smith and Theodorou, 2000). During the unfavourable season, the use of dried and highly fibrous forages may increase methane (CH_4) emission, because a high content of structural carbohydrates is related to an enhanced ruminal gas production (Johnson and Johnson, 1995).

Tannins are a class of polyphenols characterized by protein-binding ability, ubiquitous of the plant kingdom and thus easy to find in forages – especially in plant species characterising marginal areas or dry habitats – and in agricultural by-products. Ruminants' digestion could be highly influenced by dietary tannins, as they have been demonstrated to depress the overall ruminal microflora activity, affecting protein, fibre and lipid metabolism (Vasta et al., 2019). Tannins can improve the ratio of by-pass protein, potentially resulting in an enhanced intestinal absorption of amino acids (Waghorn, 2008). Moreover, they are able to reduce ruminants' CH_4 and ammonia emissions, with potential benefits for the environment (Patra and Saxena, 2011). The effects on lipid metabolism include an impairment of fatty acid biohydrogenation (BH), with potential improvement of ruminant product nutritional quality, thanks to the reduction of saturated fatty acids (SFA) and the increase of polyunsaturated fatty acids (PUFA) and BH intermediates, such as vaccenic and rumenic acid (Frutos et al., 2020).

Considering the great variability in diet composition along the year, extensive farming could benefit from dietary tannin effects in different ways according to the season.

Many factors are known to affect the activity and the effectiveness of dietary tannins, making difficult a univocal conclusion on their practical application. Tannins' complex structure causes a huge variability of bioactive capacity, even for molecules of the same group, such as hydrolysable (HT) or condensed tannins (CT) (Jayanegara et al., 2015; Terranova et al., 2018), and a dose-dependent effect on rumen metabolism is often reported (Vasta et al., 2009a; Niderkorn et al., 2012; Sarnataro and Spanghero, 2020). Furthermore, dietary tannins effects may vary according to animals' basal diet, as reported by studies where concentrate and herbage rations (Vasta et al., 2009b), linseed and soybean oil supplementations (Minieri et al., 2014) or hay and fresh herbage diets (Rufino-Moya et al., 2019) were compared. However, there are no studies available in which the interactive effects between all these three factors – tannin type, tannin dose and basal diet – were evaluated.

Considering all the above, we hypothesised that increasing doses of different tannin extracts may differently affect the rumen fermentation of different basal diets. Therefore, the aim of the present study was to investigate simultaneously the effects of different doses of two commercial tannin extracts, contrasting by their composition (CT+HT *vs* CT), on rumen fermentation and biohydrogenation when different basal diets, namely pasture and hay, were incubated *in vitro*.

2. Material and methods

The experimental procedures were conducted in accordance with the European Union Directive 2010/63/EU, reviewed by the local ethics committee (C2E2A, "Comité d'Ethique pour l'Expérimentation Animale en Auvergne") and authorised by the French Ministry for Research (no. 7138-2016092709177605-V5).

2.1. Plant material and tannin extracts for in vitro incubation

Pure vetch grassland (*Vicia sativa* L.) was grown in Agira (Sicily, Italy, 37°37' N, 14°33' E). It was harvested at vegetative stage in October 2017, corresponding to the time at which it is usually grazed. Hay was produced from the same plot, with vetch mowed at flowering stage in May 2018. Samples of vetch pasture [VP; 155 g dry matter(DM)/kg] and vetch hay (VH, 876 g DM/kg) were freeze-dried (Christ, alpha 2–4 LD plus, Osterode am Harz, Germany), ground through a 1 mm screen (Rotary Mill, Brabender GmbH, Duisburg, Germany) and then used as substrates for *in vitro* incubation.

Two types of commercial zootechnical tannin extracts (SilvaTeam, San Michele Mondovì, Cuneo, Italy) were tested: Silvafeed®ByProX (MIX), a mixture 60:40 of HT from chestnut (*Castanea sativa* Mill.) and CT from quebracho (*Schinopsis lorentzii* Engl.), respectively, and Silvafeed®Q (QUE), composed of CT from quebracho only. According to the manufacturer and to the method of Makkar et al. (1993), commercial MIX and QUE contained 773 g/kg and 823 g/kg of total tannins, which represented 90.21% and 92.01% of total phenolic compounds, respectively.

2.2. Buffered rumen fluid preparation

Three rumen cannulated sheep (Texel breed) were daily fed 1.2 kg of total mixed ration (80 % hay and 20 % concentrate) in two equal meals at 09:00 h and 17:00 h, 17 days before the beginning of the trial. Free water and salt blocks were always available. Three

times over three weeks, rumen contents were collected from each sheep immediately before the morning feeding, combined in equal proportions and squeezed through a polyester monofilament fabric (mesh opening 800 μm). The obtained rumen fluid was mixed 1:2 (v/v) in an anaerobic phosphate:carbonate (2:3 v/v) buffer solution, as described by Goering and Van Soest (1970) and modified by Niderkorn et al. (2011), and used as inoculum for the *in vitro* incubations. The procedure was carried out in order to never exceed 25 min between rumen contents sampling and fermenters inoculation.

2.3. In vitro incubation procedure and sampling

For each type of substrate (i.e. VP, VH), two types of tannin extracts (i.e. MIX, QUE) were tested at three doses each (i.e. 0, 15, 30 g of tannin/kg DM). The tannin doses applied in the present experiment are close to the values of 1–2% DM intake, referred to as no detrimental for ruminant performance by Vasta et al. (2019). For each combination of substrate, tannin extract and tannin dose, two sets of bottles were incubated to study: 1) fermentation and 2) biohydrogenation. The incubations were carried out in 2 technical repetitions and repeated 3 times over three consecutive weeks (runs) for statistical replicates. Thus, 144 bottles [2 substrates (VH, VP) × 2 tannin types (MIX, QUE) × 3 doses (0, 15, 30 g/kg) × 2 studies (fermentation, biohydrogenation) × 2 technical repetitions × 3 runs (statistical replicate)] were used in total. For each fermentation, 600 ± 0.5 mg of substrate was incubated with 40 ml of buffered rumen fluid in 120-ml serum bottles (Wheaton, Millville, NJ, USA) pre-warmed at 39 °C and flushed with N_2. After sealing with butyl rubber stoppers (Bellco Glass Inc., Vineland, NJ, USA) and aluminium crimp seals, the bottles were kept in a shaking water bath at 39 °C for 24 h. Throughout the incubation, every 2.5 min, the medium was stirred for 30 s at 600 rpm using a magnetic bar.

The gas production in bottles was determined at 3.5 and 24 h of fermentation using the pressure transducer technique (Theodorou et al., 1994) and the composition of fermentation gases was determined at 3.5 h and 24 h. At 3.5 h, after measuring the pressure and sampling gas, the fermentation gases were removed from the headspace with a syringe and without breaking the anaerobiosis.

For the study of fermentation, at the end of the incubation period (24 h), the whole content of bottles was transferred in a pre-weighted 50-ml Falcon tube and the pH was measured. After centrifugation at 3400×*g* for 10 min at 4 °C, the supernatant was sampled for volatile fatty acids (VFA) and ammonia analyses. Then, bottles were washed twice with distilled water to recover all the non-degraded particles that were transferred into the Falcon tube and the residue was used for determination of dry matter (DM).

For the study of BH, after 24 h of fermentation, the whole content of bottles was freeze-dried for determination of FA profile.

2.4. Chemical analyses

Freeze-dried samples of VP and VH and rumen digesta were analysed for DM by oven-drying at 103 °C for 48 h, and organic matter (OM) by ashing at 550 °C for 6 h in a muffle furnace. The difference between DM of plant material before the fermentation and DM of residue after 24 h of fermentation was used to calculate *in vitro* DM degradability (IVDMD). Neutral detergent fibre (using a heat stable amylase; aNDF), acid detergent fibre (ADF), both expressed inclusive of residual ash, and lignin (sa) contents were determined using the procedure reported by Van Soest et al. (1991), using a Fibre Analyzer (Ankom Technology Corporation, Fairport, NY, USA). Total crude protein (CP), ether extract (EE) and ash were determined according to the AOAC (1995) methods 976.06, 920.39 and 942.05, respectively. Pepsin-cellulase digestibility was determined according to Aufrere and Michalet-Doreau (1988).

Sequential extraction in acetone 70% (v/v) followed by methanol 80% (v/v) was used to extract total phenolic compounds and tannins from substrates, as reported by Luciano et al. (2019). Extracts were then analysed for total phenols content through Folin-Ciocalteu reaction, following Makkar et al. (1993) procedure. Total tannins were calculated as difference between total phenols and non-tannin phenols, analysed previous polyvinylpyrrolidone treatment of extracts.

The individual VFA in the supernatant fraction of the incubation medium were analysed by gas chromatography (PerkingElmer Clarus 580CPG), according to Jouany (1982), and ammonia was measured using the Berthelot reaction (Weatherburn, 1967). The composition of fermentation gases (CH_4, CO_2 and H_2) was determined by gas chromatography using a Micro-GC 3000A (Agilent Technologies, France) equipped with two columns, MS-5A using argon as carrier gas and set to 100 °C and PPU using helium and set to 75 °C. The Micro-GC was calibrated using a certified gas standard mixture (Messer, France) containing CH_4, O_2, N_2, CO_2 and H_2. Approximately 2 ml of the sampled gas was injected in the Micro GC for analysis.

The FA profile of rumen content and incubation substrates was analysed by transesterification into fatty acid methyl esters (FAME) using a combined basic followed by acid catalysis according to Alves et al. (2013), with some modifications as described by Natalello et al. (2019). Briefly, 200 mg of freeze-dried rumen digesta were weighted in a Pyrex tube containing 0.5 mg of internal standard (C19:0 as free FA), then 1.6 ml of sodium methoxide in methanol (0.5 M) were added. The solution was then incubated for 10 min at 50 °C, cooled and added with 2.4 ml of 10 % HCl solution in methanol. After incubating the solution for 15 min at 50 °C, 3.2 ml of 6% aqueous potassium carbonate and 2 ml of hexane were added. The solution was then centrifuged (1500×*g*, 10 min, 4 °C) and the supernatant was transferred to another tube: this extraction step was performed twice. The final solution was dried over anhydrous sodium sulphate and, after centrifugation, the supernatant was collected to another tube and evaporated under a stream of nitrogen. The residue was then dissolved in 0.5 ml of hexane (GC grade). The FAME were separated and quantified through gas chromatography with a Thermo Finnigan Trace GC equipped with a flame ionization detector (FID; ThermoQuest, Milan, Italy) and 100 m high-polar fused silica capillary column (SP – 2560 fused silica, Supelco, Bellafonte, PA, 100 m ×0.25 mm i.d.; film thickness 0.25 μm). Helium was used as carrier gas at a constant flow of 1 ml/min. Total FAME profile in a 2 μl sample volume, at a split ratio of 1:30, was determined following oven, injector and detector settings described by Valenti et al. (2018). Furthermore, isothermal analysis at 165 °C was performed in order to separate C18:1 isomers. Identification of individual FAME was based on the comparison with retention time of commercially available standard FAME mixture (Nu-Chek Prep Inc., Elysian, MN, USA; Larodan Fine Chemicals, Malmo,

Sweden). Individual FA were expressed as g/100 g of total FA.

2.5. Calculations and statistical analysis

The total gas, CH_4 and CO_2 productions at 24 h of fermentation were calculated as sum of measurements after 3.5 h and 24 h of incubation. The estimated BH (%) for C18:2 *c*9*c*12 (LA) and C18:3 *c*9*c*12*c*15 (ALA) were calculated according to Alves et al. (2017).

Data were analysed with IBM SPSS For Analytics using a mixed linear model that included the fixed effects of experimental substrates, tannin types and tannin doses with their interactions in a full factorial model and the random effect of runs. Results are reported as estimated marginal means. Orthogonal polynomial contrasts were used to evaluate linear and quadratic components of the response to incremental doses of each tannin extract. When significant interactions with the dose occurred, orthogonal contrasts were evaluated individually for each factor level. Differences were declared significant at $P \leq 0.050$ and considered a trend towards significance at $0.050 < P \leq 0.100$.

3. Results

3.1. Substrates chemical composition

The chemical composition of freeze-dried substrates is shown in Table 1. As expected, CP and EE content were higher in VP than VH. VH showed higher aNDF and lignin (sa) content, compared to VP. Pepsin-cellulase assay depicted a greater DM digestibility for VP than VH. VP showed higher total phenols and total tannins contents, compared to VH, although levels were low if compared to experimental supplementation with tannin extracts. FA analysis highlighted a far greater content of PUFA in VP compared to VH.

3.2. Rumen fermentation characteristics

Results for rumen fermentation parameters are shown in Table 2. For all the gas samples, after both 3.5 h and 24 h of incubation, H_2 production was under the detection limit. The substrate factor significantly affected all the parameters ($P < 0.050$). Total gas and CO_2 productions displayed a turnaround over time, with VP treatment showing the highest values after 3.5 h and the lowest values after 24 h. Incubating VH resulted in greater CH_4 production ($P < 0.050$) per g DM or DDM (degraded DM), but lower IVDMD and CO_2/CH_4 ratio compared to VP ($P < 0.001$). Acetate and butyrate proportions in VFA and the acetate/propionate ratio were higher for VH treatment, whereas all other analysed VFA proportions were higher for VP treatment ($P < 0.001$). Ammonia concentration was higher in bottles where VP was incubated, compared to VH ($P < 0.001$).

Tannin extract type did not affect gases productions, but the CO_2/CH_4 ratio after 24 h with MIX was higher than with QUE when both tannin extracts were added at 30 g/kg ($P = 0.048$; Fig. 1). The IVDMD was significantly lower for MIX compared to QUE ($P = 0.003$) and the gap between the two tannin types tended to increase ($P = 0.061$) along with the dose. A similar trend for the interaction between tannin type and dose was observed for total *iso*-VFA proportion ($P = 0.093$). A significant interaction between tannin type and

Table 1
Chemical composition of incubated freeze-dried plant substrates.

	VH	VP
Chemical composition (g/kg DM)		
DM (g/kg fresh matter)	957	940
Ether extract	9	29
Ash	59	155
Crude protein	134	245
aNDF	479	405
ADF	253	252
Lignin (sa)	47	41
Total phenols	6.41	17.1
Total tannins	2.5	4.9
Predicted DM digestibility[1] (g/g)	0.71	0.80
FA content (g/kg DM)		
C16:0	1.71	4.84
C18:0 (SA)	0.58	0.89
C18:1 *c*9 (OA)	1.05	1.13
C18:2 *c*9*c*12 (LA)	2.42	4.27
C18:3 *c*9*c*12*c*15 (ALA)	0.67	15.7

VH, vetch hay; VP, vetch pasture; DM, dry matter; aNDF, neutral detergent fibre assayed with a heat stable amylase and expressed inclusive of residual ash; ADF, acid detergent fibre expressed inclusive of residual ash; Lignin (sa), lignin determined by solubilization of cellulose with sulphuric acid; FA, fatty acids; SA, stearic acid; OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid.

[1] Calculated from pepsin-cellulase digestibility (Aufrere and Michalet-Doreau, 1988).

Table 2
In vitro rumen fermentation characteristics of rumen digesta after 24 h incubation with increasing doses of two tannin extracts in combination with different plant substrates.

	Substrate (S)		Tannin (T)		Dose (D), g/kg				SEM	Significance[1]						
	VH	VP	MIX	QUE	0	15	30	Contrasts[2]		S	T	D	S×T	S×D	T×D	S×T×D
pH	6.26	6.50	6.37	6.39	6.37[b]	6.38[a,b]	6.39[a]	L Q	0.014	***	+	**	ns	ns	ns	ns
IVDMD	53.2	59.6	55.6	57.2	59.7[a]	56.0[b]	53.5[c]	L Q	0.61	***	**	***	ns	ns	+	ns
Gas production (ml/g DM)																
Total, 3.5 h	53.8	65.9	59.9	59.6	61.4[a]	59.6[a]	58.5[b]	L	3.27	***	ns	**	ns	ns	ns	ns
CH_4, 3.5 h	9.86	9.64	9.64	9.64	10.1[a]	9.64[b]	9.19[c]	L Q	0.287	*	ns	***	ns	ns	ns	ns
CO_2, 3.5 h	43.9	56.3	50.2	50.0	51.3[a]	50.0[ab]	49.1[b]	L	3.27	***	ns	*	ns	ns	ns	ns
CO_2/CH_4, 3.5 h	4.50	5.93	5.25	5.18	5.08[b]	5.22[a]	5.34[a]	L	0.39	***	ns	**	ns	ns	ns	ns
Total, 24 h	154	146	150	150	153[a]	150[ab]	148[b]	L	0.8	***	ns	*	ns	ns	ns	ns
CH_4, 24 h	32.7	28.0	30.3	30.5	31.4[a]	30.3[b]	29.4[b]	L Q	0.75	***	ns	***	ns	ns	ns	ns
CO_2, 24 h	121	118	120	120	122[a]	119[ab]	118[b]	L	0.9	**	ns	*	ns	ns	ns	ns
CO_2/CH_4, 24 h	3.72	4.22	3.99	3.95	3.90[c]	3.97[b]	4.05[a]	na[3]	0.127	***	*	***	ns	+	*	ns
CH_4/g DDM	61.6	47.1	54.9	53.8	52.9[b]	54.7[ab]	55.6[a]	L	1.19	***	+	**	ns	ns	ns	+
End products (mmol/g DM)																
Ammonia	0.94	2.29	1.59	1.64	1.74[a]	1.61[b]	1.49[c]	L Q	0.095	***	ns	***	*	ns	ns	ns
Total VFA	6.28	6.60	6.41	6.46	6.59[a]	6.44[b]	6.28[c]	L Q	0.155	***	ns	***	ns	ns	ns	ns
VFA proportion(% total VFA)																
Acetate	66.6	64.2	65.5	65.3	65.0[b]	65.3[b]	65.9[a]	L Q	0.59	***	ns	**	*	ns	ns	ns
Propionate	17.8	19.8	18.8	18.8	18.7	18.8	18.8	na[4]	0.61	***	ns	ns	*	ns	ns	**
iso-Butyrate	1.03	1.74	1.36	1.40	1.47[a]	1.39[b]	1.29[c]	L Q	0.048	***	**	***	ns	ns	ns	ns
Butyrate	11.4	9.31	10.3	10.4	10.4	10.4	10.2		0.31	***	ns	ns	ns	ns	ns	ns
iso-Valerate	1.78	3.16	2.43	2.51	2.63[a]	2.49[b]	2.28[c]	L Q	0.073	***	**	***	*	ns	ns	ns
Valerate	1.26	1.73	1.49	1.51	1.59[a]	1.50[b]	1.40[c]	na[5]	0.103	***	ns	***	ns	*	ns	ns
Total *iso*-VFA	2.81	4.90	3.80	3.92	4.10[a]	3.89[b]	3.57[c]	L Q	0.110	***	**	***	*	ns	+	ns
Acetate/Propionate	3.75	3.26	3.52	3.49	3.49	3.50	3.52		0.138	***	ns	ns	*	ns	ns	ns

VH, vetch hay; VP, vetch pasture; MIX, mixture of hydrolysable and condensed tannins; QUE, condensed tannins; IVDMD, *in vitro* dry matter degradability; DDM, degraded dry matter; VFA, volatile fatty acids; SEM, standard error of means.

[a-c] means without a common superscript letter are significantly different.

[1] ns, non-significant; +, $P < 0.100$; *, $P < 0.050$; **, $P < 0.010$; ***, $P < 0.001$.

[2] Significance ($P < 0.05$) of linear (L) and quadratic (Q) responses to dose effect. na, not applicable.

[3] Tannin-specific contrasts for D×T interaction: MIX = LQ, QUE = L.

[4] Specific contrasts for triple (D×S×T) interaction: VH = ns, VP×MIX = LQ, VP×QUE = ns.

[5] Substrate-specific contrasts for D×S interaction: VH = ns, VP = LQ.

substrate was detected, resulting in higher acetate/propionate ratio ($P = 0.016$) and lower ammonia ($P = 0.040$) and total *iso*-VFA proportions ($P = 0.044$) for MIX compared to QUE, when both tannins were supplemented to VH substrate (Fig. 2).

Dose affected significantly almost all fermentation parameters and both the tannin extracts used were able to affect rumen fermentation. Increasing dose of tannin led to linear decrease of total gas and CO_2 productions ($P < 0.050$) and quadratic decrease of IVDMD, CH_4, total VFA and ammonia productions ($P < 0.001$). The CO_2/CH_4 ratio after 3.5 h and CH_4 production per g DMD increased linearly ($P < 0.010$) with the dose, whereas the slope of the CO_2/CH_4 ratio after 24 h rose following a different pattern according to the tannin type: quadratic for MIX, linear for QUE. The presence of both the tannin extracts significantly decreased the molar production of each VFA ($P < 0.050$, data not shown), but exerted different effects on the proportions: acetate proportion quadratically increased along with the dose ($P < 0.050$), whereas *iso*-VFA proportions quadratically decreased ($P < 0.010$). However, the addition of tannin extracts did not affect acetate/propionate ratio. A quadratic reduction of valerate proportion with increasing doses of tannin extracts in VP treatment was observed ($P = 0.022$), while there was no change for VH (Fig. 3).

A significant three-way interaction was observed for the propionate proportion ($P = 0.008$; Fig. 4): the incubation of VP with 30 g/kg of MIX resulted in the highest value, while no dose effect was observed in other combinations of tannin extracts and substrates. Moreover, the two tannin types displayed an opposite trend according to the substrate when incubated at 30 g/kg, with significantly higher value for QUE than for MIX in VH treatment and *vice versa* for VP.

3.3. Fatty acids profile of rumen digesta

Results for FA proportions are shown in Table 3. The substrate effect was significant for almost all the analysed FA ($P < 0.050$). The tannin type effect was never significant, except for C18:1 *c*9 (OA) proportion, which was lower in QUE treatment compared to MIX ($P = 0.036$). An interaction between tannin type and substrate occurred for C17:0 ($P = 0.047$), highlighting a higher proportion for QUE compared to MIX, when both were incubated with VH (Fig. 2).

Increasing dose of tannin extracts resulted in a significant reduction of most odd and branched chain fatty acids (OBCFA) proportions ($P < 0.050$), as well as C18:0 (SA; $P = 0.041$) proportion. On the contrary, OA ($P = 0.024$), LA ($P < 0.001$), ALA ($P < 0.001$) and total PUFA ($P < 0.001$) proportions increased along with the dose. Indeed, estimated BH rate of LA and ALA significantly decreased with increasing doses of tannin supplementation.

A significant interaction between substrate and dose was observed in some FA proportions: adding tannin extracts to VH resulted in a decreased C15:0 *iso* ($P = 0.006$), C16:0 *iso* ($P = 0.003$) and, consequently, total *iso*-FA ($P = 0.049$), whereas no changes were observed for VP treatment (Fig. 3). A similar interaction was observed for C18:2 *c*9*t*11 (RA) to LA ratio ($P = 0.045$; Fig. 3). The ALA proportion increased for both VH and VP treatments but following different slope: linear ($P < 0.001$) and quadratic ($P = 0.003$), respectively. Concerning proportion of C17:0 *anteiso*, adding tannin extracts did not result in significant difference when incubated with VH, whereas a significant reduction was observed with VP along with increasing dose ($P < 0.001$; Fig. 3). Interaction between tannin type and dose was never found to be significant for FA profile, as well as three-way interaction.

4. Discussion

4.1. Interaction between substrate and tannin type on indicators of nitrogen metabolism

At the best of our knowledge, this is the first study that shows the interactions on *in vitro* rumen fermentation and BH that occur when different tannin types, contrasting by their composition (CT+HT *vs* CT), are added at increasing doses in a green or dry forage substrate.

Interestingly, we observed a synergistic effect of different types of tannin on protein metabolism when they were incubated *in vitro*

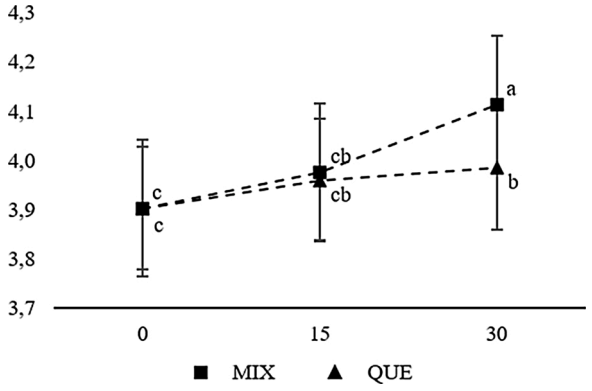

Fig. 1. Effect of interaction between tannin type and tannin dose (g/kg) on CO_2/CH_4 ratio after 24 h of *in vitro* incubation.
MIX, mixture of hydrolysable and condensed tannins; QUE, condensed tannins.
[a–c] means without a common letter differ at $P < 0.050$.

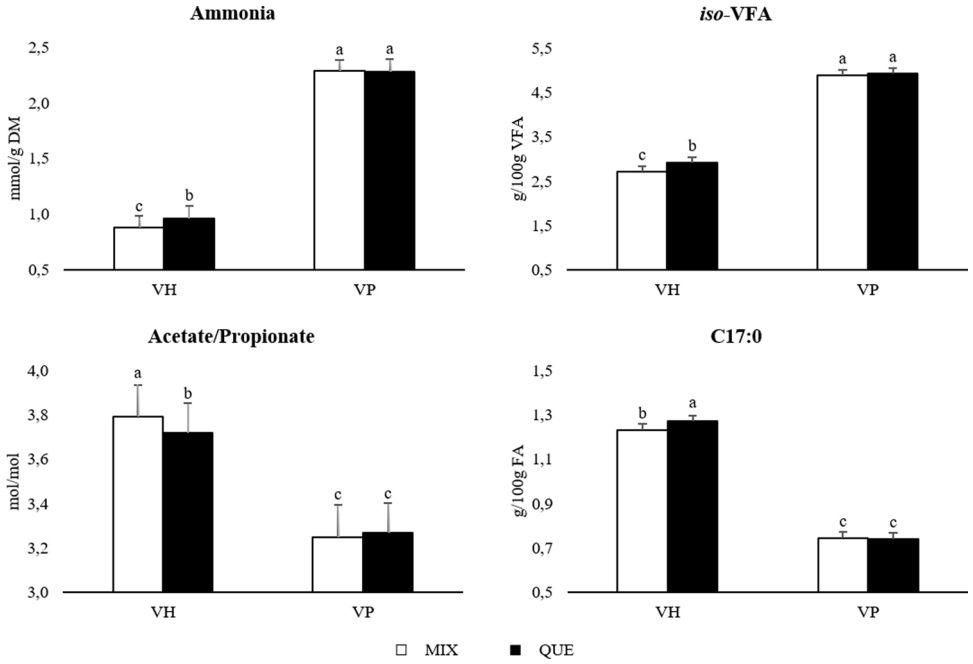

Fig. 2. Effects of interaction between substrate and tannin type on *in vitro* rumen fermentation and FA profile.
VH, vetch hay; VP, vetch pasture; MIX, mixture of hydrolysable and condensed tannins; QUE, condensed tannins.
[a–c] means without a common letter differ at $P < 0.050$.

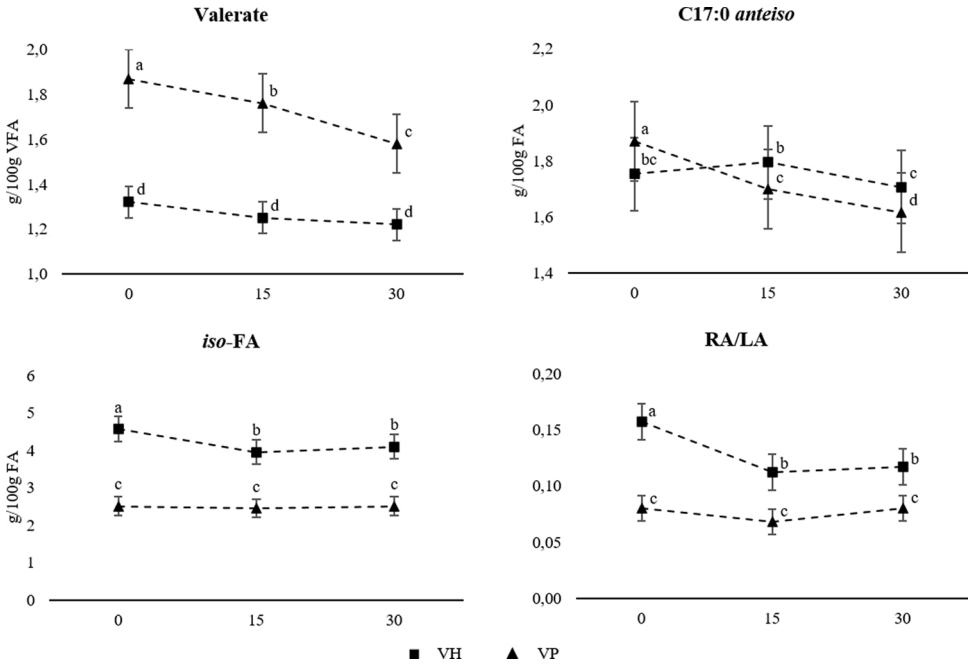

Fig. 3. Effects of interaction between substrate and tannin dose (g/kg) on *in vitro* rumen fermentation and FA profile.
VH, vetch hay; VP, vetch pasture; RA, rumenic acid; LA, linoleic acid.
[a–d] means without a common letter differ at $P < 0.050$.

with a substrate poor in protein. Indeed, adding MIX to VH treatment resulted in a higher depression of ammonia production and *iso*-VFA proportions, compared to the QUE supplementation. These two fermentation products are related to protein degradation, since they derive from protein and branched-chain amino acids deamination (Kaneda, 1991). The affinity of tannins for protein is highly

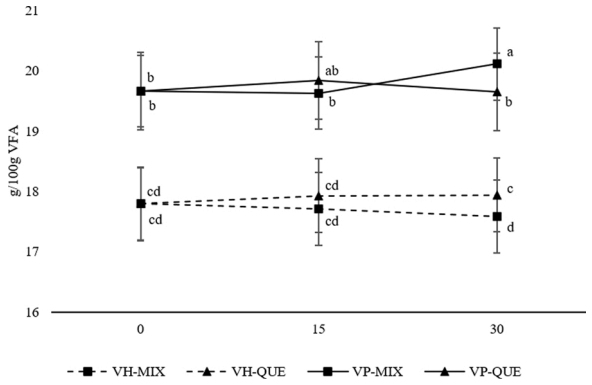

Fig. 4. Effect of interaction between substrate, tannin type and tannin dose (g/kg) on *in vitro* ruminal propionate proportion.
VH, vetch hay; VP, vetch pasture; MIX, mixture of hydrolysable and condensed tannins; QUE, condensed tannins.
[a–d] means without a superscript letter differ at $P < 0.010$.

dependent on their chemical structure (Patra and Saxena, 2011), thus tannins activity is difficult to estimate because of the wide variety of existing molecules. Many authors observed no differences between CT and HT on protein ruminal metabolism (Jayanegara et al., 2015; Aboagye et al., 2018; Costa et al., 2018; Witzig et al., 2018), therefore the results found in this experiment are unlikely simply due to the presence of HT in MIX. Moreover, Bhatta et al. (2009) observed *in vitro* a greater ammonia and *iso*-VFA reduction when incubating HT and CT together compared to HT alone, suggesting a lower effectiveness of HT in affecting protein degradation. Therefore, we suggest that the combination of different tannins, each with its own specific physicochemical characteristics, may enlarge the spectrum of possible tannin-protein bonds, leading to a synergy that enhances dietary tannin's effect on protein metabolism.

The lack of a tannin type effect in VP treatment could be due to the higher CP content in pasture, which probably requires higher doses of tannins to produce observable effects. However, *iso*-VFA proportions and ammonia production decreased along with tannin supplementation dose in both the substrates we tested, demonstrating the potential suitability of dietary tannins for decreasing the detrimental effects for the environment of the excessive protein content in green pasture.

4.2. Interaction between substrate and tannin type on indicators of carbohydrate metabolism

Another innovative result found in the present study is the reduced acetate/propionate ratio in QUE treatment, compared to MIX, in combination with the hay-based diet. Indeed, acetate proportion decreased and propionate proportion increased in VH-QUE treatment. Since acetate/propionate ratio is an indication of proportionally higher NDF digested (Getachew et al., 2004), this result suggested that QUE may have had a higher effectiveness than MIX in impairing fibre digestion in VH treatment. Similarly, Buccioni et al. (2015) observed acetate production to decrease in the rumen of sheep ingesting quebracho tannins compared to the control group, while it increased for those ingesting chestnut tannins. Waghorn (2008) suggested that HT (as those contained in chestnut) may be metabolized by rumen microorganisms to form gallic and ellagic acid, which may be further converted into acetic and butyric acid. However, this would lead to a loss of HT effectiveness, whereas in the present study MIX did not showed a lower biological activity than QUE.

The interaction between substrate and tannin on VFA proportions seems to affect also C17:0 proportion. Since linear odd-chain FA are formed by rumen microorganisms using propionyl-coenzyme A as primer (Vlaeminck et al., 2006), the higher C17:0 proportion in VH-QUE treatment may be due to the lower acetate/propionate ratio. However, no significant interaction was observed for C15:0 proportion.

We suggest that the results on VFA proportions may be attributed to a specific sensitivity to different tannin compounds of the rumen microbiota selected by the different substrates tested in the study. This could also explain the three-way interaction found for propionate proportions (Fig. 4). Indeed, basal diet is known to affect rumen population: Belanche et al. (2012) observed shifts in rumen microbiota of dairy cows in response to different dietary energy sources (fibre-rich or starch-rich) and protein levels (high or low). In addition, Costa et al. (2018) found CT and HT to affect differently fibrolytic bacteria populating rumen of cannulated sheep, with consequential effects on fermentation pathways.

4.3. Interaction between tannin type and tannin dose on gas production

The higher gas and CH_4 productions – as well as the lower IVDMD – found in VH treatments reflect the higher content of structural carbohydrates of dry forages, in agreement with literature data (Beauchemin et al., 2008; Rufino-Moya et al., 2019). The initial higher gas production in VP treatment, measured after 3.5 h of fermentation, was likely due to the higher amount of rapidly fermentable carbohydrates in fresh herbage (Cone et al., 1997).

The MIX treatment showed a stronger increase of CO_2/CH_4 ratio after 24 h of incubation when supplemented at 30 g/kg, compared

Table 3
In vitro fatty acid profile (g/100 g total FA) of rumen digesta after 24 h incubation with increasing doses of two tannin extracts in combination with different plant substrates.

	Substrate (S)		Tannin (T)		Dose (D), g/kg				SEM	Significance[1]				
	VH	VP	MIX	QUE	0	15	30	Contrast[2]		S	T	D	S×T	S×D
C12:0	0.50	0.34	0.43	0.40	0.45	0.35	0.45		0.034	***	ns	+	ns	ns
C13:0	0.23	0.13	0.18	0.18	0.19[a]	0.17[b]	0.18[c]	L	0.014	***	ns	*	ns	ns
C13:0 *iso*	0.15	0.22	0.18	0.19	0.19	0.18	0.18		0.010	***	ns	ns	ns	ns
C14:0	1.82	0.95	1.38	1.39	1.42	1.36	1.37		0.146	***	ns	ns	ns	ns
C14:0 *iso*	0.67	0.35	0.51	0.51	0.52	0.50	0.51		0.065	***	ns	ns	ns	ns
C15:0	2.82	1.81	2.30	2.33	2.41[a]	2.29[b]	2.24[b]	L	0.079	***	ns	***	ns	ns
C15:0 *anteiso*	2.96	1.60	2.26	2.30	2.38[a]	2.19[b]	2.26[ab]	L	0.069	***	ns	*	ns	ns
C15:0 *iso*	1.22	0.77	0.99	1.00	1.06[a]	0.96[b]	0.96[b]	na[3]	0.115	***	ns	**	ns	**
C16:0	19.4	14.5	17.0	17.0	16.9	16.9	17.1		0.31	***	ns	ns	ns	ns
C16:0 *iso*	1.42	0.60	1.00	1.02	1.05	0.99	0.99	na[3]	0.047	***	ns	ns	ns	**
C16:1 *c*9	0.19	0.16	0.17	0.18	0.17	0.17	0.19		0.011	*	ns	ns	ns	ns
C17:0	1.25	0.74	0.99	1.01	1.02[a]	0.99[b]	0.98[b]	L	0.021	***	+	**	*	ns
C17:0 *anteiso*	1.75	1.73	1.73	1.75	1.81[a]	1.75[b]	1.66[c]	na[4]	0.132	ns	ns	***	ns	***
C17:0 *iso*	0.83	0.57	0.69	0.70	0.72[a]	0.70[ab]	0.67[b]	L	0.137	***	ns	**	ns	ns
C17:1 *t*10	0.28	0.16	0.22	0.22	0.22	0.22	0.22		0.033	***	ns	ns	ns	ns
C18:0 (SA)	36.3	40.9	38.5	38.6	38.87[a]	38.26[b]	38.21[b]	L	1.277	***	ns	*	ns	ns
C18:1 *c*9 (OA)	2.85	3.01	2.96	2.90	2.88[b]	2.94[ab]	2.98[a]	L	0.061	***	*	*	ns	ns
C18:1 *c*11	0.49	0.38	0.43	0.43	0.44	0.42	0.43		0.017	***	ns	ns	ns	ns
C18:1 *c*12	0.26	0.23	0.25	0.24	0.26	0.23	0.24		0.012	*	ns	ns	ns	ns
C18:1 *t*6 + *t*7 + *t*8	0.18	0.34	0.25	0.26	0.25	0.26	0.25		0.036	***	ns	ns	ns	ns
C18:1 *t*9	0.21	0.31	0.25	0.26	0.28	0.25	0.25		0.039	***	ns	ns	ns	ns
C18:1 *t*10	0.36	0.43	0.40	0.38	0.40	0.39	0.38		0.064	**	ns	ns	ns	ns
C18:1 *t*11 (VA)	3.61	5.12	4.35	4.39	4.38	4.41	4.31		0.261	***	ns	ns	ns	ns
C18:2 *c*9c12 (LA)	3.15	3.94	3.58	3.51	3.31[c]	3.57[b]	3.76[a]	L Q	0.087	***	ns	***	ns	ns
C18:2 *c*9t11 (RA)	0.40	0.30	0.36	0.34	0.37	0.31	0.37		0.034	***	ns	+	ns	ns
C18:3 *c*9c12*c*15 (ALA)	2.18	4.52	3.38	3.32	3.12[c]	3.34[b]	3.59[a]	na[5]	0.148	***	ns	***	ns	**
C20:0	1.31	0.90	1.09	1.11	1.11	1.11	1.09		0.021	***	ns	ns	ns	ns
C20:2 *c*11*c*14	0.55	0.49	0.51	0.53	0.56	0.50	0.51		0.033	**	ns	ns	ns	+
C20:4 *n*-6	0.23	0.12	0.17	0.19	0.18	0.17	0.18		0.006	***	+	ns	ns	ns
C22:0	0.84	0.59	0.71	0.72	0.73	0.73	0.70		0.038	***	ns	ns	ns	ns
C23:0	0.31	0.37	0.34	0.34	0.32	0.35	0.35		0.015	***	ns	ns	ns	ns
C24:0	0.87	0.63	0.74	0.76	0.73	0.76	0.76		0.024	***	ns	ns	ns	ns

(*continued on next page*)

Table 3 (*continued*)

	Substrate (S)		Tannin (T)		Dose (D), g/kg				SEM	Significance[1]				
	VH	VP	MIX	QUE	0	15	30	Contrast[2]		S	T	D	S×T	S×D
∑ SFA	61.2	58.6	59.8	60.1	59.6	60.5	59.7		0.90	***	ns	+	ns	ns
∑ MUFA	9.53	11.3	10.5	10.4	10.5	10.4	10.4		0.43	***	ns	ns	ns	ns
∑ PUFA	6.70	10.1	8.46	8.35	8.02^{c}	8.36^{b}	8.84^{a}	L Q	0.290	***	ns	***	ns	ns
∑ OBCFA	13.5	8.90	11.2	11.2	11.7^{a}	10.9^{b}	11.0^{b}	L	0.26	***	ns	**	ns	ns
∑ OCFA	4.62	3.09	3.86	3.85	3.99^{a}	3.77^{b}	3.81^{b}	L	0.238	***	ns	*	ns	ns
∑ BCFA	8.88	5.81	7.35	7.35	7.73^{a}	7.09^{b}	7.23^{b}	L	0.184	***	ns	**	ns	ns
∑ *iso*-FA	4.21	2.49	3.36	3.33	3.54^{a}	3.20^{b}	3.30^{ab}	na[3]	0.248	***	ns	*	ns	*
∑ *anteiso*-FA	4.68	3.32	3.99	4.01	4.19^{a}	3. 89^{b}	3.92^{b}	L	0.090	***	ns	***	ns	ns
SFA/PUFA	9.19	5.83	7.42	7.60	7.84^{a}	7.58^{a}	7.12^{b}	L Q	0.345	***	ns	***	ns	ns
PUFA *n*-6/*n*-3	1.88	1.18	1.53	1.52	1.57	1.50	1.51		0.054	***	ns	ns	ns	ns
BH intermediates	5.57	8.27	6.89	6.95	7.03	6.93	6.80		0.383	***	ns	ns	ns	ns
LA BH (%)	87.5	66.3	76.8	77.0	78.1^{a}	76.9^{b}	75.7^{c}	L Q	0.86	***	ns	***	ns	ns
ALA BH (%)	68.7	89.5	78.8	79.4	80.2^{a}	79.1^{ab}	78.0^{b}	L	1.36	***	ns	**	ns	ns
RA/LA	0.128	0.076	0.103	0.102	0.118^{a}	0.090^{b}	0.098^{b}	na[3]	0.009	***	ns	**	ns	*

VH, vetch hay; VP, vetch pasture; MIX, mixture of hydrolysable and condensed tannins; QUE, condensed tannins; SA, stearic acid; OA, oleic acid; VA, vaccenic acid; LA, linoleic acid; RA, rumenic acid; ALA, α-linolenic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; OBCFA, odd- and branched-chain fatty acids; OCFA, odd- chain fatty acids; BCFA, branched-chain fatty acids; BH, biohydrogenation; SEM, standard error of means.

[a–c] means without a common superscript letter are significantly different.

[1] ns, non-significant; +, $P < 0.100$; *, $P < 0.050$; **, $P < 0.010$; ***, $P < 0.001$. T × D and S × T × D interactions were never found to be significant.

[2] Significance ($P < 0.05$) of linear (L) and quadratic (Q) responses to dose effect. na, not applicable.

[3] Substrate-specific contrasts for D×S interaction: VH = L, VP = ns.

[4] Substrate-specific contrasts for D×S interaction: VH = Q, VP = LQ.

[5] Substrate-specific contrasts for D×S interaction: VH = L, VP = LQ.

to QUE. Bhatta et al. (2009) found HT and CT together to be more effective in reducing methanogenesis compared to HT alone, hypothesizing a synergetic activity of different tannin molecules that could fits our results. The occurrence of a significant difference between the two tannin extracts only at the highest dose of supplementation suggests a threshold effect that further studies on dietary tannin dose may explain.

However, in the current experiment, tannin extracts supplementation induced a switch in fermentation pathways towards the production of CO_2 detrimental to CH_4 production, regardless of the tannin type. The lack of a decrease in CH_4 production per g of DDM suggests that tannins effect on CH_4 was mainly due to the depression of fibre degradability, although some authors found HT to be more effective than CT in reducing CH_4 emission per kg DM, probably thanks to the direct inhibition of archaea-methanogens (Denman and McSweeney, 2006; Jayanegara et al., 2009).

4.4. Interaction between substrate and tannin dose on fatty acid profile

The FA profiles of VH and VP residues after fermentation are consistent with those of the substrates. The lower proportion of OBCFA – a major marker for microbial activity (Vlaeminck et al., 2006) – in VP treatment is likely related to a dilution effect due to the greater amount of PUFA, rather than to a greater antimicrobial effect. Indeed, BH intermediates were higher for VP treatment than for VH, probably because plant endogenous lipases enhance lipolysis and following BH in green forages (Doreau et al., 2005). The reverse estimated rates of LA and ALA biohydrogenation in VH and VP are likely related to the content of these PUFA in substrates. Consistently with Maia et al. (2007), the rate of BH was higher for the prevailing PUFA in substrate, as a result of their potential toxicity for ruminal microorganisms.

The decrease of RA/LA ratio in the presence of tannin extracts observed in the present study indicates a slowdown of the first steps of BH, in agreement with literature (Carreño et al., 2015; Campidonico et al., 2016; Natalello et al., 2020). However, it occurred only with tannin supplementation in VH treatment, probably because of the lower proportion of LA in VH compared to VP. Also, whereas SA proportion decreased with tannin supplementation, in accordance with LA and ALA rate of BH, the proportions of VA and RA were not affected, suggesting an overall depression of the further steps of BH.

Similar to RA/LA ratio, C15:0 *iso*, C16:0 *iso* and, consequently, total *iso*-FA proportions decreased only when tannin extracts were added to VH. This effect could be linked to the lower CP content of VH, since *iso*-FA are synthesised by ruminal bacteria using *iso*-VFA, products of protein degradation (Kaneda, 1991). On the contrary, C17:0 *anteiso* proportion decreased along with tannin supplementation dose only in VP treatment, interestingly following a similar pattern compared to valerate proportion, thus suggesting a relation between the two. The *in vitro* reduction of valerate by adding tannin in fresh forage substrate and the lack of an effect in dried forages was also observed by Rufino-Moya et al. (2019). However, C17:0 *anteiso* bacterial synthesis seems rather related to 2-methylbutyrate (Kaneda, 1991), a branched VFA not investigated in the present experiment, reported to often coelute with *iso*-valerate in gas-chromatographic analysis (Roman-Garcia et al., 2016).

On the basis of these results, we hypothesize that incubation substrates tested in this experiment selected different microorganisms with different sensitivity to tannins activity. Indeed, although it was not investigated in this experiment, microbial population is the main responsible for rumen FA profile and BH (Lourenço et al., 2010) and diet is likely the major factor affecting rumen microbiota composition (Ellison et al., 2017).

5. Conclusions

The *in vitro* effectiveness of tannin extracts may change according to substrate composition. In particular, with a substrate poor in protein and/or rich in fibre such as hay, tannins exert a more evident effect on rumen metabolism. Therefore, if these results will be confirmed in further *in vivo* studies and on farm, a lower dose of dietary tannins could be effective in seasons when animals are mostly fed dry forages, compared to season of green pasture availability. Moreover, further research should investigate the tannin sensitivity of the different rumen microorganism species selected by hay-based or pasture-based diets.

In the present experiment, CT or a mixture of CT and HT exerted similar effects on BH, whereas they affected differently fermentation parameters. The mixture of CT and HT was found to be more effective in protecting proteins from degradation, probably thanks to an enlarged binding spectrum, especially in the presence of hay (a low protein substrate). This could mean that mixtures of tannins should be preferred in grazing period, when diet is rich in degradable protein, in order to minimize ammonia emissions, despite higher dose may be necessary. In the context of extensive husbandry facing seasons of different pasture availability, this study leads the way to *in vivo* trials with potentially important practical implications.

Authorship contributions

Conception and design of study: VN, AP, MC, RM, GL, BV, AN.
Acquisition of data: VN, AT, RM.
Analysis and/or interpretation of data: RM, VN.
Drafting the manuscript: RM.
Revising the manuscript critically for important intellectual content: VN, AP, MC, GL, BV, AN.
Approval of the version of the manuscript to be published: VN, AP, MC, RM, GL, BV, AN, AT.

Declaration of Competing Interest

The author wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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