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RESEARCH ARTICLE

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The effect of rosemary (*Rosmarinus officinalis* L.) essential oil on digestibility, ruminal fermentation and blood metabolites of Ghezel sheep fed barley-based diets

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Abstract

This study was conducted to evaluate the effects of rosemary essential oil (REO) on feed digestibility, ruminal fermentation and blood metabolites of Ghezel sheep. Four male sheep with average body weight 46 ± 2.0 kg were used in a 4 × 4 Latin square design. Treatments were control (no REO added), 100 mg d^{-1} of REO (low), 200 mg d^{-1} of REO (medium) and 400 mg d^{-1} of REO (high). Sheep were fed the 4 diets for 4 periods of 21 days (14 days as adaptation and 7 days for sample collection). The results showed that digestibility of dry matter, neutral-detergent fiber, acid-detergent fiber and crude protein were not affected by REO feeding ($p > 0.05$). The concentration of ammonia-N across sampling times was lower ($p < 0.05$) at low REO dosage compared with control. The molar proportion of acetate and butyrate across sampling times were lower at low REO dosage compared with control ($p < 0.05$). Total volatile fatty acids (VFA) concentrations at 4 h after morning feeding were reduced ($p < 0.05$) by adding 100 mg of REO d^{-1} to diet compared with the control, whereas medium REO dosage increased ($p < 0.05$) total VFA concentrations at 4 h post feeding compared with the control. The addition of REO had no effect on total protozoa counts across sampling times ($p > 0.05$). Supplementation with REO had no effect on plasma concentrations of glucose, triglyceride, cholesterol, total protein and albumin ($p > 0.05$). The results of this study indicate that, although a medium dose of REO may have positive effect on rumen fermentation, a low dose of REO may have adverse effects on ruminal fermentation.

Additional key words: volatile fatty acids; ammonia-N; protozoa.

Introduction

Nutrition of ruminants is controlled by the microbial fermentation that happens in the foregut. This fermentation could be improved in many ways; such as by improving fibre digestion as well as by decreasing protein degradation which, if modified, might increase efficiency of energy and N utilization, thus increasing the livestock production. Since ruminal fermentation is completely microbial in nature, should be manipulated by selective antimicrobial agents such as antibiotics. The use of essential oils (EO) in livestock nutrition has been expanded after the ban of the use of antibiotic as growth promoters, including the ionophores (OJEU, 2003).

The EO are blends of secondary metabolites that are commonly extracted by steam distillation or solvent extraction (Gershenzon & Croteau, 1991; Greathead, 2003). Chemically, they are characterized as having a very diverse composition, nature and activities (Calsamiglia *et al*., 2007). Many of EO and their active components have strong antimicrobial activities against a wide range of microorganisms, including bacteria, protozoa and fungi (Benchaar *et al*., 2008), and can be used in modulating the competition among different microbial populations with the objective of improving the efficiency of energy and protein utilization in the rumen (Calsamiglia *et al*., 2007). The antimicrobial activity of EO has been attributed to terpenoid (monoterpenoids and sesquiterpenoids) and

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Abbreviations used: ADF (acid-detergent fiber); CP (crude protein); DM (dry matter); EO (essential oils); HAP (hyper-ammonia producing bacteria); NDF (neutral-detergent fiber); OM (organic matter); REO (rosemary essential oil); VFA (volatile fatty acids).

phenolic compounds (Helander *et al*., 1998; Chao *et al*., 2000). In recent years, a number of studies have been published on the effects of EO on rumen microorganism and rumen metabolism. Most of these studies are short-term *in vitro* culture incubations or in situ incubations and only a few have been conducted *in vivo* to evaluate the influence of EO on ruminant metabolism.

Rosemary *(Rosmarinus officinalis* L) is a species of Mediterranean origin, which is well known around the world as a common spice for culinary purposes (Ventura *et al*., 2011). The rosemary EO (REO) contains 1, 8-cineol, camphor, bornyl acetate, α- and β-pinene, limonene, camphene, terpineol and verbenone (Baratta *et al*., 1998; Burt, 2004). Natural polyphenols found in the leaves of *R. officinalis* have potential therapeutic benefits, because of their potent antioxidant activity and their anticarcinogenic and antiviral properties, observed in vitro and in human liver (Savoini *et al*., 2003). No data are available on the effects of the inclusion of REO in sheep diets on feed digestibility and rumen metabolism. Furthermore, research is needed to determine REO effects *in vivo*. Hence, this study was conducted to assay the potential of using REO to improve feed digestibility, ruminal fermentation and some blood metabolites in Ghezel sheep.

Table1. Ingredients and chemical composition of the basal diet (control)

Item	Amount
Ingredient (g kg^{-1} DM)	
Alfalfa hay	500
Barley straw	100
Barley grain	294
Wheat bran	100
Vitamin- mineral premix[1]	3.5
Salt	2.5
Chemical composition[2]	
DM (g kg^{-1} of feed)	894
CP (g kg^{-1} DM)	112
aNDF (g kg^{-1} DM)	437
ADF (g kg^{-1} DM)	247
EE (g kg^{-1} DM)	25
Calcium (g kg^{-1} DM)	7.1
Phosphor (g kg^{-1} DM)	3.5

[1] Vitamin-mineral premix contained per kilogram of DM: 11,250 IU of vitamin A; 2,250 IU of vitamin D_3; 25 mg of vitamin E; and 10 mg of $CuSO_4$ $5H_2O$. [2] DM = dry matter. CP = crude protein. aNDF = neutral detergent fiber assayed with a heat stable alpha amylase. ADF = acid detergent fiber. EE = ether extract.

Material and methods

Animal and diets

Four Ghezel sheep with average body weight of 46 ± 2 kg were used in a 4 × 4 Latin square design. Diets were offered to the animals twice daily (08:00 and 20:00 h) at a daily rate of 55 g of DM kg^{-1} of BW$^{0.75}$. This level of intake was estimated to meet the energy maintenance requirements of the experimental sheep (NRC, 1985). Sheep were fed a barley-based diet not supplemented (control) or supplemented with 100 mg d^{-1} of REO (low), 200 mg d^{-1} of REO (medium) and 400 mg d^{-1} of REO (high).The REO was supplied in two daily doses mixed with 0.1 kg of concentrate before feeding to guarantee consumption of the whole dose. Control sheep received the same amount of concentrate without REO. The REO (purity > 99%) was purchased from Barij Essential Oils Company of Iran, Kashan. The main essential oil of REO was 1, 8 Cineole (18%). Ingredients and chemical composition of basal diet are shown in Table 1.

Experimental procedure and measurements

The experiment was conducted at the experimental animal farm of the Department of Animal Science of the University of Urmia (Urmia, Iran). All experimental procedures for this study were approved by the Animal Care and Use Committee of the University of Urmia. The trial comprised four 21-d periods, with a 14-d adaptation period and 7-d collection period. On days 20 and 21 of each experimental period, rumen fluid and blood samples were taken, respectively.

Sheep were individually fed in metabolic cages over the 21-d periods. Feces were collected from each animal daily at 08:00 during the first 5 days of each collection period and were immediately frozen at –20°C until analysis. Ruminal fluid was collected using an orogastric tube at 0 and 4 h after the morning feeding and filtrated through four layers of cheesecloth. Samples were collected into 50-mL plastic tubes and the pH was measured immediately

after sampling with a pH-meter (Metrohm, USA). Filtrate samples (50 mL) were acidified to pH = 2 with 1 mL of 50% H_2SO_4 and frozen at –20°C for determination of volatile fatty acids (VFA) and ammonia-N concentrations (Ipharraguerre *et al*., 2006). For protozoa counts, a 10 mL aliquot of ruminal fluid was immediately mixed with an equal amount of 10% formalin and transferred to the laboratory. Samples were stored at room temperature in the dark until counting.

At 0 and 4 h after morning feeding, blood samples were collected from the jugular vein into 10 mL vacuum tubes containing heparin. Samples were centrifuged (3,000 g × min for 20 min at 4°C) and the plasma was frozen at –20°C until analysis.

Chemical analysis

The dry matter (DM) content of feeds and feces samples was determined by oven-drying at 105°C for 48 h (AOAC, 1990; method 930.15). Ash content of samples was determined after 5h of incineration at 500°C in a muffle furnace, and the organic matter (OM) content was calculated as the difference between 100 and the percentage of ash (AOAC, 1990; method 942.05). Concentration of total N was determined by combustion assay (AOAC, 1990; method 990.03) and crude protein (CP) was calculated as N × 6.25. The ether extract content was determined using a Soxhlet System Apparatus (Electromantle ME1000, UK) according to AOAC (1990; method 920.39). The neutral-detergent fiber (NDF) and acid-detergent fiber (ADF) content was determined as described by Van Soest *et al*. (1991) using of sodium sulfite and heat stable α-amylase. The concentration of VFA in the rumen liquor was analyzed by gas chromatography (Shimadzu, GC-17A, Japan) with a FFAP capillary column (30 m × 250 μm i.d., 0.25 μm film thickness, DB-wax-123-7032) and a flame ionization detector (FID). The gas flow rate for nitrogen was 1.0 mL min^{-1}. The temperature program used was the following: 60-200°C (20°C min^{-1}, 10 min), injector 250°C, detector 300°C and the injection was performed by split mode at a ratio of 1:30. The analysis time was approximately 15 min.

Ruminal ammonia-N was measured by spectrophotometry as described by Conway (1950). The total number of protozoa was enumerated microscopically (Ogimoto & Imai, 1981) using a Neubauer improved Bright Line Hematocytometer (Hausser Scientific, Horsham, PA, USA). Each sample was counted twice, and if the average of the duplicates differed by more than 10%, the counting were repeated.

Plasma concentrations of glucose, triglyceride, cholesterol, high density lipoprotein (HDL), albumin and total protein were determined enzymatically by Pars Azmon kits (Pars Azmon Co., Iran) using an autoanalyzer (Alcyon 300, UK).

Statistical analysis

Data of digestibility were analyzed using the GLM procedure of SAS (2002). The effect of rumen content sampling time (hours post-feeding) was analyzed by repeated measures, using the MIXED procedure of the SAS (2002). The statistical model included the fixed effects of treatment, period, hours post-feeding and their interactions with treatment, while random effect was sheep within treatment. Means were separated using the 'pdiff' option of the 'LSMEANS' statement of the MIXED procedure. Differences were declared as significant at $p < 0.05$ and trends were discussed at $0.05 \leq p \leq 0.10$.

Results

In vivo digestibility and ruminal fermentation

The effect of REO on nutrient digestibility in sheep is summarized in Table 2. Digestibility of DM, NDF, ADF and CP was not ($p > 0.05$) affected by adding different levels of REO to diets.

The ruminal pH not differ ($p > 0.05$) among the different amounts of additive across sampling times. Ruminal concentrations of ammonia-N (Table 3) decreased after feeding and were lower ($p < 0.05$) at low REO dosage compared to control and at 400 mg d^{-1} REO compared with 200 mg d^{-1}, with no differences between diets including 200 or 400 mg d^{-1} of REO and the control one. At 4 h after morning feeding, total VFA concentrations (Table 3) were reduced ($p < 0.05$) by adding 100 mg d^{-1} REO to diet compared with three other treatments, whereas supplementation with 200 mg d^{-1} REO significantly increased ($p < 0.05$) total VFA concentrations versus

Table 2. Effects of rosemary essential oil (REO) on apparent digestibility (%) of nutrients in sheep

	REO dose level (mg sheep^{-1} d^{-1})				SEM	*p* value
	0	100	200	400		
DM	60.7	60.0	63.9	60.2	1.19	0.181
OM	63.3^{ab}	62.5^{b}	66.8^{a}	63.1^{b}	0.72	0.024
NDF	55.5	55.1	59.5	55.4	1.42	0.213
ADF	40.6	44.2	51.6	44.5	2.55	0.100
CP	59.8	57.3	60.9	58.4	1.59	0.498

SEM = standard error of the means. DM: dry matter, OM: organic matter, NDF: neutral detergent fiber, ADF: acid detergent fiber, CP: crude protein. $^{a, b}$ Within a row, means without a common superscript letter differ significantly ($p < 0.05$).

Table 3. Effect of different levels of REO on ruminal fermentation of sheep

	Time (h)	REO dose level (mg sheep^{-1} d^{-1})				SEM	Effects (*p* value)		
		0	100	200	400		Time	Treatment	Treatment × Time
pH	Mean	6.94	7.02	6.88	6.97	0.10	<0.001	0.562	0.404
	0	7.09	7.15	7.11	7.17				
	4	6.79	6.89	6.64	6.77				
Ammonia-N (mg dL^{-1})	Mean	7.7^{ac}	4.3^{b}	8.8^{a}	5.4^{bc}	1.05	0.103	0.002	0.954
	0	8.2	4.7	8.9	5.9				
	4	7.3	3.8	8.6	5.0				
Total VFA (mM)	Mean	45.5	37.6	48.4	43.8	1.94	<0. 001	0.002	0.027
	0	28.9	24.5	29.0	27.8				
	4	62.1^{b}	50.7^{c}	67.7^{a}	59.8^{b}				
Acetate (mM)	Mean	33.5^{a}	28.1^{b}	37.7^{a}	32.3^{ab}	1.36	<0.001	0.001	0.192
	0	21.4	18.3	22.9	20.1				
	4	45.5	37.9	52.4	44.4				
Propionate (mM)	Mean	6.2	5.2	6.1	5. 9	0.40	<0. 001	0.736	0.140
	0	4.1	3.6	4.0	3.8				
	4	8.3	6.9	8.1	7.9				
Butyrate (mM)	Mean	4.7^{a}	3.4^{c}	3.9^{bc}	4.1^{ab}	0.27	<0.001	0.010	0.302
	0	3.1	2.3	2.5	2.7				
	4	6.3	4.4	5.2	5.4				
Acetate/Propionate (mol mol^{-1})	Mean	5.39	5.43	6.18	5.62	0.59	0.597	0.965	0.541
	0	5.29	5.31	5.87	5.64				
	4	5.49	5.54	6.5	5.6				
Total protozoa (×10^{5} mL^{-1})	Mean	3.5	3.2	3.6	3.5	1.32	<0.001	0.378	0.517
	0	3.0	2.7	3.2	2.9				
	4	4.0	3.7	4.0	4.0				

SEM = standard error of the means . $^{a, b, c}$ Within a row, means without a common superscript letter differ significantly ($p < 0.05$).

the control. The 100 mg d^{-1} REO diet promoted lower acetate concentrations ($p < 0.05$) compared to 200 mg d^{-1} of REO and control diets. With regards to butyrate, sheep fed diets containing REO had lower ($p < 0.05$) butyrate concentration than control group except for the 400 mg d^{-1} of REO diet. However, no effect ($p > 0.05$) of varying the REO dosage was observed on acetate to propionate ratio. The inclusion of RO oil did not affect ($p > 0.05$) total protozoa count. In general, except for propionate, which was not affected

Table 4. Effect of different levels of REO on plasma metabolites

	Time (h)	REO dose level (mg sheep^{-1} d^{-1})				SEM	Effects (p value)		
		0	100	200	400		Time	Treatment	Treatment × Time
Glucose (mg dL^{-1})	Mean	67.1	72.3	68.5	68.4	2.52	0.852	0.725	0.857
	0	68.3	72.3	67.8	67.3				
	4	66.0	72.3	69.3	69.5				
Triglyceride (mg dL^{-1})	Mean	17.6	19	16.8	16.3	2.51	0.411	0.772	0.694
	0	17.5	17.3	16.8	16.5				
	4	17.8	20.8	16.8	16.8				
Cholesterol (mg dL^{-1})	Mean	62.6	65.4	63.6	64.6	4.23	0.014	0.927	0.668
	0	66.8	67	67.3	66.0				
	4	58.5	63.8	60.0	63.3				
HDL (mg dL^{-1})	Mean	22.12	21.00	21.37	22.12	1.48	0.537	0.839	0.251
	0	21.75	21.00	23.25	21.75				
	4	22.50	21.00	19.50	22.50				
Total protein (g dL^{-1})	Mean	8.00	8.06	7.98	7.70	0.42	0.332	0.822	0.947
	0	7.97	8.02	7.87	7.57				
	4	8.02	8.10	8.10	7.82				
Albumin (g dL^{-1})	Mean	4.91	4.60	4.73	4.58	0.33	0.241	0.454	0.427
	0	4.90	4.60	4.40	4.52				
	4	4.90	4.60	5.07	4.65				

SEM = standard error of the means. HDL = high density lipoprotein.

($p > 0.05$) by REO supplementation, other end-products of ruminal fermentation were reduced when sheep were fed 100 mg d^{-1} of REO compared to the control group.

Blood parameters

Sampling time and supplementation with REO had no effect ($p > 0.05$) on any plasma parameter of energy and protein metabolism (Table 4). The Treatment × Time post-feeding interaction was not significant ($p > 0.05$) for blood metabolites.

Discussion

At a medium dose (200 mg d^{-1}) of REO, total VFA concentration increased and there was a trend to improvement in digestibility of ADF in total tract ($p \leq 0.10$). However, the opposite occurred at a low dose (100 mg d^{-1}), as total VFA concentrations and molar proportion of acetate and butyrate decreased. In agreement with our results, Chaves *et al.* (2008) reported higher total VFA concentration for growing lambs fed diets supplemented with 200 mg d^{-1} of carvacrol or cinnamaldehyde compared with those fed a control diet. Benchaar *et al.* (2008) reported that VFA concentrations may decrease as a result of the antimicrobial effect of EO, which could be dose dependent. On the basis of these data, it seems that REO at dosage of 100 mg d^{-1} could inhibit the ruminal fermentation of sheep.

Data on the effects of REO on *in vivo* digestibility and ruminal fermentation are scarce. Castillejos *et al.* (2008) showed that REO at 5 and 50 mg L^{-1} had no effect on final pH and VFA concentrations *in vitro*. In order to compare the results from the present study to results from *in vitro* studies, it is necessary to estimate the concentration of REO in the rumen fluid of the sheep. Assuming a 10-L rumen volume (Hristov *et al.*, 2008), the 100 mg d-1 dose would have resulted in about 10 mg/l dose *in vivo*. Because the effects of EO in ruminants depend on experimental conditions and dose (Benchaar *et al.*, 2008), *in vitro* studies are not necessarily indicative of what happens *in vivo*. The average ruminal pH (across all sampling times) was within the optimum pH range (6.7±0.5) to maintain normal function of cellulolytic organisms (Van Soest, 1994).

Wallace *et al.* (2002) reported that rate of ammonia-N production from amino acids in the rumen fluid decreased with EO. In the present study, the effects of the low REO diet may be due to the effect of 1,8 cineol. McEwan *et al.* (2002) reported that addition of EO reduced the number and diversity of hyper-ammonia producing (HAP) bacteria, resulting in reduced rate of ammonia production from amino acids. The HAP bacteria are present in low numbers in the rumen (<0.01 of the rumen bacterial population), but they have a very high deamination activity (Russell *et al.*, 1988). In agreement with our results, Wallace (2004) reported that the number of HAP bacteria was reduced by 77% in sheep receiving a low protein diet supplemented with EO at 100 mg d^{-1}. The decrease of the amino acids degradation could benefit the host by supplying additional amino acids for absorption.

As ruminal protozoa have proteolytic and deaminating activities, they have a negative role on N utilization by ruminants (Williams & Coleman, 1992; Benchaar *et al.*, 2008). Ando *et al.* (2003) showed that feeding 200 g d^{-1} (30 g kg^{-1} of total dietary DM) of peppermint (*Mentha piperita* L.) to Holstein steers decreased the total number of protozoa, but this response did not occur in the current study in which the amounts of REO ingested were 100, 200 and 400 mg d^{-1}. Similar to present results, Newbold *et al.* (2004) and Benchaar *et al.* (2007) reported that ruminal protozoa counts were not affected when sheep and dairy cows were fed 110 and 750 mg d^{-1} of EO, respectively. It seems that EO and their components have no marked effect on numbers and/or activity of ruminal ciliate protozoa.

Plasma concentrations of glucose, triglyceride, cholesterol, HDL, total protein and albumin were not affected by REO feeding, which implies that the amount of REO required to modify the non-ruminal metabolism is higher than the amount required to modify the rumen metabolism.

As conclusion, supplementation of diet with rosemary essential oil (REO) affected ruminal fermentation of feed in a dose-dependent manner in sheep. A low dose of REO (100 mg d^{-1}) decreased the ruminal total volatile fatty acids, acetate, butyrate and ammonia-N concentration, which are negative effects. It seems that the optimal level of REO that should be included in the diet of sheep is the medium level (200 mg d^{-1}). Based on the result of present study we speculated that the medium level of REO had more adaption effects to rumen environment than the two other levels. However, the results regarding supplementation of REO as feed additives to ruminant diets are very scarce and the current work is preliminary. Hence, it needs to be supported with further studies involving challenge experiments with using different levels of REO and other nutrition parameters.

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