Small Ruminant Research 126 (2015) 10–18

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Use of *Rosmarinus officinalis* in sheep diet formulations: Effects on ruminal fermentation, microbial numbers and in situ degradability☆

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ARTICLE INFO

Article history:
Available online 31 January 2015

Keywords:
Rosmarinus officinalis
Ruminal bacteria
Ruminal degradability
Sheep

ABSTRACT

This study investigated the effects of rosemary dietary supplementation on the rumen degradability of alfalfa hay, the ruminal bacterial population and rumen metabolism in sheep. The experiment was designed as a 4 × 4 Latin square balanced for carry-over effects using four ruminally cannulated sheep fed a basal diet consisting of 1.5 kg/day of alfalfa hay supplemented (400 g/day) with one of four different concentrates as follows: (1) control (CTR); (2) CTR with the addition of 1.75% rosemary essential oil adsorbed on an inert support (EO); (3) CTR with the addition of 2.50% dried and ground rosemary leaves (RL); and (4) same as (3), except that rosemary leaves were included in the concentrate before pelleting (RL pellet). No effects were recorded in the volatile fatty acid or lactic acid production. Ruminal ammonia concentration tended to be lower ($P = 0.077$) with the RL pellet diet. The crude protein degradability, after incubation times of 2 and 48 h, decreased ($P < 0.001$) in sheep fed with the RL pellet diet. The dry matter degradability followed the same trend, with lower ($P < 0.001$) rates of disappearance resulting from the RL pellet diet after 24 h of incubation. The pelleting process may influence the availability of the active compounds of rosemary leaves, allowing greater antibacterial activity. Supplementation of sheep diets with rosemary leaves could potentially be used to manipulate ruminal degradation patterns.

1. Introduction

Since the complete ban of antibiotic ionophores for use as growth promoters in the European Union (Directive 1831/2003/CEE, European Commission, 2003), an increased interest in alternative means of manipulating rumen fermentation has emerged.

Plants and plant extracts that contain secondary metabolites, such as essential oils (EOs), have been shown to stimulate the immune system, thereby enhancing the resistance of animals to inflammatory and infectious

☆ This paper is part of a supplement issue entitled "S.I.P.A.O.C. Congress 2012". Guest Edited by Antonello Carta, Maria Teresa Manfredi, Giuseppe Moniello and Massimo Trabalza-Marinucci.

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http://dx.doi.org/10.1016/j.smallrumres.2015.01.018
0921-4488/

diseases. Selective antibacterial activities, as well as antioxidant and radical-scavenging properties, have also been demonstrated in EOs (Gladine et al., 2007; Matkowski et al., 2008).

As a result of such activity, the addition of certain plant extracts to the rumen environment (even at relatively low concentrations) can cause changes in the ruminal microbial fermentation such as an inhibition of deamination and methanogenesis, resulting in lower ammonia N (NH_3-N), methane and acetate, as well as higher propionate and butyrate concentrations (Busquet et al., 2005b; Calsamiglia et al., 2005; Cardozo et al., 2006). Moreover, the inhibition of protein degradation in the rumen can potentially increase the intestinal supply of amino acids to the animal host (Wallace, 2004).

One potential plant of interest for its EOs is rosemary (*Rosmarinus officinalis* L.), an evergreen perennial shrub that belongs to the Lamiaceae family. Rosemary has been widely used as a spice and is known for its antimutagenic, chemopreventive and antibacterial properties (Oluwatuyi et al., 2004). The major components of rosemary essential oils, such as the monoterpenoids α-pinene, β-pinene, camphene, 1–8 cineole, camphor, borneol, bornyl acetate and verbenone, are known for their antimicrobial properties against both gram-positive and gram-negative bacteria (Santoyo et al., 2005; Jiang et al., 2011; Tavassoli et al., 2011). In addition, the powerful antioxidant activity of the phenolic diterpenes contained in rosemary leaves (carnosol, carnosic acid, rosmanol, epirsomanol, isorosmanol, methyl carnosate and rosmarinic acid) has been well documented (Cuvelier et al., 1996; Ibañez et al., 2003; Bozin et al., 2007).

The aim of this study was to evaluate the effect of including different forms of rosemary on ruminal degradability of alfalfa hay, microbial fermentation and bacterial population numbers in sheep diets.

2. Materials and methods

2.1. Animals, diets and experimental design

The experiment was designed as a 4 × 4 Latin square balanced for carry-over effects using four ruminally cannulated sheep over four 21 day periods, including 14 days of adaptation, followed by 7 days of sample collection.

Four Bergamasca × Appenninica sheep (6 years old, with a mean body weight of 60.5 ± 3.4 kg at the beginning of the experiment) fitted with permanent ruminal cannulas were used in this experiment. The study was conducted in accordance with the European recommendations for the protection of animals used for scientific purposes (EU Directive 2010/63/EU, September 22, 2010). The animals were kept in stalls equipped for individual feeding to allow for the four different dietary treatments and free access to water.

The basal diet consisted of 1.5 kg/day of alfalfa hay (13.5% crude protein, CP; 50.6% neutral detergent fibre, NDF; 36.7% acid detergent fibre, ADF and 5.3% lignin sa) in two equal meals, supplied at 8:00 and 16:00 h. The animals were randomly assigned to one of the four different pelleted concentrates (400 g/day) as follows: (1) control (CTR); (2) CTR with the addition of 1.75% rosemary essential oil adsorbed on inert support (EO); (3) CTR with the addition of 2.50% dried and ground rosemary leaves (RL); and (4) same as (3), except that the rosemary leaves were included in the concentrate before pelleting (RL pellet). The composition of the concentrates was as indicated in Table 1. To minimise variations due to plant composition, all experimental concentrates were prepared in a single batch, and the supplements, such as rosemary leaves (6.0% CP; 4.0% crude fat, CF; 7.8% ash; 34.8%, NDF; 24.4% ADF and 12.7% lignin sa) and adsorbed essential oils (95.55% ash and 3.12% CF), were from a single batch.

The choice to use whole leaves (subjected or not subjected to technological treatment) or just the EOs of the whole leaves was motivated by the need to evaluate the effects, if any, exerted by the different concentrations and availabilities of the active compounds of rosemary. The amounts of phytoderivatives included in the experimental concentrates were calculated to give the same dose of EOs to the animals. To define the yield of EOs, both supplement (ground dried leaves and inert support-adsorbed EOs) and the concentrate samples were hydrodistilled in a clevenger-type apparatus in accordance with the European Pharmacopoeia directions. The EOs were collected and dehydrated on anhydrous sodium sulphate and the content (calculated as g/kg) was expressed as the mean of three separate extractions.

The pelleting process was divided into two phases as follows: the conditioning of feed ingredients (25 °C) and the real pelleting (45–50 °C). The entire process lasted 40 s. To facilitate the introduction and mixing of the plant supplement to both the EO and RL diets, all feeds were subjected to a rough grinding process.

2.2. Measurements and analyses

2.2.1. Feed phytochemical composition

Feed samples and plant supplements (2 g) were powdered for ultrasound-assisted extraction in methanol at room temperature (23 °C) for 1 h (2 × 30 min). The samples were centrifuged, and the extracts were diluted to a final volume (100 mL) with a solvent. The freshly prepared extracts were directly used for quantification of the main secondary metabolite classes. Total phenolics were determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965). The total phenolic content was expressed as milligrams of gallic acid equivalent per gram of dry extract.

The flavonoid content was estimated by the $AlCl_3$ method, using rutin as the standard (Lamaison and Carnat, 1990). Total flavanol and proanthocyanidin contents were quantified by n-butanol hydrochloride and p-dimethylaminocinnamaldehyde assays, respectively (Porter et al., 1986, 1989).

The total antioxidant capacity assay was performed using the improved 2,2′-azinobis-3 ethylbenzothiazoline-6-sulphonic acid (ABTS) assay. The radical cation decolourisation assay was performed as described by Re et al. (1999).

Rosmarinic and carnosic acids were identified and quantified following a previously described method with slight modifications (Almela et al., 2006). For the quantitative determination of rosmarinic and carnosic acid, powdered samples were first defatted with ethyl ether at room temperature. The supernatant was removed, and the residue was dried at 30 °C. The residue was then extracted in methanol in an ultrasonic bath (2 × 30 min). The supernatants were collected, adjusted to final volume, filtered (0.45 μm pore filter) and directly injected into the HPLC.

The chromatographic system consisted of a binary system pump (Jasco PU-2080, Tokyo, Japan) and a diode array detector (Jasco MD-2010, Tokyo, Japan) equipped with a reversed-phase Kinetex C18 column (250 mm × 4.5 mm, 5 μm particle size; Phenomenex, Torrance, USA). The mobile phase consisted of 0.3% formic acid (A) and methanol (B) using an isocratic elution (70% A; 10 min), followed by a linear gradient programme up to 50% of A in 20 min. The flow rate was 1 mL/min.

Peak identification was performed by direct comparison of retention times and UV–vis spectra with those of pure standards (Sigma Aldrich, St. Louis, MO, USA), which were used at known concentrations to build the calibration curves (R^2 = 0.997 and 0.998 for rosmarinic and carnosic acids, respectively) used for quantification. For the quantitative determination, peaks of carnosic and rosmarinic acids were detected at 260 and 330 nm, respectively.

All phytochemical determinations were performed in triplicate.

2.2.2. In situ degradability

Hay degradability estimates were determined using the in situ nylon bag technique. Nylon bags (pore size of 50 ± 15 μm; dimensions of 5 cm × 10 cm; Ankom Technology Corporation, Fairport, New York, USA) were filled with 1.5 g of alfalfa hay (14.0% CP; 1.2% CF; 51.9% NDF; 36.2% ADF and 5.1% lignin sa) ground through a 2 mm screen (bag surface area ratio of 15 mg/cm^2). Fourteen bags per treatment were tied on a main line, which was suspended in the rumen and secured at the ruminal cannula. The bags were incubated for 2, 4, 12, 24, 48, 72 and 120 h. At the end of each incubation time, two bags were randomly removed from the rumen and washed under running tap water until the rinse water was

Table 1
Ingredients (% as fed basis) and chemical composition (g/100 g) of the concentrates used in the experimental diets.

Item	Concentrates			
Ingredients	CTR	RL pellet	RL	EO
Wheat bran	40.00	30.00	39.00	39.30
Wheat flour middlings	17.80	24.30	17.35	17.49
Corn grain	10.00	10.00	9.75	9.82
Sunflower meal	14.90	14.90	14.53	14.64
Soybean meal	5.00	6.00	4.87	4.91
Calcium carbonate	4.20	4.20	4.09	4.13
Dehydrated alfalfa meal	3.50	3.50	3.41	3.44
Beet protein concentrate	2.00	2.00	1.95	1.96
Sugar cane molasses	2.00	2.00	1.95	1.96
Vitamin-mineral supplement[a]	0.60	0.60	0.60	0.60
Rosemary leaves	–	2.50	2.50	–
Rosemary essential oil	–	–	–	1.75
Chemical composition				
Analysed				
Dry matter	92.88	92.78	92.84	92.96
Crude protein	18.40	18.44	18.09	18.08
Crude fat	3.08	3.21	3.12	3.05
Ash	9.84	9.98	9.79	11.24
NDF	29.65	29.52	29.79	29.13
ADF	10.65	11.17	10.99	10.47
Lignin sa	3.14	2.99	3.38	3.08
Ca	0.80	0.70	0.79	0.79
P	0.75	0.68	0.75	0.74
Na	0.28	0.27	0.27	0.28
Calculated				
Lys	0.70	0.71	0.68	0.69
Met	0.29	0.29	0.28	0.28
Met + Cys	0.58	0.58	0.56	0.57
Choline	0.14	0.14	0.14	0.14

CTR: control; RL pellet: CTR with the addition of 2.50% dried and ground rosemary leaves included in the concentrate before pelleting; RL: CTR with the addition of 2.50% dried and ground rosemary leaves; EO: CTR with the addition of 1.75% rosemary essential oil adsorbed on an inert support.

[a] Supplied per kilogram of diet: Vitamin A, 18,000 I.U. (retinol); Vitamin D3, 2100 I.U.; Vitamin E, 21 mg (α-tocopheryl acetate); Fe, 29 mg; Co, 0.75 mg; Mn, 39 mg; Zn, 150 mg; Se, 0.06 mg.

clear. An additional two bags per treatment, washed by the procedure outlined above, were used for the zero hour (0 h) values to determine the washing loss. Washed nylon bags were dried in a forced-air drying oven at 60 °C for 48 h, and weighed for determination of dry matter (DM) disappearance. The rumen-incubated residues of each bag were analysed for CP (up to 48 h of incubation), NDF and ADF composition.

2.2.3. Feed composition

Hay, concentrates and contents of each bag incubated in the rumen were analysed to determine chemical composition. DM was evaluated following AOAC (2000) method 934.01. CP was determined by measuring the total nitrogen according to AOAC (1990) Kjeldahl method 954.01 and then converting this value to the protein content by multiplying by 6.25. CF and ash were determined by AOAC methods 920.39 and 942.05, respectively (AOAC, 1990). NDF, ADF and lignin sa were analysed according to Van Soest et al. (1991). The NDF and ADF concentrations were analysed in the presence of sodium sulphite without α-amylase treatment and are expressed inclusive of residual ash.

2.2.4. Ruminal fermentation and microbial populations

The procedures for preparation of media and the anaerobic cultural techniques used in this study were as previously described by Dehority (1969) and Dehority et al. (1989).

Ruminal content samples were collected from each rumen-cannulated sheep (before morning feeding) for the evaluation of pH (Hanna Instruments, Woonsocket, USA), the total bacterial population, the amylolytic bacterial subpopulation and the cellulolytic bacterial subpopulation according to the most probable number (MPN) procedures (Dehority et al., 1989). Growth of the total bacterial population and the amylolytic bacterial subpopulation was determined by an increase in visual turbidity and a decrease in pH after 7 days of incubation at 39 °C. In general, the decrease in pH with this bacterial culture medium ranged from approximately 0.2–0.45 (Dehority et al., 1989). Moreover, the growth of the cellulolytic bacterial subpopulation was estimated by the degradation of a cellulose filter paper strip after 14 days of incubation at 39 °C. The results were expressed as $\log_{10}$ bacterial number per gram of DM of rumen content. Further ruminal content samples were collected and frozen at −80 °C for later determination of volatile fatty acid (VFA), NH_3-N and lactic acid contents.

The VFA content was determined according to the procedure of Filípek and Dvořák, (2009). Fifteen gram of ruminal content was filtered through two layers of cheesecloth and centrifuged at 3500 rpm for 15 min at 4 °C. After centrifugation, 200 μL of metaphosphoric acid (25% aqueous solution) and 200 μL of internal standard (3-methyl valeric acid; 1 g/L aqueous solution) were added to 1 mL of the supernatant. After 25 min of centrifugation (6000 rpm at 4 °C), 1 mL of the clear supernatant was added to 150 μL of ethanol and analysed by means of gas chromatography.

The gas chromatographic analysis was performed using a Carlo Erba FTV 4300 gas chromatograph equipped with a DB-FFAP fused silica capillary column (30 m × 0.25 mm i.d.; film thickness of 0.25 μm; J&W, Agilent technologies, Palo Alto, CA, US) and a flame ionisation detector (FID). The carrier gas was helium at a flow rate of 1 mL/min. The oven temperature programme was 1 min at 60 °C, followed by an increase to 200 °C at a rate of 5 °C/min. The injector temperature was 270 °C, and the detector temperature was 300 °C. The sample was injected into a split/splitless system (split ratio 1:10). Peaks were identified by comparison with known standards. Quantification of VFA was performed using 3-methyl valeric acid as the internal standard.

For lactic acid and NH_3-N analyses, the rumen contents were filtered through four layers of cheesecloth and centrifuged at 6000 rpm for 90 min at 4 °C. Both an UV method (Enzyplus EZA 891+, D/L-lactic acid; Raisio Diagnostics, Rome, Italy) and an enzymatic method (Ammonia Ultra 17660, Sentinel Diagnostic, Milan, Italy) for lactic acid and

Table 2
Main secondary metabolite components detected in the experimental concentrates and plant supplements.

	Experimental diets				SEM	*P* value	Supplements	
	CTR	RL pellet	RL	EO			Rosemary essential oil	Rosemary leaves
Total phenols (mg/g d.e. as gallic acid)	72.48[a]	61.60[b]	70.98[a]	71.85[a]	1.10	<0.001	NA	33.03 ± 2.94
Flavonoids (mg/g d.e. as rutin)	6.08[b]	6.43[a]	6.20[b]	6.11[b]	0.04	<0.001	NA	20.63 ± 1.48
Flavanols [mg/g d.e. as (−) epicatechin]	3.00	2.95	2.87	2.94	0.07	0.604	NA	2.05 ± 0.11
Oligomeric proanthocyanidins (mg/g d.e. as cyanidin hydrochloride)	0.37	0.42	0.40	0.40	0.03	0.646	NA	0.41 ± 0.05
Total Antioxidant Activity (ABTS test)	20.09[b]	21.45[a]	19.65[b]	17.70[c]	0.33	<0.001	NA	20.19 ± 1.26
Rosmarinic acid (μg/g DM)	0.011[b]	0.336[a]	0.087[b]	0.014[b]	0.028	<0.01	NA	13.84 ± 0.23
Carnosic acid (μg/g DM)	ND	1.013	1.002	ND	0.084	0.692	NA	43.16 ± 2.03
Essential oil content (g/kg)	ND	0.052	0.049	0.050	0.001	0.355	3.01 ± 0.21	2.12 ± 0.16

CTR: control; RL pellet: CTR with the addition of 2.50% dried and ground rosemary leaves included in the concentrate before pelleting; RL: CTR with the addition of 2.50% dried and ground rosemary leaves; EO: CTR with the addition of 1.75% rosemary essential oil adsorbed on an inert support; DM: dry matter.
NA: not applicable; ND: not detected.
Means with different letters within a same row differ significantly.

NH_3-N determination, respectively, were then applied according to the manufacturer's instructions.

2.2.5. Calculations and statistical analyses

The percent disappearance of DM, CP, NDF and ADF at each incubation time was calculated from the proportion remaining after incubation in the rumen.

The disappearance rate was fitted to the following equation (Ørskov and McDonald, 1979):

$$P = a + b(1 - e^{-ct})$$

where P is the disappearance rate at time t (%); a is the soluble or rapidly degradable fraction (%); b is the insoluble but potentially degradable fraction (%); c the fractional rate constant indicating the degradation rate of fraction b per hour; t the incubation time (h). The maximum extent of degradation (total potentially degradable fraction) would be indicated by '$a+b$', and '$100-(a+b)$' was considered to be the undegradable fraction.

The a, b and c parameters were obtained by fitting the data using Neway software (Rowett Research Institute, Aberdeen, UK).

The effective degradability values were then calculated using the following equation (McDonald, 1981):

$$P = a + \frac{bc}{c+k}$$

where k is the estimated rate of outflow from the rumen (assumed to be equal to 0.02 h^{-1}).

All data concerning phytochemical composition, in situ degradability, ruminal bacterial numbers and ruminal metabolism were analysed by ANOVA, as a 4 × 4 Latin square using the MIXED procedure (SAS Institute Inc., 2010). The statistical model included sheep, period, dietary treatment, and residual error. Fixed effects included period and dietary treatment. Sheep was the random effect. Volatile fatty acids (when expressed as a percentage) and degradability data were arcsine-transformed prior to statistical analysis. Overall differences between the means were evaluated using a Tukey test. Data were reported as least squares means ± standard error. Differences were considered to be significant when $P \leq 0.05$ and trends were discussed if $0.05 < P < 0.10$.

3. Results

3.1. Feed phytochemical composition

The phytochemical composition of the experimental concentrates and the plant supplements is set out in Table 2. The secondary metabolites profile of rosemary leaves were in accordance with the literature data (Shan et al., 2005; Rodríguez-Rojo et al., 2012; Kasparavičienė et al., 2013; Meziane-Assami et al., 2013). The supplementation of the concentrates with either rosemary essential oil or leaves determined variations, in terms of chemical composition, that are coherent with the composition of the plant supplements.

The RL pellet concentrate contained the lowest ($P < 0.001$) amount of phenols and the highest quantity of flavonoids ($P < 0.001$) and rosmarinic acid ($P < 0.01$), compared with the other experimental feeds (Table 2). In addition, the RL pellet showed the highest ($P < 0.001$) total antioxidant activity. No differences were detected in flavanols, proanthocyanidins or essential oil contents among the diets. Similar amounts of carnosic acid were detected in both concentrates (RL and RL pellet) supplemented with rosemary leaves.

3.2. In situ degradability

The CP disappearance of alfalfa hay incubated within the rumen of sheep fed with the RL pellet diet was lower ($P < 0.001$) after incubation times of 2 and 48 h, compared with the other treatments (Table 3). After 24 h of incubation, the recorded CP disappearance for the RL pellet diet was lower than those for the CTR and RL treatments, but did not differ from that of the EO diet (Table 3). The alfalfa DM disappearance followed the same trend, with lower ($P < 0.001$) rates of disappearance resulting from the RL pellet diet after 24 h of incubation (Table 3). In contrast, the degradability of the NDF and ADF fractions was not affected by the dietary treatment (average degradability after 120 h of incubation was 41.15% and 38.02% for NDF and ADF, respectively).

The effective degradability of forage CP was numerically lower for sheep fed the RL pellet diet (Table 3). The value of the 'a' fraction for the CP tended to be lower ($P = 0.083$) for the RL pellet diet, compared to the other diets (Table 3).

Table 3
Effects of experimental diets on in situ rumen degradability (%) of alfalfa hay.

	Experimental diets				SEM	*P* value
	CTR	RL pellet	RL	EO		
In situ rumen degradability						
CP						
0	55.26^{c}	56.21^{b}	57.96^{a}	57.24^{a}	0.22	<0.001
2	51.29^{a}	43.05^{b}	53.12^{a}	52.19^{a}	1.17	<0.001
4	46.33^{b}	42.94^{b}	51.47^{a}	53.52^{a}	1.27	<0.001
12	61.85^{ab}	57.90^{b}	64.03^{a}	65.72^{a}	1.22	<0.01
24	65.07^{b}	61.50^{c}	68.34^{a}	63.19^{b,c}	0.55	<0.001
48	67.35^{ab}	57.37^{c}	70.27^{a}	64.70^{b}	1.26	<0.001
DM						
0	34.40^{b}	35.66^{ab}	36.06^{a}	35.84^{a}	0.31	<0.001
2	40.85	36.77	38.29	38.82	1.39	0.262
4	38.06	40.03	41.11	43.42	1.47	0.115
12	48.93	49.20	51.75	53.78	1.38	0.077
24	57.13^{a}	53.49^{b}	57.90^{a}	58.94^{a}	0.74	<0.001
48	58.23^{ab}	53.84^{b}	60.54^{a}	57.89^{ab}	1.40	<0.05
72	55.81	56.20	59.01	58.01	1.40	0.356
120	53.53	55.26	55.19	56.35	1.66	0.692
Degradation kinetics						
DM						
a	31.97	31.66	30.58	31.03	2.78	0.984
b	24.49	23.30	27.86	26.68	2.55	0.596
c	0.11	0.11	0.13	0.15	0.02	0.278
a + b	56.46	54.97	58.43	57.71	1.57	0.451
CP						
a	43.21	19.52	42.87	45.46	7.45	0.083
b	28.23	40.18	51.03	31.80	14.91	0.714
c	0.09	0.26	0.14	0.18	0.08	0.545
a + b	71.44	59.70	93.89	77.26	13.83	0.403
Effective degradability						
DM	52.29	51.37	54.60	54.68	1.30	0.233
CP	64.24	56.80	65.35	62.90	3.62	0.382

DM: dry matter; CP: crude protein; CTR: control; RL pellet: CTR with the addition of 2.50% dried and ground rosemary leaves included in the concentrate before pelleting; RL: CTR with the addition of 2.50% dried and ground rosemary leaves; EO: CTR with the addition of 1.75% rosemary essential oil adsorbed on an inert support.
Means with different letters within a same row differ significantly.
a: the soluble or rapidly degradable fraction; b: the insoluble but potentially degradable fraction; c: the fractional rate constant indicating the degradation rate of fraction b per hour; a+b: total potentially degradable fraction.

3.3. Ruminal fermentation and microbial populations

There were no differences in total VFA concentration or the proportions between the four experimental diets (Table 4). The total, cellulolytic and amylolytic ruminal bacterial numbers were not affected by the dietary supplementation (Table 5). The pH and lactic acid values were similar among the diets (Table 4). The NH_3-N concentrations in rumen fluid tended ($P = 0.077$) to be lower with the RL pellet diet (Table 4).

4. Discussion

The phytochemical analysis revealed that even limited changes in concentrate composition (2.5 and 1.75% of rosemary leaves and essential oil, respectively) can markedly modify the quantitative content of the secondary metabolites. As most of the secondary metabolites were already present in the basic ingredients of the CTR concentrate, the most noticeable effects were observed for the metabolites contained at higher concentrations such as phenols, flavonoids and rosmarinic acid. Differences in phytochemical composition were observed between the two concentrates (RL and RL pellet) supplemented with rosemary leaves. It can be speculated that the manufacturing technology, particularly the pelleting process, may affect the stability, distribution or availability of the active ingredients.

To current knowledge, this is the first study to compare the effects of different types of rosemary derivatives on ruminal microbial population, fermentation and in situ degradability. The most noticeable effect was the decrease in the alfalfa hay CP and the DM ruminal degradability when sheep were fed the RL pellet diet. More interestingly, a marked effect on the rapidly degradable fraction of the CP was observed. Under the same dietary treatment, the ruminal NH_3-N tended to be lower, compared with the other experimental diets.

According to other studies, rosemary oil shows a fairly broad range of activity against the gram-positive and gram-negative bacteria (Smith-Palmer et al., 1998; Hammer et al., 1999; Elgayyar et al., 2001; Santoyo et al., 2005) with the gram-positive strains being more sensitive than the gram-negative strains (Smith-Palmer et al., 1998; Pintore et al.,

Table 4
Effects of experimental diets on ruminal metabolism.

	Experimental diets				SEM	*P* value
	CTR	RL pellet	RL	EO		
VFA (mmol/L)						
Total VFA	89.46	90.60	84.91	88.48	6.57	0.932
Acetic	67.62	68.28	64.68	67.13	5.18	0.962
Propionic	11.15	11.45	10.86	11.15	0.88	0.971
Butyric	6.33	6.37	5.42	6.17	0.55	0.619
Isobutyric	1.41	1.41	1.32	1.34	0.06	0.624
n-Valeric	0.84	0.91	0.75	0.77	0.08	0.471
Isovaleric	1.87	1.95	1.64	1.71	0.12	0.345
Capronic	0.16	0.17	0.13	0.14	0.03	0.806
Heptanoic	0.06	0.06	0.09	0.09	0.03	0.799
VFA (%)						
Acetic	75.59	75.40	76.23	75.89	0.68	0.832
Propionic	12.46	12.57	12.72	12.59	0.33	0.954
Butyric	7.04	7.07	6.38	6.91	0.46	0.697
Isobutyric	1.60	1.55	1.58	1.54	0.08	0.960
n-Valeric	0.95	1.00	0.87	0.87	0.06	0.413
Isovaleric	2.12	2.15	1.96	1.95	0.13	0.605
Capronic	0.18	0.19	0.15	0.15	0.03	0.710
Heptanoic	0.06	0.06	0.11	0.10	0.03	0.637
Ruminal pH	6.72	6.80	6.85	6.83	0.11	0.844
NH_3-N (mmol/L)	3.93	2.58	5.23	4.55	0.58	0.077
Total lactic acid (mmol/L)	5.48	6.77	6.46	6.58	0.70	0.589
D-lactic acid (mmol/L)	4.13	3.72	5.05	4.83	0.75	0.598
L-lactic acid (mmol/L)	1.34	3.05	1.41	1.76	0.95	0.585

VFA: volatile fatty acid; NH_3-N: ammonia; CTR: control; RL pellet: CTR with the addition of 2.50% dried and ground rosemary leaves included in the concentrate before pelleting; RL: CTR with the addition of 2.50% dried and ground rosemary leaves; EO: CTR with the addition of 1.75% rosemary essential oil adsorbed on an inert support.

2002). Using five EOs (clove oil, eucalyptus oil, garlic oil, oregano oil and peppermint oil) in vitro at three different doses (0.25, 0.50 and 1.0 g/L), Patra and Yu (2012) recorded a linear decrease in growth of archaea, protozoa and major cellulolytic bacteria (i.e., *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus*) with increasing essential oil doses.

As shown by other researchers, EOs and their components affect ruminal N metabolism in a dose-dependent manner (McIntosh et al., 2003; Busquet et al., 2006; Newbold et al., 2006). Nevertheless, contradictory results have been reported. So, for example, McEwan et al. (2002) and Newbold et al. (2004) used a commercial mixture of EOs and observed a decrease in the degradation of protein-rich substrates incubated in dacron bags within the rumen. This effect on protein degradation is highly selective and depends on the substrate used (Molero et al., 2004).

Busquet et al. (2006) demonstrated that some EOs and their main components (anethol, benzyl salicylate, carvacrol, carvone, cinnamaldehyde, and eugenol) significantly inhibit NH_3-N concentration at high doses (3 g/L), and they also reported that these effects are lower at moderate doses (0.3 g/L) and absent at low doses (0.03 g/L). Evaluating the growth of the predominant species of ruminal bacteria at different concentrations of essential oils, Wallace et al. (2002) demonstrated that the growth of some hyper-ammonia-producing bacteria (*Clostridium sticklandii*, *Prevotella ruminicola* and *Peptostreptococcus anaerobius*) is inhibited. In another trial, Wallace et al. (2002) demonstrated that the numbers of hyper-ammonia-producing bacteria decreased by 77% in sheep receiving a low protein diet supplemented with a commercial mixture of EOs. The most sensitive species are *Prevotella* spp. (involved in all of the steps of protein catabolism) (Wallace et al., 1997) and *Ruminobacter amylophilus* (highly active starch and protein digester) (Stewart et al., 1997). Russell et al. (1991) observed that hyper-ammonia-producing bacteria may be responsible for most ruminal deamination

Table 5
Comparison of total, cellulolytic and amylolytic ruminal bacterial numbers (log_{10} bacteria per g) in MPN media.

	Experimental diets				SEM	*P* value
	CTR	RL pellet	RL	EO		
Ruminal bacterial numbers						
Total	9.13	8.95	8.89	9.03	0.15	0.718
Cellulolytic	8.62	7.85	8.19	8.08	0.39	0.579
Amylolytic	8.82	9.00	8.83	8.89	0.16	0.839

CTR: control; RL pellet: CTR with the addition of 2.50% dried and ground rosemary leaves included in the concentrate before pelleting; RL: CTR with the addition of 2.50% dried and ground rosemary leaves; EO: CTR with the addition of 1.75% rosemary essential oil adsorbed on an inert support.

processes (up to 50% of the total), even though they are present in extremely low numbers (1% of the rumen bacterial population).

The effects on the hyper-ammonia-producing bacteria may explain the reduction of CP degradability of alfalfa hay recorded when feeding sheep the RL pellet diet. These results may be related to the higher content of flavonoids and rosmarinic acid observed in the RL pellet concentrate. These compounds have been shown to have antimicrobial activity against gram-positive bacteria, gram-negative bacteria and yeasts (Bais et al., 2002; Moreno et al., 2006) and to have high antioxidant power (Soares et al., 1997; Zhang et al., 2012).

In accordance with present results, no effect on fibre degradation was observed by Newbold et al. (2004). However, in contrast, Patra and Yu (2012) reported a linear decrease in the apparent degradability of DM and NDF with increasing doses of various EOs, excluding garlic oil.

Only a few studies have shown that the use of EO dietary supplementation increases ruminal total VFA concentration indicating an improved feed digestion (Castillejos et al., 2005; Benchaar et al., 2007). The effect of EOs on total VFA production may depend on the composition of the diet and on the dose of EOs (Busquet et al., 2006; Benchaar et al., 2007). In their in vivo studies, Newbold et al. (2004) and Beauchemin and McGinn (2006) did not record any effects on total VFA concentration or proportions using a commercial mixture of EO compounds in sheep (0.11 g/day) or cattle (1 g/day) diets. Busquet et al. (2006) and Castillejos et al. (2006) reported no effects of various EOs on ruminal fermentation in 24 h batch cultures. However, at the highest concentration (3 and 5 g/L of culture fluid), most treatments decreased total VFA concentration. Similar results have been reported by Patra and Yu (2012), with a decrease in total VFA concentration caused by clove oil and oregano oil but not by garlic oil, eucalyptus oil or peppermint oil. All of the EOs differed in altering the molar proportions of acetate, propionate, and butyrate. Castillejos et al. (2008) evaluated the effects of ten EOs, including rosemary oil, in in vitro 24 h batch cultures of diluted rumen fluid. These researchers showed that the properties of rosemary oil at the highest dose (0.5 g/L) are similar to those of monensin with the ability to increase propionate and valerate proportions as well as to reduce acetate proportions, butyrate proportions and the acetate to propionate ratio, without changing the total VFA concentration.

According to a number of experimental reports (Cardozo et al., 2004, 2006; Busquet et al., 2005a), there is an adaptive response to EO supplementation at the bacterial and/or population level at least in the in vitro systems. In some conditions, the rumen microbial population may be able to chemically reduce active components of EOs to inert alcohols (Chizzola et al., 2004). This response would be particularly evident when using low levels of EOs. If this response was confirmed in vivo, it would represent a major challenge for the commercial application of these supplements.

Benchaar et al. (2008) concluded that the lack of changes in total VFA concentration can be considered acceptable, if it is associated with other desirable modifications of rumen metabolism (such as decreased NH3-N concentration, decreased methane production, or a change in the molar proportions of VFA).

5. Conclusions

The rosemary derivatives evaluated in this study did not seem to induce marked changes in ruminal metabolism. For this reason, the antibacterial, chemopreventive and antioxidant properties of rosemary may be used safely and reliably in ruminant nutrition.

However, results indicate that rosemary leaves subjected to the pelleting process can inhibit CP degradation of alfalfa hay, particularly that of the rapidly degradable fraction. These effects may positively influence ruminant nutrition and potentially improve the efficiency of nitrogen utilisation in ruminants. Further experiments are needed to determine rosemary compound properties and their mode of action within the rumen.

Conflict of interest

None of the authors has any financial or personal interest that would inappropriately influence or bias the contents of this paper.

Acknowledgements

This research was sponsored by the IZSUM 004/09 RC project funded by the Italian Ministry of Health (MinSal). The authors would like to thank E. Cassetta and M. Ricci for help in laboratory analyses and G. Ponti, A. Mazzoccanti and G. Covarelli for assistance and care of the animals. Conagit S.p.A. and APA-CT srl are gratefully acknowledged for providing technical support and advice in formulating experimental feeds. This work is part of a PhD research project in “Animal Health, Livestock Production and Food Safety”, University of Perugia, XXVIII cycle.

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