Effect of thymol and carvacrol on nutrient digestibility in rams fed high or low concentrate diets

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Summary

Published data on the effects of essential oils (EO) on *in vivo* nutrient digestibility in sheep are contradictory. In 2 experiments, the effect of thymol and carvacrol on nutrient digestibility was studied in sheep fed with high (70%) or low (52%) concentrate diets, using incomplete Latin Square designs. The essential oils were mixed with the concentrate portion of the diet at the rate of 0.0, 0.3, or 0.6 g per kg dry matter (DM) diet. Supplementation of thymol had no significant effect on digestibility of dry matter (DM), organic matter (OM), crude protein (CP) and acid detergent fiber (ADF). The main effect of thymol on neutral detergent fiber (NDF) and ether extract (EE) digestibility and on nitrogen balance (NB) was significant ($P<0.05$), but within each level of dietary concentrate no significant differences were observed for these measurements. Overall, ruminal ammonia concentration was higher ($P<0.05$) in both HCD and LCD lambs receiving 0.3 mg thymol per kg diet. Supplementation of carvacrol had no significant effect on nutrient digestibility. The main effect of carvacrol on ruminal ammonia levels and NB was significant, but within each level of dietary concentrate no significant differences were observed in ammonia levels and NB. Inclusion of 0.3 g/kg diet DM of carvacrol or thyme was more effective than 0.6 g/kg diet DM in terms of NB but neither dose affected nutrient digestibility. Future research should determine the long-term effects of essential oils on digestibility and performance in sheep, before recommendation can be made for their use under practical husbandry conditions.

Key words: Carvacrol, Digestibility, Essential oils, Sheep, Thymol

Introduction

Concern has grown over the use of antibiotics as feed additives because of emergence of resistant bacteria, and antibiotic residues in livestock products. This has stimulated research for replacing antibiotics with natural products such as essential oils (EO) (Jounay and Morgavi, 2007). Because of their antimicrobial activity (Dorman and Deans, 2000), plant extracts or their active components have been used to study their effects in ruminants (Patra and Saxena, 2010; Patra, 2011). Most studies were *in vitro* and of short duration (Klevenhusen *et al*., 2012). At high doses, EO reduced ammonia nitrogen concentration and methane production; however, in most cases they decreased ruminal fermentation rate. Additionally, data on antimicrobial activity of EO and their effects on nutrient utilization and ruminant performance were not consistent (Benchaar *et al*., 2008).

Using *in vitro* gas production technique, a dose-dependent response in ruminal fermentation pattern was reported for several essential oils in sheep (Kamalak *et al*., 2011; Baraka and Abdl-Rahman, 2012; Talebzadeh *et al*., 2012). In an *in vivo* study, Khalesizadeh *et al*. (2011) no effect of garlic oil (Allium sativa) or tumeric powder was found (Curcuma longa Linn) on apparent digestibility in sheep. *In vivo* studies addressing the effects of EO in sheep are scarce (Chaves *et al*., 2008a, b, 2011; Klevenhusen *et al*., 2011) and more studies are needed before firm conclusions can be made on the *in vivo* effects of EO in this species. Therefore, two experiments were carried out to study the effect of the EO of Thymus vulgaris L. (thymol) or Satureja montana L. (carvacrol) on nutrient digestibility in sheep fed with high or low concentrate diets. Thymus vulgaris essential oil is a mixture of monoterpenes, consisting of the natural terpenoid thymol and its phenol isomer carvacrol (Zarzuelo and Crespo, 2002). A wide range of activities has been ascribed to essential oils including antimicrobial, and antibacterial effects (Baser, 2008).

Materials and Methods

The animals were housed and cared for according to guidelines in the Guide for the Animal Care and Use of Experimental Animals, Shiraz University.

Experiment 1

Six healthy rams (3-year-old; 35.8 ± 4.0 kg) were housed in metabolic crates (100 × 100 cm). The experiment was conducted as an incomplete Latin Square design, and the rams were fed with one of the 6 diets during each 17-d period (10 d for adaptation to the diet and 7 d for data collection). Two isonitrogenous and

isoenergetic basal diets (Table 1) containing 52% (low-concentrate diet, LCD) or 70% (high-concentrate diet, HCD) concentrates were prepared to meet the daily requirements for maintenance and a live weight gain of 250 $g.d^{-1}$ (NRC, 2007). Thymol (Barij Co., Iran) was mixed with water, sprayed on the concentrate portion of the diets and mixed thoroughly. Control diets were sprayed with water only; therefore, diets contained 0.0, 0.3, or 0.6 g EO per kg dry matter (DM). The diet was fed as a total mixed diet. Fresh batches of the diet were prepared at 5-d intervals to minimize the loss of EO. Fresh clean water was freely available. The average daily weight gain during the experiment was 280 ± 10 g.

Experiment 2

The design of this experiment was similar to that of the first experiment but carvacrol (supplied by Khorraman Pharmaceutical Co., Lorestan, Iran) was used in lieu of thymol. Six healthy rams (3-year-old; 35.4 ± 4.6 kg) were used in this experiment. The average daily weight gain during the experiment was 280 ± 30 g. The mean initial live weights and average daily gains were not different (P>0.80) between the two experiments.

Feeding and sample preparation for analysis

Diets were fed twice daily (morning and afternoon). Daily feed (about 2 kg DM) was adjusted based on the amount of orts in the morning; it was increased by 100 g if orts were ≤100 g, and decreased by 100 g whenever orts were ≥200 g. When all feed had been consumed, the diet was increased by 200 g (Forbes, 2007).

A 100-g sample of the diet was taken daily during the 7-d data collection period; the samples were mixed and a 100-g sub-sample was stored in sealed bags until analysis. Daily samples of feces (10% of daily fecal output) or urine (100 ml) were kept at -20°C. These were then mixed at the end of data collection period and sub-samples of feces (100 g) and urine (100 ml) were stored at -20°C until analysis. Urine sample was transferred into a plastic container containing 10% (v/v) sulfuric acid (pH = 3.0) to minimize ammonia loss. Fecal samples (100 g) were dried to a constant weight in a forced-air oven at 60°C for 48 h, and then sieved through a 1-mm screen for subsequent chemical analysis. Apparent nutrient digestibility was calculated as the nutrient intake not recovered in feces.

Ruminal fluid was collected before adaptation period, and at the beginning and end of sample collection; the collections were made prior to the morning feeding (*time zero*; $t = 0$) and at 2 and 4 h post-feeding by an electric vacuum pump equipped with a tube (length: 1.5 m; diameter: 5 mm). Ruminal fluid was filtered through four layers of cheesecloth and pH was measured using a digital pH-meter (Jenway Model 3510, Camlab, Cambridge, UK). For determination of ruminal NH_3-N, 5 ml of strained ruminal fluid was immediately acidified with 1 ml of 25% (w/v) meta-phosphoric acid and frozen at -20°C. Nitrogen balance was calculated by subtracting nitrogen excreted in the feces and urine from total nitrogen intake.

Chemical analysis

Feed and fecal samples were ground and milled through a 1-mm screen. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined sequentially using thermo-stable alpha-amylase and sodium sulfite (Van Soest *et al*., 1991). Crude protein (CP, N × 6.25, method No. 984.13), ether extract (EE, method No. 954.02), dry matter (DM, method No. 930.15), and ash (method No. 942.05) contents were measured according to AOAC (1990). The organic matter (OM) content was calculated as the difference between sample DM weight and ash content. Frozen urine samples were placed in a refrigerator, allowed to thaw overnight, and centrifuged at 12,000 g for 20 min at 4°C. Urinary nitrogen content was determined using micro-Kjeldahl instrument (PEPCO, Iran). Ruminal NH_3-N concentration in the supernatant (after centrifugation at 11269 × g, 20 min., 4°C) was determined spectrophotometrically by the phenol-hypochlorite reaction method (Broderick and Kang, 1980).

Statistical analysis

Each experiment was arranged as an incomplete Latin Square design containing 6 treatments, six rams (columns) and 4 periods (rows). The data were analyzed using the mixed procedure (SAS, 2003), and mean comparison was performed using the least squares means procedure adjusted for Tukey's test (P=0.05).

Table 1: Dietary ingredients and chemical composition

Item	Low-concentrate diet (HCD)[1]	High-concentrate diet (LCD)[1]
Ingredients (%DM)		
Alfalfa hay	40	20
Wheat straw	8	10
Concentrate mix	52	70
Composition (g/kg DM)		
Crude protein[2]	127	124
Calcium[3]	1.00	0.91
Phosphorus[3]	0.39	0.45
Metabolizable energy (Mcal/kg)[3]	2.24	2.39

[1] Contained 0.5% vitamin-mineral premix supplying per 100 g: 500,000 IU vitamin A; 10,000 IU vitamin D_3; 100 mg vitamin E; 180 mg Ca; 90 mg P; 2000 mg Mn; 3000 mg Fe; 300 mg Cu; 100 mg Co; 3000 mg Zn; 55 g Na; 19 g Mg. [2] Based on chemical analysis of individual feedstuffs. [3] Based on NRC (2007)

Results

Experiment 1

Supplementation of thymol had no effects on digestibility of DM, OM, CP and ADF (Table 2). The main effect of thymol on NDF and EE digestibility and on NB was significant, but within each level of dietary concentrate no significant differences were observed for NB, and NDF and EE digestibilities. Overall, ruminal ammonia concentration was higher (P<0.05) in both HCD and LCD lambs receiving 0.3 mg thymol per kg diet (Table 3).

A diet by day interaction effect was recorded for ruminal fluid pH. Ruminal pH values in rams feeding on HCD were not affected by thymol, but the lower level of thymol in LCD resulted in an increase in ruminal fluid pH on day 10 of the experiment. However, on day 17, there was no difference between diets in ruminal pH values (Table 4).

Experiment 2

Carvacrol did not affect nutrient digestibility (data not tabulated). The main effects of carvacrol on ruminal ammonia levels and NB (Table 5) were significant, but within each level of dietary concentrate no significant differences were observed in ammonia levels and NB.

Diet by day interaction affected ruminal fluid pH (Table 6) and ammonia concentration (Table 7). In LCD(0.3) rams, ruminal pH was lower on day 1 (pH = 6.73) compared with day 10 (pH = 7.10) and 17 (pH = 7.12). There was no significant effect of diets on ruminal pH on day 1 and day 17; however, on day 10 LCD(0.3) rams recorded a higher pH value compared with LCD(0.0) and LCD(0.6) rams (Table 6). Only on day 17 was there a significant effect of diets on ruminal ammonia concentration (Table 7). Lower ammonia concentration was recorded in rams feeding the LCD(0.6) diet. For the same diet, ruminal ammonia on day 17 was lower than on days 1 and 10.

Table 2: Effect of thymol (mg/kg DM diet) on digestibility in rams fed high (HCD) or low (LCD) concentrate diets

Diet	C:R ratio	Thymol	Apparent digestibility (%)					
			Dry matter	Organic matter	Crude protein	ADF	NDF	Ether extract
HCD(0.0)	70:30	0.0	67.2	70.0	65.0	58.8	74.8^{a}	88.1^{a}
HCD(0.3)	70:30	0.3	66.7	69.8	61.1	57.2	65.1^{ab}	80.6^{a}
HCD(0.6)	70:30	0.6	62.2	66.3	56.5	63.7	65.2^{ab}	81.4^{a}
LCD(0.0)	52:48	0.0	67.6	70.5	70.9	58.9	61.8^{ab}	38.1^{b}
LCD(0.3)	52:48	0.3	67.4	70.5	68.3	56.4	59.0^{b}	46.5^{b}
LCD(0.6)	52:48	0.6	63.6	67.6	68.8	67.3	55.7^{b}	45.9^{b}
SEM	-	-	2.38	2.21	3.01	4.34	3.53	6.31
P-value	-	-	NS	NS	NS	NS	0.02	0.0003

$^{a, b}$ Within columns, mean values with common superscript(s) do not differ (P>0.05). C:R ratio: Concentrate to roughage ratio, ADF: Acid detergent fiber, NDF: Neutral detergent fiber, and NS: P>0.05

Table 3: Effect of thymol (mg/kg DM diet) on ruminal fluid pH and ammonia (mg/dL), and nitrogen balance (g/day) in rams fed high (HCD) or low (LCD) concentrate diets

Diet	C:R ratio	Thymol	pH	Ammonia	Nitrogen balance
HCD(0.0)	70:30	0.0	6.93^{ab}	19.6^{c}	18.3^{ab}
HCD(0.3)	70:30	0.3	6.92^{ab}	21.2^{a}	16.4^{b}
HCD(0.6)	70:30	0.6	6.85^{b}	19.9^{bc}	15.6^{b}
LCD(0.0)	52:48	0.0	6.83^{b}	19.7^{bc}	21.8^{a}
LCD(0.3)	52:48	0.3	7.03^{a}	20.9^{a}	22.1^{a}
LCD(0.6)	52:48	0.6	6.96^{ab}	20.2^{b}	22.7^{a}
SEM	-	-	0.05	0.12	1.71
P-value	-	-	0.05	0.05	0.05

$^{a, b}$ Within columns, mean values with common superscript(s) do not differ (P>0.05). C:R ratio: Concentrate to roughage ratio, and NS: P>0.05

Table 4: Diet by day interaction effect on ruminal fluid pH in rams fed high (HCD) or low (LCD) concentrate diets containing thymol (mg/kg DM)

Diet	C:R ratio	Thymol	Day 1	Day 10	Day 17
HCD(0.0)	70:30	0.0	7.04^{aA}	6.76^{bB}	6.97^{aA}
HCD(0.3)	70:30	0.3	6.75^{bA}	6.93^{bB}	7.05^{aA}
HCD(0.6)	70:30	0.6	6.75^{bA}	6.76^{bA}	7.05^{aA}
LCD(0.0)	52:48	0.0	6.66^{bA}	6.88^{bA}	6.97^{aA}
LCD(0.3)	52:48	0.3	6.73^{bA}	7.18^{aA}	7.13^{aA}
LCD(0.6)	52:48	0.6	6.99^{aA}	6.76^{bA}	7.09^{aA}

$^{a, b}$ Within columns, mean values with common superscript(s) do not differ (P>0.05; SEM=0.07). $^{A, B}$ Within rows, mean values with common superscript(s) do not differ (P>0.05; SEM=0.07). C:R ratio: Concentrate to roughage ratio

Table 5: Effect of carvacrol (mg/kg diet DM) on ruminal fluid pH and ammonia (mg/dL), and nitrogen balance (g/day) in rams fed high (HCD) or low (LCD) concentrate diets

Diet	C:R ratio	Carvacrol	pH	Ammonia	Nitrogen balance
HCD(0.0)	70:30	0.0	6.86	20.1^{ab}	15.6^{b}
HCD(0.3)	70:30	0.3	6.90	21.2^{a}	21.6^{b}
HCD(0.6)	70:30	0.6	6.86	20.1^{ab}	23.5^{b}
LCD(0.0)	52:48	0.0	6.08	20.3^{ab}	30.5^{ab}
LCD(0.3)	52:48	0.3	7.01	21.9^{a}	41.2^{a}
LCD(0.6)	52:48	0.6	6.95	19.68^{b}	31.3^{ab}
SEM	-	-	0.04	0.02	3.5
P-value	-	-	NS	0.05	0.0031

[a, b] Within columns, mean values with common superscript(s) do not differ (P>0.05). C:R ratio: Concentrate to roughage ratio, and NS: P>0.05

Table 6: Diet by day interaction effect on ruminal fluid pH in rams fed high (HCD) or low (LCD) concentrate diets containing carvacrol (mg/kg diet DM)

Diet	C:R ratio	Carvacrol	Day 1	Day 10	Day 17
HCD(0.0)	70:30	0.0	6.89	6.75^{b}	6.95
HCD(0.3)	70:30	0.3	6.72	6.93^{ab}	7.06
HCD(0.6)	70:30	0.6	6.76	6.77^{b}	7.06
LCD(0.0)	52:48	0.0	6.66	6.85^{ab}	7.02
LCD(0.3)	52:48	0.3	6.73^{B}	7.10^{aA}	7.12^{A}
LCD(0.6)	52:48	0.6	7.00	6.76^{b}	7.09

[a, b] Within columns, mean values with common superscript(s) do not differ (P>0.05; SEM=0.07). [A, B] Within rows, mean values with similar superscript(s) do not differ (P>0.05; SEM=0.07). C:R ratio: Concentrate to roughage ratio

Table 7: Diet by day interaction effect on ruminal fluid ammonia concentration in rams fed high (HCD) or low (LCD) concentrate diets containing carvacrol (mg/kg diet DM)

Diet	C:R ratio	Carvacrol	Day 1	Day 10	Day 17
HCD(0.0)	70:30	0.0	20.0	19.8	20.6^{a}
HCD(0.3)	70:30	0.3	21.2	21.1	21.2^{a}
HCD(0.6)	70:30	0.6	20.3	19.9	20.2^{a}
LCD(0.0)	52:48	0.0	20.1	20.2	20.5^{a}
LCD(0.3)	52:48	0.3	20.8	21.0	20.9^{a}
LCD(0.6)	52:48	0.6	20.7^{A}	20.8^{A}	17.5^{bB}

[a, b] Within columns, mean values with common superscript(s) do not differ (P>0.05; SEM=0.4). [A, B] Within rows, mean values with common superscript(s) do not differ (P>0.05; SEM=0.4). C: R ratio: Concentrate to roughage ratio

Body weight change during the experiment

The mean live weight at the beginning and the end of the experiment did not differ between the animals in the Exp. 1 and Exp. 2.

Discussion

In the present study, carvacrol did not modify nutrient digestibility, and thymol only affected the NDF and EE digestibility. Klevenhusen *et al*. (2011) reported that garlic and its principal component diallyl disulfide improved digestibility in sheep but did not reduce methane production.

Direct (Noirot *et al*., 2007) and indirect (Klevenhusen *et al*., 2012) factors affect the response to EO but it is difficult to examine many influencing factors simultaneously. Response to EO in ruminants varies with the species, type and dose of EO, diet composition, and whether the effect was investigated under the *in vivo* or *in vitro* condition. Because of a large number combinations of essential oils (type and dosage) and concentrate levels in the present study it was not feasible to apply all combinations in a single experiment. Therefore, two separate experiments were designed using rams of the same age and live weight, which could have resulted in different responses to EO.

Both doses of thymol, but the lower dose of carvacrol, improved NB in LCD compared with the HCD. While both doses of thymol resulted in higher ammonia concentration in both HCD and LCD, neither dose of carvacrol affected ammonia concentration compared with their respective controls in HCD and LCD groups. In both experiments, higher NB was found with LCD diets. This was despite the findings that ruminal ammonia levels showed little difference between LCD and HCD. This might have been due to better dietary protein utilization.

EO dose is an important determinant of ruminal fermentation. It is difficult to limit the dose for *in vivo* studies, and some studies have used the highest possible doses. Lower doses were applied *in vitro* (a mean of 0.10 g/kg diet DM; Khiaosa-ard and Zebeli, 2013) than *in vivo* (a mean of 4.5 g/kg diet DM; Klevenhusen *et al*., 2012). Although low doses were used in *in vivo*

experiments the results of a meta-analysis (Khiaosa-ard and Zebeli, 2013) revealed potential benefits of EO as ruminal fermentation modifiers. Essential oils are known to have antimicrobial and antibacterial effects although their precise mechanism of action is not known (Baser, 2008; Klevenhusen *et al*., 2012). In the rumen, essential oils seem to increase propionate at the expense of acetate without reducing total VFA production. Detrimental effects of high doses of EO were observed in both *in vitro* (Klevenhusen *et al*., 2012) and *in vivo* (Khiaosa-ard and Zebeli, 2013).

The low dose of thymol increased pH values in LCD by 0.2 units, but carvacrol did not affect ruminal fluid pH. In line with unchanged VFA production in published works, the changes in pH were also small which is beneficial in terms of rumen health (Khiaosa-ard and Zebeli, 2013).

There may also be an association between EO and dietary composition such as NDF and CP (Klevenhusen *et al*., 2012). Although *in vitro* studies indicated a benefit in terms of protein degradation in the rumen, (Klevenhusen *et al*., 2012) such effect was not observed by Khiaosa-ard and Zebeli (2013) in their meta-analysis of the published data; this was corroborated by the finding that EO did not affect ammonia production in the rumen.

The effects of EO in beef cattle have been more pronounced and predictable than in small ruminants because beef cattle are fed with more constant concentrate diets (low fiber and NDF) and have lower ruminal fluid pH values (Khiaosa-ard and Zebeli, 2013). Although administration of EO for short periods may affect ruminal fermentation pattern, their effects on animal performance and feed efficiency may require longer duration.

Inclusion of 0.3 g/kg diet DM carvacrol or thymol oils in high-concentrate diets fed to sheep was more effective than 0.6 g/kg diet DM in terms of N-balance but neither dose affected the nutrient digestibility. Future research should aim at studying the long-term effects of essential oils on digestibility and performance in sheep, before recommendation can be made for their use under practical husbandry conditions.

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