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The effect of Mediterranean thyme (*Thymbra spicata* L. var. spicata) essential oil on fattening performance and ruminal parameters in lamb

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ABSTRACT. The aim of this study was to investigate the effect of dietary supplementation of *Thymbra spicata* L. var. spicata (TS) essential oil (10 or 15 µl · kg^{-1} live weight (LW)) on growth performance (LW, LW gain, feed intake) and rumen parameters (volatile fatty acids (VFA)) and ammonia levels, and pH) in lambs. The experiment lasted 56 days, including 14 days of adaptation and 42 of experimental period. The TS essential oil was extracted from the leaves harvested at the beginning of plant flowering and contained: 66.86% carvacrol, 12.18% *p*-cymene, 10.73% γ-terpinene and 2.77% thymol. The addition of TS essential oil to concentrate feed did not affect final LW, LW gain, feed intake or feed conversion ratio ($P > 0.05$). Molar concentrations (mmol · l^{-1}) of total VFA and acetic (A), butyric (B) and propionic (P) acids, and levels of pH and ammonia-nitrogen (NH_3-N) in rumen fluid were also not affected by TS essential oil inclusion ($P > 0.05$). However, the proportion of A and (A+B):P ratio in rumen fluid VFA increased linearly ($P < 0.01$) with increasing TS essential oil doses, whereas the P proportion decreased linearly ($P = 0.008$). So, it could be suggested that TS essential oil enhanced the concentration of selected VFA in rumen fluid through a positive effect on feed digestion in the rumen. In particular, the linear increase in A and (A+B):P ratio could have enhanced fibrolytic effect in the rumen ecosystem. It was concluded that different doses and longer terms of use of TS essential oil should be further investigated in *in vivo* studies.

Introduction

In southern Europe and the Eastern Mediterranean, several species of *Origanum, Satureja, Thymbra, Thymus* and *Corydothymus* due to their similar smell are known as thyme (Başer, 1995). Besides bay leaf, thyme is the most often exported aromatic plant from Turkey (Ünlü et al., 2009). In the flora of Turkey, 31 taxa consisting of 23 species of *Origanum* are registered (Önenç, 2008). *Thymbra, Origanum,*

Satureja, Thymus and *Corydothymus* genera contain carvacrol, thymol or both phenols that are the main components of their essential oils (Başer et al., 1994).

Thymbra spicata (TS) L. belongs to the *Lamiaceae* family and 4 taxa of this plant exist in Turkey, namely *Thymbra spicata* var. spicata and intricata, *Thymbra sintenisii* var. sintenisii and isaurica with essential oil content of 1.0–3.4%, 1.4–2.7%, 1.5%, and 1.6%, respectively (Başer, 2002). The major component of their essential oils is carvacrol (Başer, 2002; Ünlü et al., 2009). The TS essential oil was reported to consist of 53.1% oxygenated compounds, 25.7% monoterpene hydrocarbons, 4.4% sesquiterpenes and 14.1% *p*-cymene (Hancı et al., 2003). The leaves of TS have been traditionally used in meat products and beverages, but also as ingredients of certain medicines due to their antimicrobial and antiseptic properties (Hancı et al., 2003; Ünlü et al., 2009).

Supplementing essential oils and aromatic plants to ruminant diets improve the digestion, resulting in reduced methanogenesis and nitrogen excretion. Besides, the antioxidative effect, such substances contribute to the stability and palatability of animal diet and so, due to reduced oxidation, an improved shelf-life and quality of animal products (Franz et al., 2010). In our previous study the effect of TS essential oil at different levels (40, 80, 120, 160 and 200 mg · l^{-1} of rumen liquids) on *in vitro* gas production of dairy and beef cattle fed total mixed rations was evaluated (Baytok et al., 2013). It was demonstrated that TS essential oil, especially at the level of 80 mg · l^{-1}, exerts a positive effect on the *in vitro* feed digestibility (Baytok et al., 2013). Nowadays, it is very important to reduce ruminal methane emission in order to prevent global warming and gross energy losses of feed (Kara, 2015; Kara et al., 2015). In the aforementioned study, the 80 and 120 mg · l^{-1} doses of TS essential oil were found to reduce methane production by 9.45 and 34.90%, respectively (Baytok et al., 2013).

The purpose of the present study was to determine the effect of TS essential oil dietary supplementation, which exerts an *in vitro* anti-methanogenic effect, on growth performance (live weight (LW), LW gain, feed intake) and rumen parameters (volatile fatty acids and ammonia levels, and pH) in lambs. The obtained results may be helpful in providing information about TS essential oil usage in livestock.

Material and methods

The study was approved by The Local Ethics Committee of Erciyes University (ERU-HADYEK), Kayseri (Turkey) on 14 December 2011 (No. 11/134).

Plant samples and extraction

In the study, *Thymbra spicata* L. var. spicata plants naturally grown in the province of Hatay (Turkey) were used for essential oil extraction. The plants were collected in the flowering stage and samples were further dried at 35 °C in the laboratory. Essential oil was extracted by using steam distillation, and its chemical composition was determined by gas chromatography-mass spectrometry (GC-MS) technique. Analysis of the essential oil was carried out using an ISQ™ Single Quadrupole GC-MS System (Thermo Fisher Scientific, Waltham, MA, USA) equipped with autosampler and TR-5MS capillary column (5% phenyl polysilphenylene-siloxane, length 30 m, diameter 0.25 mm, film thickness 0.25 μm). Helium (99.9%) was used as the carrier gas at a flow rate of 1 ml · min^{-1}; ionization energy was 70 eV. Mass range was m/z 1.2–1100. The used data acquisition method was scan mode. The MS transfer line temperature was 250 °C, MS ionization source temperature was 220 °C, and the injection port temperature was 220 °C. The samples were injected with 1:250 split ratio. The injection volume was 1 μl. The oven temperature was programmed to increase from 50 °C to 220 °C at a rate 3 °C · min^{-1}. The structure of each compound was identified by comparison to its mass spectrum (Wiley, Hoboken, NJ, USA) and data were handled using the Xcalibur Software (Thermo Fisher Scientific, Waltham, MA, USA).

Animal management

In the study, 15 male 3-month old lambs belonging to the Akkaraman breed which is a local sheep breed in Turkey's Central Anatolia Region were used. The animals were kept in the Sheep Research Unit of the Agricultural Research Farm of Erciyes University in the Kayseri Province (Turkey). The experiment lasted 56 days: 14 days of adaptation and 42 days of experimental period.

Adaptation period

The animals were housed in individual boxes (200 cm × 180 cm × 120 cm; height, length, width, respectively) equipped with feeders dispensing forage and concentrate feed separately, and an automatic drinker. The lambs were fed twice a day during adaptation and experimental periods. In adaptation period that lasted 2 weeks lambs got accustomed to the experimental diet. The level of concentrate feed was increased gradually during adaptation period. At day 1, they were fed only lucerne hay (100% of diet), and at the end of this period (day 14) – diet containing 70% concentrate feed (pelleted form) and 30% lucerne hay.

All lambs were treated against internal and external parasites.

Experimental period

The animals were housed in the same boxes as in adaptation period. Through the whole experimental period, all lambs were fed diet containing about 70% of concentrate feed (Table 1) and 30% of lucerne hay. The diets were prepared by calculating the daily needs of lambs for dry matter and energy according to National Research Council (NRC, 2007). Diet and water were offered *ad libitum*.

Table 1. Ingredient composition of concentrate feed

Ingredients	Amount, %
Barley	34.0
Wheat	22.0
Maize	20.0
Wheat bran	10.0
Cotton seed meal (31% CP)	5.4
Sugar beet molasses	5.0
Limestone (38% Ca)	2.5
Di-calcium phosphate	0.5
Salt	0.6

The lambs were separated according to their LW into three groups; control – without TS essential oil dietary supplementation, and two treatment groups fed diet supplemented with 10 or 15 µl of TS essential oil per kg of LW (Table 2). These levels of supplementation appeared to exert a positive effect on the digestion of concentrate feed in our previous study (Baytok et al., 2013). Essential oils were added to the concentrate feed daily using an automatic pipette before lambs feeding. Concentrate feed with TS essential oil was consumed readily. These calculations were carried out individually for each lamb in the treatment groups (Table 2). The TS essential oil was stored in a sealed thermos for protection against sunlight and high temperature.

Table 2. Calculation[1] of daily amount of *Thymbra spicata* essential oil, µl · day^{-1}

Week	Experimental groups (*Thymbra spicata* essential oil)	
	10 µl · kg^{-1} LW (n = 5)	15 µl · kg^{-1} LW (n = 5)
as daily amount, µl · day^{-1}		
1	384	572
2	402	600
3	418	621
4	430	642
5	453	677
6	476	712

[1] – calculations were fitted individually for each lamb. Values are the average of 5 lambs for both treatment groups; LW – live weight

The individual forage or concentrate feed intake of lambs was determined daily [feed intake, g = (provided feed, g − feed residues, g)]. Live weight was determined weekly and LW gain was calculated.

Rumen fluid characteristics

At the end of the study, samples of the rumen fluid from each lamb were collected into 2 sterile glass bottles (50 g per bottle) using a stomach tube 3 h after the morning feeding.

The pH value of the rumen fluid was determined using a digital pH meter (Mettler Toledo S220; Mettler Toledo, Greifensee, Switzerland). The ammonia-N (NH_3-N, mg · l^{-1}) concentration of the rumen medium was estimated by a distillation method (Makkar and Becker, 1996). Briefly, after centrifugation at 1000 *g* for 15 min the samples were subjected to the distillation with potassium hydroxide (2 N) without prior acid digestion. The liberated NH_3 was collected in flasks with boric acid and titrated with diluted hydrochloric acid (0.1 N) (Souza et al., 2010).

Ruminal volatile fatty acids (VFA) [acetic (A), propionic (P) and butyric (B) acids, mmol · l^{-1} of rumen fluid] concentration was assessed by GC (TRACE™ 1300 GC, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a flame ionisation detector as described by Erwin et al. (1961). The ruminal fluid was filtered through 4 layers of cheesecloth, mixed with 25% (w/v) meta-phosphoric acid and kept frozen (−20 °C) for the further analysis of VFA. The frozen samples were thawed at 4 °C and centrifuged. The analysis was performed under the following conditions: wax column (TraceGOLD™ TG-WaxMS, length 60 m, diameter 0.25 mm, film thickness 1.25 µm); injector and detector temperature 240 °C; stove heat program, from 80 °C (1 min hold) to 240 °C rising at 10 °C · min^{-1} and held for 20 min at 240 °C; flow speed 15 psi; detector 70 eV; ionization type, EI; carrier gas: helium (20 ml · min^{-1}); sample injected 1 µl. Identification of constituents was carried out with the help of the retention times of standard substances (Fluka – Sigma Aldrich, St. Louis, MO, USA). Data were handled using the Xcalibur Software (Thermo Fisher Scientific, Waltham, MA, USA).

The concentration of selected and total VFA was determined as mmol · l^{-1}. The individual acids proportions as the % in VFA and the (A+B):P ratio were also calculated.

Chemical analysis

The samples of concentrate feed and lucerne hay were milled through a 1-mm sieve (MF 10 basic Microfine grinder drive, IKA®-Werke GmbH & Co. KG, Staufen, Germany) for further use in chemical

analysis. The analyses of concentrations of dry matter (DM), ash, crude protein (CP), and diethyl ether extract (EE) were provided according to AOAC International (2012), methods 934.01, 942.05, 954.01 and 920.39, respectively. The neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) contents were determined in approximately 1.0 g of samples by using a fibre analyser (FIWE3, VELP Scientifica, Usmate, Italy) according to Van Soest et al. (1991) method. The NDF was determined using 0.5 g of sodium sulphite and 200 µL of thermo-stable α-amylase (aNDF) (Megazyme, Wicklow, Ireland). Total aNDF, ADF and ADL contents were corrected for ash (aNDFom, ADFom and ADL, respectively). The total condensed tannins (TCT) content of the samples was determined by the butanol-HCl method according to Makkar et al. (1995) using a spectrophotometer (UviLine 8100, SI Analytics, Mainz, Germany). Analyses were carried out in duplicate (Table 3).

Table 3. Chemical composition of concentrate feed and lucerne hay

Indices, % DM	Concentrate feed	Lucerne hay
Crude protein	12.70	10.23
Ash	7.82	8.61
Ether extract	3.13	1.30
Crude fibre	8.89	36.34
aNDFom	28.87	48.81
ADFom	12.47	41.27
HC	16.40	7.54
ADL	3.27	8.67
TCT	0.32	0.61

aNDFom – neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; ADFom – acid detergent fibre expressed exclusive of residual ash; HC – hemicellulose (aNDFom − ADFom); ADL – acid detergent lignin determined by solubilisation of cellulose with sulphuric acid; TCT – total condensed tannin

Statistical analysis

The experimental data were firstly subjected to Levene's test to detect the variance homogeneity. One-way variance analysis (ANOVA) was implemented for homogeneous variances by General Linear Model procedures to test treatment differences. Data were analysed according to the following statistical model:

$$Yij = \mu ij + Si + ei$$

where: Yij – general mean common for each parameter under investigation, μij – general mean common of TS essential oil for each parameter under investigation, Si – effect of TS essential oil on the observed parameters, ei –standard error term.

The means were compared by Tukey's multiple range test at $P < 0.05$. The data were presented as mean ± standard error of mean.

Analyses were performed using SPSS 17.0 software (IBM Corp., Chicago, IL, USA).

Results

Essential oil of TS harvested at the beginning of flowering was determined to contain 66.86% carvacrol, 12.18% *p*-cymene, 10.73% γ-terpinene, 2.77% thymol and several other components (Table 4).

Table 4. Chemical composition of *Thymbra spicata* L. essential oil

Compounds	%	RI[1]	RT[2]
α-pinene	0.56	1028	3.64
α-phellandrene	0.64	1033	3.71
camphene	0.06	1073	4.36
β-pinene	0.10	1113	5.16
δ-3-carene	0.05	1155	6.10
β-myrcene	1.04	1170	6.51
α-terpinene	1.48	1184	6.90
dl-limonene	0.17	1202	7.43
β-phellandrene	0.12	1212	7.69
γ-terpinene	10.73	1252	8.86
p-cymene	12.18	1276	9.69
α-terpinolene	0.05	1286	10.04
oct-1-en-3-ol	0.11	1454	16.17
trans sabinene hydrate	0.05	1465	16.59
cis sabinene hydrate	0.03	1547	19.73
linalool	0.03	1551	19.91
trans caryophyllene	1.28	1589	21.39
4-terpineol	0.53	1598	21.79
isoborneol	0.21	1694	25.36
d-carvone	0.02	1728	26.55
anethole	0.04	1826	30.05
caryophyllene oxide	0.65	1968	34.87
spathulenol	0.15	2125	39.56
thymol	2.77	2218	41.80
carvacrol	66.86	2239	42.61
naphthalene[3]	0.08	2281	44.26

[1] RI – retention index; RT – retention time; [2] RT – retention time; [3] naphthalene,1,2,3,4,4a,5,6,7-octahydro-4a-methyl

There was no negative effect of both levels of TS essential oil dietary supplementation on LW, LW gain, feed intake (concentrate feed and lucerne hay) and feed conversion ratio ($P > 0.05$; Table 5).

The pH values, NH_3-N level (mg N · l^{-1}), and molarities of B, P and VFA (mmol · l^{-1}) in rumen fluid were not affected ($P > 0.05$) by the TS essential oil addition at both levels (10 and 15 µl · kg^{-1}). On the other hand, molarity of A ($P = 0.024$), the individual proportion (%) of A ($P < 0.001$) and (A+B) : P ratio ($P = 0.009$) in the VFA of rumen fluid increased linearly with the increasing dose of TS essential

Table 5. Effect of *Thymbra spicata* L. essential oil on some performance parameters in lambs

	Control group	Experimental groups (*Thymbra spicata* essential oil)		*P*-value
		10 µl · kg^{-1} LW	15 µl · kg^{-1} LW	
Live weight, kg				
initial of experimental period	38.41 ± 1.78	38.52 ± 2.36	38.14 ± 1.41	0.989
week 3 of experimental period	43.03 ± 1.66	43.51 ± 1.94	42.83 ± 1.84	0.964
week 6 of experimental period	47.62 ± 2.01	47.18 ± 2.05	47.48 ± 1.53	0.986
Live weight gain, g · day^{-1}				
average of first 3 weeks	220.20 ± 0.67	237.60 ± 0.53	223.30 ± 0.62	0.891
average of last 3 weeks	218.70 ± 0.19	214.90 ± 0.18	221.50 ± 0.14	0.156
Concentrate feed intake, g · day^{-1}				
average of first 3 weeks	1016.31 ± 12.32	1027.90 ± 9.95	1015.42 ± 11.72	0.696
average of last 3 weeks	1084.20 ± 9.08	1086.41 ± 10.36	1086.00 ± 9.67	0.986
Lucerne hay intake, g · day^{-1}				
average of first 3 weeks	466.00 ± 17.77	458.00 ± 16.62	475.00 ± 7.41	0.722
average of last 3 weeks	611.00 ± 10.77	619.00 ± 9.53	624.00 ± 9.66	0.661
Feed conversion ratio*, g · g^{-1}				
average of first 3 weeks	6.73 ± 0.16	6.25 ± 0.21	6.67 ± 0.18	0.452
average of last 3 weeks	7.75 ± 0.42	7.93 ± 0.29	7.72 ± 0.34	0.738

* – calculated as dry matter intake (concentrate feed + lucerne hay)/ live weight gain; LW – live weight

Table 6. Effect of *Thymbra spicata* L. essential oil on concentrations of organic acids, NH_3-N and pH value in lamb rumen fluid

Indices	Control group	Experimental groups (*Thymbra* spicata essential oil)		*P*-value	
		10 µl · kg^{-1} LW	15 µl · kg^{-1} LW	linear	quadratic
Molarities in rumen fluid, mmol · l^{-1}					
acetic acid	47.90 ± 0.97	47.80 ± 0.82	50.36 ± 1.24	0.024	0.319
propionic acid	26.74 ± 0.68	26.14 ± 0.26	26.82 ± 0.66	0.928	0.385
butyric acid	8.91 ± 0.25	8.92 ± 0.30	9.74 ± 0.40	0.103	0.333
VFA[1]	83.55 ± 1.86	82.86 ± 1.37	86.91 ± 2.15	0.224	0.317
Individual proportions in total VFA, %					
acetic acid	57.34 ± 0.12	57.69 ± 0.05	57.94 ± 0.03	<0.001	0.629
propionic acid	32.00 ± 0.16	31.55 ± 0.21	30.86 ± 0.32	0.008	0.679
butyric acid	10.67 ± 0.14	10.76 ± 0.19	11.21 ± 0.32	0.135	0.543
(A+B):P[2]	2.13 ± 0.02	2.17 ± 0.02	2.24 ± 0.03	0.009	0.652
pH	6.91 ± 0.19	7.04 ± 0.13	7.06 ± 0.15	0.270	0.356
NH_3-N, mg N · l^{-1}	15.68 ± 0.40	17.88 ± 1.51	18.62 ± 1.69	0.154	0.664

[1] VFA – violate fatty acids = acetic + propionic + butyric acids as mmol · l^{-1} in rumen fluid; [2] (A+B):P = (acetic acid + butyric acid)/ propionic acid as mmol · l^{-1}; LW – live weight

oil. In addition, the individual proportion (%) of P ($P = 0.008$) in rumen fluid decreased linearly with the levels of TS essential oil addition (Table 6).

Discussion

The essential oils (or secondary metabolites) extracted from aromatic plants have different biological activities in human and animal organisms depending on the genus of the aromatic plant and the amount of secondary metabolites (Benchaar et al., 2006; Ali et al., 2015; Calo et al., 2015). In the present study, the most common component in the essential oil of TS at the early flowering stage was carvacrol (66.86%). Other high compounds were terpinolen isomeric hydrocarbons (α-terpinene + γ-terpinene, 10.73 + 1.48%, respectively) and *p*-cymene aromatic hydrocarbon (12.18%). İnan et al. (2011) demonstrated that the levels of TS essential oil components varied from 53.55 to 64.53% for carvacrol, from 14.29 to 19.45% for γ-terpinene and from 7.06 to 10.34% for *p*-cymene according to the plant

developmental stage and this was confirmed in our study. Ünlü et al. (2009) stated that the TS essential oil was characterised by a high content of the phenolic carvacrol (60.39%) and other major compounds as monoterpenic hydrocarbons: γ-terpinene (12.95%) and *p*-cymene (9.61%). So, the most common secondary compounds in TS essential oil are the monoterpenic hydrocarbons: γ-terpinene and *p*-cymene, which has been confirmed by the results of previously mentioned and current study.

Tümen et al. (1994) studied the compositions of *Thymbra spicata* var. spicata and *Thymbra spicata* var. intricata (endemic in Turkey) which vary in different regions of Turkey and show that the composition of TS essential oil show large variations in the relative concentration of major components: carvacrol (9.10–76.86%), γ-terpinene (5.35–22.33%) and *p*-cymene (5.49–23.54%). In the studies carried out on TS it was observed that the chemical composition of essential oil might depend on harvesting seasons, geographical locations, processing, storage conditions and parts of the plants.

The plant extracts used in ruminant nutrition are considered as natural manipulators of rumen fermentation and digestibility due to their physiological and pharmacological effects. In this study, the addition of TS essential oil at the levels of 10 and 15 µl per kg LW of lambs did not adversely affect LW or LW gain. The intake of concentrate feed and lucerne hay was similar which is in accordance with the results of other *in vivo* studies examining thyme oil and active compounds of this genus (carvacrol, thymol, γ-terpenene and *p*-cymene) (Bampidis et al., 2005; Chaves et al., 2008; Wang et al., 2009; Vakili et al., 2013). Simitzis et al. (2008) also observed that supplementation of concentrate feed oregano essential oil (1 ml · kg^{-1}) did not affect LW or LW gain. Biricik et al. (2016) found that supplementation of carvacrol (100 or 300 mg · kg^{-1}), thymol (100 or 300 mg · kg^{-1}) or their mixtures (100 or 300 mg · kg^{-1}) to the diet of fattening lambs did not affect feed intake, LW, LW gain and feed conversion ratio.

In the present study, the individual molarities of B, P and total VFA, NH_3-N concentration and the pH value of rumen were not influenced after the supplementation with TS essential oil. Wang et al. (2009) also reported that the addition of oregano oil at the dose of 250 mg · d^{-1} into sheep diet did not affect the ruminal pH and A:P ratio, but increased VFA content and decreased NH_3-N concentration. Chaves et al. (2008) found that carvacrol (purity >98%) supplementation at the level of 0.2 g · kg^{-1} DM reduced ruminal pH, increased VFA and did not change A:P ratio or NH_3-N level in lambs. Besides, thyme essential oil (5 g · d^{-1} · $calf^{-1}$) supplementation to growing calves diet (15% lucerne hay and 85% concentrate feed) did not affect pH value, concentration of NH_3-N and molar concentration of VFA in rumen fluid; whereas decreased the molar proportion of acetate and A:P ratio, and increased the molar proportion of propionate (Vakili et al., 2013). In another study, addition of *Origanum vulgare* oil (rich in carvacrol) into lamb diet increased pH value, NH_3-N concentration, and the molar concentration of VFA in rumen fluid (Biricik et al., 2016). The differences observed in these studies could be attributed to such factors as the method of essential oil supplementation, the time of addition (just before feeding), the content of the active compound, the ambient temperature and the amount of other active compounds present in the diet.

Conclusions

Thymbra essential oil has a positive effect on the digestion of the diet, thus increasing the concentration of selected volatile fatty acids in the rumen liquid. In particular, a linear increase in the molarity of acetic acid and (acetic + butyric):propionic acid ratio in rumen fluid of lambs fed diet supplemented with *Thymbra spicata* essential oil may indicate an increase in fibrolitic activity in the rumen ecosystem. It may be advisable that these essential oils which are characterized by high carvacrol, *p*-cymene, γ-terpinene contents can be used as a functional additive in lamb diets. Nevertheless, antiprotozoal and antimicrobial properties of *Thymbra spicata* essential oil in rumen should be further investigated by using different doses and in further *in vivo* studies of greater duration.

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