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Supplementation of *Acacia mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep

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Abstract. The objective of this experiment was to assess the effects of a partial replacement of ryegrass (*Lolium perenne*) by red clover (*Trifolium pratense*) or alfalfa (*Medicago sativa*) supplemented with 0 or 41 g *Acacia mearnsii* extract (containing 0.615 g/g condensed tannins)/kg dietary dry matter on nitrogen turnover and methane release by sheep, using the respiration chamber technique. Across all variables, there was no significant interaction between basal diet and tannin supplementation. The partial replacement of the grass by the legumes remained without effect on the amounts of nitrogen excreted through faeces or urine. Nitrogen and energy utilisation was lower ($P < 0.05$) with ryegrass–alfalfa than with ryegrass alone, and methane release (kJ/MJ gross energy intake) was higher ($P < 0.05$) with ryegrass–red clover than with ryegrass alone. Tannin supplementation decreased ($P < 0.05$) ruminal ammonia concentration and urinary nitrogen excretion without affecting body nitrogen and energy retention, and reduced ($P < 0.001$) methane release by 13% on average. The results suggest that supplemented *Acacia mearnsii* tannins can be useful in mitigating methane and potential gaseous nitrogen emissions, whereas a replacement of grass by legumes obviously shows no advantage in this respect.

Additional keywords: energy expenditure, feed additives, grass–legume associations, lucerne, ruminant.

Introduction

There is growing interest in the use of grass–legume associations and in the identification of efficient natural feed additives, particularly in organic farming systems. One major goal is to improve the metabolic protein supply of the animals without the need of exogenous mineral nitrogen (N). Ideally, such production strategies should aim at limiting environmentally harmful emissions such as methane and N. However, there is little and contradictory information available on methane release from ruminants fed grass–legume diets compared with grass-alone diets, and this information was mostly assessed by indirect techniques. Mbanzamihigo *et al*. (2002), using ethane (C_2H_6) as a tracer gas, found no effect of dietary legume proportion on methane emission of sheep fed fresh ryegrass–white clover diets. Murray *et al*. (2001), using a ventilated polythene tunnel, observed 30% higher methane emissions from sheep on ryegrass–white clover pastures than on ryegrass alone, but in this study forage consumption was not determined and an associated increase in dry matter (DM) intake could have been the major source of variation. In contrast, McCaughey *et al*. (1999), using the sulfur hexafluoride (SF_6) tracer-gas technique, observed 25% lower methane yields (71 *v.* 95 kJ/MJ of gross energy intake) for cattle grazing grass–alfalfa pastures than for cattle grazing grass-alone pastures. Finally, Benchaar *et al*. (2001), using a modelling approach, predicted that, due to differences in the chemical composition, methane losses expressed as a proportion of gross energy intake were 28% higher for alfalfa hay than for timothy hay.

Forage grasses and legumes from temperate regions are often rich in rumen-degradable protein, which may result in a low efficiency of N utilisation and high metabolic energy costs for N excretion with urine. Positive responses in body N retention to supplementary by-pass protein have been documented for fresh forage-based diets (Fraser *et al*. 1990), indicating inadequate metabolic protein supply in ruminants fed on these types of diets. Reducing methane emission and ruminal protein degradation in grass and legume-based diets could also result in decreased metabolic energy losses and gaseous nitrogen emissions from manure during storage and application. Tannins, prevalent in many plants, may reduce ruminal protein degradation and increase duodenal protein flow when provided at moderate doses of 20–45 g/kg of forage dry matter (Min *et al*. 2003). Results from recent experiments furthermore suggest that tannins in the diet

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might reduce ruminal methane production (Waghorn *et al*. 2002; Hess *et al*. 2003, 2004) but, given at higher doses, they may also adversely affect animal performance (Norton 2000). However, as far as is known, no quantitative direct *in vivo* measurements of methane have been performed concerning the effects of condensed tannins and temperate legume supplements and their interaction.

The hypotheses tested in the present study were: (*i*) that the partial replacement of ryegrass with red clover or alfalfa would increase the metabolic protein supply of ruminants without affecting methane release, and (*ii*) that the addition of a commercially available product based on condensed tannins would also improve metabolic protein supply and, simultaneously, suppress methanogenesis.

Materials and methods

Experimental forages

The experimental haylages were prepared from pure swards of ryegrass (*Lolium perenne* cv. Lacerta), red clover (*Trifolium pratense* cv. Leisi), and alfalfa (*Medicago sativa* cv. Sanditi). The 3 swards were established in spring 2002 at the research station Chamau of the Swiss Federal Institute of Technology (Zug, Switzerland, 47°12′N, 8°24′E; 394 m elevation, 8.8°C annual mean temperature, 1170 mm annual precipitation). The experimental plots were fertilised with 100 kg of total N per hectare (in the form of liquid cattle manure) before establishment. The forage of the second cut (4 weeks of regrowth) was intensively field-wilted to ~700 (650–770) g/kg DM and conserved without additives in small, high-pressure bales weighing ~25 kg. There was no rainfall during harvest. Due to the high DM content of the wilted forages, the fermentation activity was low, which is reflected by the very low concentration of lactic acid and the high terminal pH of the 3 haylages (Table 1). In order to prevent spoilage after being opened, bales were stored in the refrigerator from then on. All haylages presented high crude protein (CP) contents (>200 g/kg DM). The grass was higher in neutral detergent fibre (NDF) and lower in acid detergent fibre (ADF) than the legumes, with alfalfa presenting the highest content of acid detergent lignin.

Table 1. Analysed composition (g/kg dry matter) and pH of the experimental forages ($n = 6$, unless otherwise stated)

	Grass	Red clover	Alfalfa
Dry matter (g/kg)	768	743	654
Organic matter	871	862	868
Crude protein	243	222	213
Neutral detergent fibre	459	361	423
Acid detergent fibre	264	271	341
Acid detergent lignin	27	39	72
Hemicellulose[A]	195	90	82
Cellulose[B]	237	232	269
Lactic acid[C]	1.43	0.71	1.01
Ammonia[C]	0.47	0.70	1.13
pH[C]	6.20	5.82	5.81

[A]Calculated as difference between neutral detergent fibre and acid detergent fibre.
[B]Calculated as difference between acid detergent fibre and acid detergent lignin.
[C]$n = 1$.

Experimental procedure

Six growing, castrated male lambs of the Swiss White Hill breed with an initial bodyweight (BW) of 25.4 (±2.1) kg were allocated to 6 dietary treatments in a 6 × 6 Latin-square design with a 3 × 2-factorial arrangement ($n = 6$). Treatments consisted of 3 basal diets as one of the main factors: (*i*) ryegrass alone, (*ii*) ryegrass and red clover (1 : 1, DM basis) and (*iii*) ryegrass and alfalfa (1 : 1, DM basis), with the latter 2 being mixed directly before feeding. All 3 diets were evaluated with or without the addition of 41 g/kg of dietary DM of a crude tannin extract (stated content of condensed tannins of 0.725 g/g DM; Weibull Black, Tanac S.A., Montenegro, Brazil). The actually measured content of condensed tannins was 0.615 g/g DM as determined by the butanol/HCl procedure described by Terrill *et al*. (1992), which is equivalent to a supplementation of 25 g condensed tannins/kg dietary DM. The extract had been obtained from the bark of *Acacia mearnsii* through extraction with hot water, vacuum evaporation and subsequent spray-drying. Each animal received the 6 diets in a different sequence. Daily, 75 g of forage DM were offered per kg of metabolic bodyweight ($BW^{0.75}$), which was ~10% above the assumed voluntary intake. The feeds were provided in one meal per day at 09 00 hours. The animals had free access to fresh water and its consumption was measured. They received 10 g/day of a commercial mineralised salt mixture for growing sheep. The experiment was carried out in accordance with the Swiss guidelines for animal welfare and the experimental protocol was approved by the Veterinary Department of Zurich, Switzerland.

The 6 experimental periods lasted for 21 days each, including 12 days of adaptation to the respective experimental diets, 8 days (Days 13 through 20) of complete collection of faeces and urine, 2 days (Days 19 and 20) of quantitative measurement of gaseous exchange (two 22.5-h runs) in dual open-circuit respiration chambers, and a final day (Day 21) reserved for rumen fluid sampling. The respiration chambers (5.44 m^3 each) were aluminium-glass constructions allowing intervisibility of the sheep, and were air-conditioned (av. temp. 18°C, relative humidity 60%, air flow 8.3 m^3/h). The animals were weighed directly before feeding in the morning on Days 3, 5, 7, 9, 11, 13 (for adjustment of the daily DM supply), and Day 21 of every experimental period. The average of Days 13 and 21 was used as bodyweight in data evaluation, and feed DM supply was kept constant during that period. From Days 13 through 21, feed refusals were recorded daily before the next feed portions were offered, and samples of refusals and of each diet component were collected and stored at −20°C. Prior to laboratory analyses, samples of refusals and of diet components were pooled across days within period. All samples were lyophilised and ground in a laboratory mill to pass a 1-mm screen. During the collection periods, faeces and urine were collected completely and separately once per day and stored at –20°C. The urine was immediately acidified in 3 M sulfuric acid to avoid N volatilisation. Subsamples consisting of fresh, non-acidified urine were obtained once daily for subsequent analysis of carbon (C) content. At the end of the collection periods, faeces and urine were pooled across days within period for each animal and stored at −20°C until chemical analyses. Part of the faeces were lyophilised for 48 h and ground in a laboratory mill to pass a 1-mm screen for the determinations of DM, total ash, gross energy (GE), and fibre content. The remaining part was stored at −20°C for all other analyses. On Day 21 of every period, 4 h after feeding, rumen fluid samples were taken through the oesophagus by a flexible stomach tube and strained through 4 layers of compress gauze. In these samples, pH, ammonia concentration, and microbial counts were determined immediately, and subsamples of 1.8 mL were preserved for the analysis of volatile fatty acids (VFA) by adding 0.2 mL of mercuric chloride solution (12.5 g/L) before storage at −20°C. On Days 7 and 21, blood samples were drawn from the jugular vein 4 h after feeding, heparinised, centrifuged at 2000*G* for 20 min at 4°C, and stored at −20°C until being analysed.

Laboratory analyses

Feeds, refusals, and lyophilised faeces were analysed for DM (105°C for 12 h), total ash (500°C for 3 h), NDF, and ADF (Van Soest *et al*. 1991). Values of NDF and ADF were corrected for ash content, and a heat-stable α-amylase was used for NDF determination, but no sodium sulfite was added to the NDF solution. Additionally, feeds were subject to analysis of acid detergent lignin using the sulfuric acid method (Robertson and Van Soest 1981). Nitrogen (CP = 6.25 × N) was determined in feeds, refusals, non-lyophilised faeces, and acidified urine, with an automatic C/N analyser (CN-2000, Version 2.2, Leco Instrumente GmbH, Kirchheim, Germany). With the same analyser, the C content of feeds, refusals, non-lyophilised faeces, and non-acidified urine was measured. Acidified urine was analysed for allantoin by high-performance liquid chromatography (HPLC), using the method of Rosskopf *et al*. (1991). Gross energy contents of feeds, refusals, and lyophilised faeces were assessed through an anisothermic calorimeter (C 700 T System, IKA-Analysentechnik GmbH, Heitersheim, Germany). Additionally, the fresh haylages were analysed for their concentration of lactic acid using gas-liquid chromatography (HP-5890 series II, Hewlett Packard, Waldbronn, Germany) according to Alén *et al*. (1985), and water extracts were prepared to measure pH and ammonia concentration with a potentiometer (model 632, Metrohm, Herisau, Switzerland) equipped with the respective electrodes.

Ruminal fluid pH and ammonia concentration were determined as described above for the haylages. Immediately after collection, ciliate protozoa and bacteria were microscopically enumerated using 0.1- and 0.02-mm-deep Bürker counting chambers (Blau Brand, Wertheim, Germany), respectively. Determination of VFA was performed on a gas chromatograph (GC Star 3400 CX, Varian, Sugarland, TX, USA) according to Tangerman and Nagengast (1996). Blood plasma concentration of urea was analysed by means of a commercial kit (Roche Diagnostics, Basle, Switzerland) on a COBAS INTEGRA automatic analyser (Roche Diagnostics, Basle, Switzerland). The data on the 2 blood samples taken per animal and period were combined for further data analysis. In the respiration chamber units, the detectors used were an Oxymat 6 (Siemens AG, Karlsruhe, Germany) for oxygen and a Binos 1001 (Fisher-Rosemount, Baar-Walterswil, Switzerland) for carbon dioxide and methane.

Calculations and statistical analysis

Urinary energy was calculated by the equation of Hoffmann and Klein (1980) from concentrations of C and N in urine. Data on gaseous exchange were used for calculation of methane energy loss and energy expenditure (total heat production) by the equations of Brouwer (1965). Total energy retention (RE) was computed as metabolisable energy (ME) less energy expenditure. Tannins were assumed to be indigestible and completely excreted with the faeces (Terrill *et al*. 1994). The amounts of nutrients consumed and faecally excreted were adjusted accordingly in the tannin-supplemented sheep before the calculation of the coefficients of apparent total tract digestibility. The values therefore represent the digestibilities of the forage part of the diet. Variables based on daily amounts were mostly given in relation to $BW^{0.75}$ in order to account for certain differences in BW and the associated level of feed allocation. Data were subjected to analysis of variance for a 6 × 6 Latin-square design with a 3 × 2-factorial arrangement of the treatments, using the GLM procedure of SAS (1996). Basal diets (ryegrass alone, ryegrass and red clover, ryegrass and alfalfa), tannin treatments, and interaction between basal diets and tannin treatments were considered as main effects. Experimental period and animal were treated as additional sources of variation. Because the interactions were never significant ($P > 0.05$), data are presented as overall basal diet means and tannin treatment means, respectively, together with the probability of error (P) and the standard errors of the means (s.e.m.). Multiple comparisons among diet means were performed by Tukey's studentised range test.

Results

Both legume addition to the basal diet and tannin supplementation reduced ($P < 0.05$) water consumption of the sheep. Feed refusals were generally small, and DM (972 g per animal and day on average) and organic matter (OM) intakes were not different with or without legume

Table 2. Intake and apparent nutrient digestibilities in lambs fed three different basal diets either with or without addition of tannins ($n = 12$ per basal diet and $n = 18$ per tannin treatment)

Within rows, values followed by the same letter are not significantly different at $P = 0.05$

	Basal diet			Tannin		s.e.m.	*P* level		
	Grass alone	Grass/clover	Grass/alfalfa	−	+		Diet (D)	Tannin (T)	D × T
Bodyweight (BW, kg)	34.7	34.5	34.2	33.7y	35.2x	0.49	0.69	0.002	0.50
Daily intake of tap water ($g/BW^{0.75}$)	340a	321b	318b	342x	310y	5.6	0.001	<0.001	0.13
Daily nutrient intake ($g/kg\ BW^{0.75}$)									
Dry matter	70.8	69.9	69.3	68.7y	71.3x	0.94	0.26	0.003	0.67
Organic matter	62.5	61.4	61.1	60.4y	62.9x	0.82	0.22	0.001	0.84
Crude protein	17.1a	16.1b	15.8b	16.4	16.3	0.25	<0.001	0.74	0.90
Neutral detergent fibre	32.2a	28.3c	30.1b	30.3	30.1	0.35	<0.001	0.65	0.78
Acid detergent fibre	18.6b	18.6b	20.6a	19.2	19.3	0.28	<0.001	0.57	0.85
Hemicellulose	13.6a	9.7b	9.5b	11.1	10.8	0.17	<0.001	0.65	0.85
Apparent total tract digestibility									
Organic matter	0.750a	0.740a	0.707b	0.739x	0.725y	0.0072	<0.001	0.029	0.63
Crude protein	0.695	0.686	0.691	0.729x	0.652y	0.0092	0.57	<0.001	0.78
Neutral detergent fibre	0.795a	0.766b	0.679c	0.766x	0.727y	0.0099	<0.001	<0.001	0.48
Acid detergent fibre	0.778a	0.727b	0.644c	0.747x	0.685y	0.0108	<0.001	<0.001	0.11
Hemicellulose	0.825a	0.850a	0.765b	0.802y	0.824x	0.0126	<0.001	0.041	0.074

supplementation (Table 2). Tannin addition increased both DM ($P < 0.01$) and OM ($P < 0.001$) intakes, but this only slightly in absolute terms. Intakes of CP, NDF, and hemicellulose were higher ($P < 0.05$) with ryegrass alone than with the legume-supplemented diets, whereas ADF intake was increased ($P < 0.05$) by alfalfa supplementation. Apparent OM and hemicellulose digestibilities were similar with ryegrass alone and with red clover supplementation and lower ($P < 0.05$) with alfalfa supplementation. Digestibilities of NDF and ADF were highest ($P < 0.05$) with ryegrass alone, intermediate with red clover, and lowest ($P < 0.05$) with alfalfa. Apparent CP digestibility was similar ($P > 0.05$) across all basal diets. The addition of tannins did not decrease daily nutrient intake but reduced ($P < 0.05$) the apparent digestibility of all nutrients investigated except that of hemicellulose.

Ruminal fluid pH did not differ significantly among treatments and averaged 7.12 (Table 3). However, it has to be acknowledged that the ruminal fluid samples could have been contaminated with saliva and these results therefore have to be interpreted with caution. Compared with ryegrass alone, ruminal fluid ammonia concentration was increased ($P < 0.05$) with alfalfa supplementation but remained unaffected by red clover supplementation. With the addition of tannins, the ruminal fluid ammonia concentration was decreased ($P < 0.05$) by 9% on average. Total VFA concentration was similar ($P > 0.05$) in all dietary treatments and averaged 109.7 mmol/L. Legume supplementation did not affect ($P > 0.05$) either the molar proportion of the major VFA or the acetate-to-propionate ratio. The addition of tannins decreased ($P < 0.05$) the molar proportion of acetate and the acetate-to-propionate ratio and increased ($P < 0.05$) the proportion of propionate. Treatment effects on microbial counts in ruminal fluid were not significant, except for holotrich ciliate counts, which were decreased ($P < 0.05$) by the addition of tannins.

The daily N intake (per kg $BW^{0.75}$) reflected the differences in N content of the forages and was the same ($P > 0.05$) with and without tannins (Table 4). The type of basal diet had no effect ($P > 0.05$) on daily faecal or urinary N excretions, but body N retention was lower ($P < 0.05$) with alfalfa supplementation than with ryegrass alone and intermediate with red clover supplementation. In contrast, the addition of tannins clearly increased ($P < 0.001$) daily N excretion via faeces, decreased ($P < 0.001$) excretion with urine, and had no significant effect ($P > 0.05$) on the amount of N retained. The urinary N as a proportion of total N excreted remained unchanged ($P > 0.05$) with legume supplementation, but decreased ($P < 0.001$) with the addition of tannins. When expressed as a proportion of total dietary N intake, N excretions in faeces were not affected ($P > 0.05$) by legume supplementation, but the proportion of N excreted in urine was increased and the proportion of N retained was decreased ($P < 0.05$) with alfalfa supplementation. Tannins resulted in a shift of relative N excretion from urine to faeces at the same proportionate N retention. Blood urea N concentration was not affected ($P > 0.05$) by the type of basal diet but decreased ($P < 0.01$) when tannins were added to the diet. The urinary excretion of allantoin per kg of $BW^{0.75}$ was lower ($P < 0.05$) with alfalfa supplementation than with red clover and was decreased ($P < 0.05$) by the tannins.

Table 3. Ruminal fluid properties and microbial counts in lambs fed three different basal diets either with or without addition of tannins ($n = 12$ per basal diet and $n = 18$ per tannin treatment)

Within rows, values followed by the same letter are not significantly different at $P = 0.05$

	Basal diet			Tannin		s.e.m.	*P* level		
	Grass alone	Grass/clover	Grass/alfalfa	−	+		Diet (D)	Tannin (T)	D × T
Rumen fluid properties									
pH	7.16	7.14	7.07	7.13	7.12	0.067	0.43	0.83	0.17
Ammonia (mmol/L)	19.3b	18.3b	21.7a	20.7x	18.9y	0.90	0.004	0.020	0.13
Volatile fatty acids									
Total (mmol/L)	102.9	116.3	109.9	107.2	112.2	5.64	0.081	0.30	0.32
Acetate (mol %)	63.0	64.2	64.5	65.2x	62.6y	0.75	0.13	<0.001	0.91
Propionate (mol %)	26.9	26.0	24.9	25.1y	26.8x	0.90	0.095	0.034	0.98
n-Butyrate (mol %)	5.4	6.1	5.8	5.5y	6.1x	0.27	0.052	0.013	0.89
Iso-butyrate (mol %)	0.80	0.69	0.81	0.74	0.80	0.106	0.45	0.46	0.92
n-Valerate (mol %)	2.21ab	1.95b	2.34a	2.01y	2.32x	0.114	0.008	0.003	0.38
Iso-valerate (mol %)	1.63a	1.08b	1.63a	1.49	1.41	0.089	<0.001	0.29	0.19
Acetate-to-propionate ratio	2.36	2.50	2.65	2.64x	2.37y	0.109	0.054	0.006	0.95
Microbial counts									
Bacteria ($10^{-9} \times N$/mL)	6.98	6.16	7.14	6.38	7.14	0.662	0.30	0.18	0.44
Ciliate protozoa ($10^{-5} \times N$/mL)	3.23	2.67	2.72	2.60	3.15	0.425	0.36	0.13	0.34
Holotrichs	0.16	0.14	0.07	0.17x	0.08y	0.055	0.23	0.044	0.81
Entodiniomorphs	3.08	2.53	2.65	2.43	3.07	0.417	0.41	0.075	0.29

Table 4. Nitrogen balance, blood urea concentration and urinary excretion of allantoin in lambs fed three different basal diets either with or without addition of tannins ($n = 12$ per basal diet and $n = 18$ per tannin treatment)

BW, Bodyweight. Within rows, values followed by the same letter are not significantly different at $P = 0.05$

	Basal diet			Tannin		s.e.m.	*P* level		
	Grass alone	Grass/clover	Grass/alfalfa	−	+		Diet (D)	Tannin (T)	D × T
N intake (g/kg $BW^{0.75}$·day)	2.74a	2.58b	2.53b	2.62	2.62	0.040	<0.001	0.99	0.90
Nitrogen excreted (g/kg $BW^{0.75}$·day)									
Faeces	0.84	0.81	0.79	0.71y	0.92x	0.030	0.25	<0.001	0.99
Urine	1.48	1.43	1.49	1.57x	1.36y	0.029	0.096	<0.001	0.72
Total	2.32	2.24	2.28	2.28	2.28	0.048	0.28	0.99	0.88
N retention (g/kg $BW^{0.75}$·day)	0.42a	0.34ab	0.25b	0.34	0.34	0.041	0.004	0.98	0.59
Urinary N (mg/g N excreted)	638	638	656	689x	596y	7.6	0.088	<0.001	0.95
Nitrogen balance (g/kg N intake)									
Faeces	307	314	312	271y	351x	9.2	0.57	<0.001	0.78
Urine	540b	554b	589a	600x	519y	10.7	0.002	<0.001	0.65
Retention	153a	132ab	99b	130	130	15.1	0.017	0.99	0.64
Blood urea N (mg/dL)	17.9	17.3	17.4	18.0x	17.1y	0.35	0.26	0.003	0.61
Daily urinary excretion of allantoin N (mg/kg $BW^{0.75}$)	63.4ab	64.1a	59.2b	64.3x	60.2y	1.92	0.038	0.016	0.66

At a similar GE intake level, the intakes of digestible energy (DE) and ME were highest ($P < 0.05$) with ryegrass alone, intermediate with red clover, and lowest ($P < 0.05$) with alfalfa supplementation (Table 5). Supplementary tannins decreased ($P < 0.05$) DE intake but did not affect ($P > 0.05$) ME intake. Energy losses through faeces increased and losses through urine decreased ($P < 0.05$) with alfalfa supplementation, but were not affected ($P > 0.05$) by red clover. Energy expenditure (total heat production) was higher ($P < 0.05$) with ryegrass alone than with the legume-supplemented diets. With the ryegrass alone, energy retention (RE) was higher ($P < 0.05$) compared with the alfalfa-supplemented diet. Tannins increased ($P < 0.001$) energy losses through faeces and decreased energy losses through urine ($P < 0.001$) and methane ($P < 0.01$). Despite increased ($P < 0.001$) total energy losses, tannins neither affected ($P > 0.05$) energy expenditure nor RE. The utilisation of GE (DE/GE, ME/GE, RE/GE) declined ($P < 0.05$) with alfalfa supplementation but was not affected by red clover. When expressed as a proportion of GE intake, DE and ME were decreased ($P < 0.001$) by the addition of tannins, which was not the case for RE ($P > 0.05$). Treatment effects on utilisation of ME for RE were not significant.

Table 5. Balance and utilisation of energy in lambs fed three different basal diets either with or without addition of tannins ($n = 12$ per basal diet and $n = 18$ per tannin treatment)

BW, Bodyweight. Within rows, values followed by the same letter are not significantly different at $P = 0.05$

	Basal diet			Tannin		s.e.m.	*P* level		
	Grass alone	Grass/clover	Grass/alfalfa	−	+		Diet (D)	Tannin (T)	D × T
Energy intake (kJ/kg $BW^{0.75}$·day)									
Gross energy (GE)	1359	1321	1322	1306y	1362x	183	0.078	0.001	0.86
Digestible energy (DE)	940a	909b	865c	919x	891y	128	<0.001	0.015	0.43
Metabolisable energy (ME)	780a	746b	711c	750	741	125	<0.001	0.41	0.28
Energy loss (kJ/kg $BW^{0.75}$·day)									
Faeces	419b	413b	457a	388y	472x	137	0.009	<0.001	0.81
Urine	95a	93ab	87b	98x	85y	24	0.015	<0.001	0.50
Methane	66	70	67	71x	64y	19	0.099	<0.001	0.72
Energy expenditure	587a	566b	557b	575	565	84	0.006	0.18	0.15
Total loss	1167	1141	1169	1131y	1187x	212	0.37	0.005	0.81
Retention (RE, kJ/kg $BW^{0.75}$·day)	192a	180ab	154b	175	176	134	0.026	0.93	0.75
Utilisation of GE (kJ/kJ)									
DE/GE	0.693a	0.688a	0.655b	0.703x	0.654y	0.0076	<0.001	<0.001	0.53
ME/GE	0.574a	0.564a	0.538b	0.574x	0.544y	0.0078	<0.001	<0.001	0.32
RE/GE	0.142a	0.136ab	0.116b	0.133	0.129	0.0099	0.043	0.60	0.76
Utilisation of ME (kJ/kJ)									
RE/ME	0.245	0.240	0.217	0.231	0.235	0.0150	0.13	0.73	0.73

Legume supplementation decreased ($P < 0.05$) daily O_2 consumption, but only supplementation of alfalfa, and not of red clover, significantly decreased CO_2 release (Table 6). The addition of tannins had no effect ($P > 0.05$) on either O_2 consumption or CO_2 release. Methane release (kJ/MJ GE intake) was higher ($P < 0.05$) with red clover supplementation than with grass alone. When related to OM and NDF digested, methane release was higher ($P < 0.05$) with any legume supplementation. Methane release decreased ($P < 0.001$) by 13% when tannins were added to the diet. This decline was quite similar in extent, with 15, 13, and 11% when supplementing the tannins to the ryegrass-alone, the ryegrass–clover and the ryegrass–alfalfa diet, respectively. The addition of tannins also decreased methane release relative to OM digested ($P < 0.01$), whereas the effect on methane release per unit of NDF digested was not significant.

Discussion

The present study focussed on 2 feeding measures that are applicable in forage-based production systems and are presumed to have beneficial environmental effect: (*i*) addition of forage legumes (known to improve farm N balance in zero- or low-mineral N fertiliser systems) and (*ii*) supplementation of tannins. The haylages, although not being intensively fermented during ensiling, were of high quality and were well consumed. This procedure allowed the offering of a constant quality throughout the experiment, whereas hay production from pure swards of legumes would have been impractical due to leaf losses.

Effects of legume supplementation

In contrast to other studies (McCaughey *et al*. 1999; Broderick *et al*. 2000), dry matter intake was independent of legume supplementation. The limited excess of DM offered over that effectively consumed (+5 to +9%) might not have been sufficient to allow the full expression of potentially different voluntary DM intakes. Fibre digestibility (NDF and ADF) was lower with legume supplementation, and this decrease was more pronounced with the alfalfa cultivar used than with the red clover. Digestibilities of OM and hemicellulose followed a similar pattern. This was probably related to the considerably higher content of lignin in alfalfa compared with that in the grass and the red clover cultivars, despite the same regrowth period before harvest in all forages. However, other studies illustrate that this could be characteristic for red clover and alfalfa in general. Accordingly, Varga *et al*. (1985) reported a lower digestibility of cell wall components of alfalfa than of grass silage, and Broderick *et al*. (2000) found lower apparent digestibilities of OM, NDF, ADF, and hemicellulose when replacing red clover with alfalfa.

Due to the slightly higher N content of the ryegrass compared with the red clover and the alfalfa, the N intake was larger with the grass-alone diet than with legume supplementation. This, however, did not result in a greater faecal N loss, a phenomenon explained by the dependence of apparent N digestibility on fermentable OM rather than on dietary N content, as outlined in the concept of the 'standard crude protein digestibility' (Schwarting and Kaufmann 1978). When supplemented with alfalfa, the sheep had higher ruminal ammonia concentrations and higher urinary N excretions relative to N intake than with the other forages. Reasons for that could have been a high proportion of non-protein nitrogen in alfalfa (Broderick *et al*. 2000) and the observed limited availability of fermentable energy as the prerequisite for microbial protein synthesis. This is obvious from both the lower OM and fibre degradation (fermentable energy) and the lower urinary allantoin excretion (indicative of microbial protein synthesis; Makkar and Chen 2004) compared with the other treatments. Overall, the partial replacement of ryegrass with alfalfa decreased the efficiency

Table 6. Gaseous exchange of lambs fed three different basal diets either with or without addition of tannins ($n = 12$ per basal diet and $n = 18$ per tannin treatment)

BW, Bodyweight. Within rows, values followed by the same letter are not significantly different at $P = 0.05$

	Basal diet			Tannin		s.e.m.	*P* level		
	Grass alone	Grass/clover	Grass/alfalfa	−	+		Diet (D)	Tannin (T)	D × T
Gaseous exchange (L/day)									
Oxygen consumption	396a	382b	375b	383	386	4.3	0.003	0.34	0.28
Carbon dioxide release	415a	406a	391b	403	405	5.5	<0.001	0.53	0.54
Methane release	23.8	25.1	23.9	25.1x	23.4y	0.74	0.17	0.010	0.74
Respiratory quotient	1.05b	1.06a	1.04b	1.05	1.05	0.004	<0.001	0.34	0.64
Relative methane release[A]									
L/kg DM intake	23.6	25.4	24.5	26.1x	22.9y	0.72	0.066	<0.001	0.63
kJ/MJ GE intake	48.6b	53.1a	50.8ab	54.4x	47.3y	1.52	0.027	<0.001	0.74
L/kg OM digested	36.4b	39.9a	40.1a	40.3x	37.4y	1.19	0.008	0.007	0.59
L/kg NDF digested	65.2b	81.7a	83.3a	78.1	75.4	2.36	<0.001	0.18	0.40

[A]DM, dry matter; OM, organic matter; NDF, neutral detergent fibre; GE, gross energy.

of N utilisation for body protein synthesis and red clover supplementation was only slightly inferior to ryegrass-alone feeding. The same holds true for GE utilisation because protein retention made up a considerable proportion of total energy retention in the growing lambs.

The average daily methane production of 24.3 (±3.97) L per animal (50.9 (±5.22) kJ/MJ of GE intake) across all forage types, although being based on forage-alone diets, was lower than the mean value reported in the literature for growing lambs with a BW of 25–45 kg (72.2 kJ/MJ; reviewed by Pelchen and Peters 1998). However, it was within the range of 46–57 kJ/MJ reported by Ulyatt *et al*. (1997) and Mbanzamihigo *et al*. (2002) for sheep grazing ryegrass- and clover-based pastures. Little is known about the effect of forage legume supplementation on digestive tract methane release in ruminants, and the few *in vivo* studies carried out yielded contradictory results. Whereas Mbanzamihigo *et al*. (2002) found no effect of the dietary legume proportion on methane emission of sheep grazing ryegrass–white clover pastures, Murray *et al*. (2001) observed considerably higher methane emissions on ryegrass–white clover pastures than on ryegrass alone. Waghorn *et al*. (2002) reported lower methane yields (g/kg of DM intake) for sheep fed exclusively alfalfa or red clover than for sheep fed ryegrass–white clover pasture. Similarly, Varga *et al*. (1985) and McCaughey *et al*. (1999) observed lower methane yields for cattle fed alfalfa silage or grass–alfalfa pastures than for cattle fed grass alone. If not the result of inconsistencies of the various methane measurement techniques applied (ranging from relatively rough estimates based on wind tunnels or tracer gas techniques to precise measurements in respiration chambers), this apparent contradiction between studies is probably related to the contrasting chemical composition of the forages used in the different experiments. In the studies of Varga *et al*. (1985), McCaughey *et al*. (1999), and Waghorn *et al*. (2002), the grass-based diets contained much less CP and much more NDF than the experimental legumes, which was not the case in the present experiment. Thus variations in the chemical composition (and, with that, fertilisation and maturity at harvest, etc.) within a given temperate forage species may be at least as important in determining the extent of methanogenesis during digestion as the differences among forage species. The results obtained in the present study indicate that the partial replacement of ryegrass by red clover or alfalfa in diets for ruminants will not reduce digestive tract methane release when the grass fed is of excellent quality. In relation to productivity (represented here by body N retention), methane emissions were even clearly higher with legume supplementation.

Effects of tannins

Forage intake was not negatively affected by the addition of *Acacia mearnsii* tannins, which is in agreement with results from previous studies that indicated that forage intake was not depressed when tannin concentration in the total diet remained below 50 g/kg DM (Barry and McNabb 1999). With higher tannin concentrations (>60 g/kg), significant decreases in feed intake have been reported (Bhatta *et al*. 2002).

It is known that tannins modify digestion and utilisation of nitrogenous compounds in ruminants by different mechanisms (Min *et al*. 2003). There is general agreement that tannins decrease ruminal protein degradation, mainly through the formation of tannin–protein complexes that are minimally degraded by ruminal microbes (Reed 1995; Broderick and Albrecht 1997). In the present study, tannins decreased ruminal ammonia and blood urea concentration, suggesting that less protein was degraded in the rumen. Similar observations have been made *in vitro* (Hess *et al*. 2003, 2004) and *in vivo* (Carulla *et al*. 2001) when supplementing tanniniferous forages. Whether or not this tannin-bound feed protein, escaping the rumen, is digested and absorbed by the animal depends on a range of factors (Mueller-Harvey and McAllan 1992; Min *et al*. 2003). Consequently, contradictory results on the effect of tannins on protein digestion and absorption in the lower gut are described. Although some studies have demonstrated an overall improvement of N utilisation due to an increase in bypass feed protein and in absorption of essential amino acids (Min *et al*. 2003), others failed to show a positive effect on N balance but demonstrated that tannins can decrease apparent total tract digestibility of crude protein (Norton 2000). The latter was also the case in the present study. However, because condensed tannins also may increase endogenous N losses (Norton 2000), it is unclear to what extent the increased faecal N losses were really due to a decreased true digestibility of the feed protein.

There is evidence from *in vitro* studies that tannins may increase the efficiency of microbial protein synthesis in the rumen (Makkar *et al*. 1995*b*; Getachew *et al*. 2001). In contrast, most *in vivo* studies have shown that with tannins the efficiency of microbial protein synthesis in the rumen is suppressed (Ben Salem *et al*. 2002) or remains unaffected (McNeill *et al*. 2000; Carulla *et al*. 2001; McSweeney *et al*. 2001; Min *et al*. 2003). In the present study, the decrease by 6% found in the excretion of allantoin in the tannin-supplemented sheep suggests that the rumen microbial protein synthesis was reduced by the tannins, which could explain the lack of effect on body N retention.

Tannin addition decreased the counts of holotrich ciliate protozoa without affecting the counts of entodiniomorphs. Although effects of tannins on rumen protozoal counts are variable (Makkar 2003), a certain evidence exists for lower protozoal numbers in the presence of tannins (Yáñez Ruiz *et al*. 2004), and holotrichs seem to be

more susceptible in that respect than entodiniomorphs (Makkar *et al*. 1995*a*).

The higher faecal N loss as caused by tannin supplementation was completely compensated by a reduction in urinary N, most likely resulting from a lower ammonia absorption from the rumen. This was associated with a decreased urine volume with the consequence of a lower water consumption. The shift in N excretory pattern from urine to faeces as a result of feeding with tannins is of practical relevance because urinary N is prone to ammonia emission during manure storage (Śliwiński *et al*. 2004). Previous own studies (Śliwiński *et al*. 2004), feeding very low tannin doses to cows, indicated that this dietary measure might have the potential to decrease ammonia N emission from animal excreta. The favourable effects of tannins in this respect could be 2-fold: (*i*) reducing the amount of easily volatile urine N and (*ii*) continuing their protein-binding activity during manure storage.

Condensed tannins may also affect fibre degradation (Reed 1995). In the present investigation, tannin supplementation suppressed NDF and ADF digestibilities without decreasing hemicellulose digestibility. This suggests that the degradation of cellulose (a major part of ADF) was reduced by condensed tannins, which could be related to a selective suppression of cellulolytic bacteria by condensed tannins (McSweeney *et al*. 2001). The reduced fibre digestion was associated with the expected shift in the VFA profile from acetate to propionate at a constant total VFA concentration in ruminal fluid. Similar shifts in the acetate-to-propionate ratio have been observed previously with tannin-supplemented diets (Makkar *et al*. 1995*b*) and may also explain why the reduced fibre digestion did not adversely affect energy retention and utilisation. However, it has to be acknowledged that by using the detergent extraction techniques, as applied in this study, the *in vivo* digestibility of cell-wall constituents of tanniniferous feeds will not always be accurately determined (Makkar *et al*. 1995*c*).

There was a clearly depressed methane production in lambs supplemented with ~25 g condensed tannins/kg DM. These results compare favourably with indirect evidence obtained from previous studies. Hess *et al*. (2003, 2004) observed *in vitro* that the inclusion of the tropical legume *Calliandra calothyrsus* (270 g of condensed tannins/kg DM) in a grass-based diet suppressed methane production relative to OM degraded by over 30%, and that this was probably due to the tannins in this legume. In accordance with this, *in vivo* studies showed that the methane production, estimated by the SF_6 technique, of cattle (Woodward *et al*. 2001) and sheep (Waghorn *et al*. 2002) fed on Lotus (26–80 g of condensed tannins/kg DM) was lower than when fed on forages of similar quality but free of tannins. These studies, however, do not allow the separation of the effects of the tannins and possibly other plant species characteristics, particularly the properties of the fibre and its resulting mode of degradation. The reasons for the lower methane production from ruminants fed tanniniferous diets are not yet well understood. In the present investigation the addition of condensed tannins influenced daily methane release in a similar way as it affected NDF digestibility, suggesting that the inhibition of methanogenesis by tannins was primarily the result of a suppressed fibre degradation. However, a direct effect of condensed tannins on ruminal methanogens cannot totally be excluded (Field *et al*. 1989). Furthermore, tannins decrease the degradation of nutrients in the rumen, which then may be degraded in the hindgut. This could have contributed to a lower methane emission too, because fermentation in the hindgut differs from that in the rumen by a lower methane production per unit of fermented nutrients (Fievez *et al*. 1999).

Interaction of legume and tannin supplementation

The absence of significant interactions for all variables evaluated illustrates that the effects of legume and tannin supplementation were independent from each other. Thus the tannin effect was similar in all diets despite the contrasting properties of the nutrients, particularly the protein, in the different haylages.

Conclusions

The two temperate forage legumes used in this study did not improve the grass-based diet with respect to metabolic protein supply of ruminants. Moreover, legume supplementation, if effective at all, enhanced methane emission (e.g. relative to OM digested), indicating that this feeding strategy will not contribute to limit methane emissions from livestock farming systems. Legumes, however, have other environmental benefits such as the associated reduction in mineral fertiliser N expenditure. By contrast, supplementing *Acacia mearnsii* tannins at a level of ~0.025 of the diet significantly reduced methane emission (kJ/MJ of GE intake) by 13% on average. In the present study, a shift in N excretory pattern from easily volatile urine to faeces due to tannin supplementation was noted, thus showing that this feeding strategy has a 2-fold environmental benefit irrespective of the forages. However, as with legume supplementation, the hypothesis of an improvement in metabolic protein supply could not be confirmed. It remains to be investigated whether or not the effect is the same when using other tannin sources at the same level or using moderately tanniniferous legumes, where tannin–nutrient complexes are already established in feed.

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