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Effect of thyme essential oil on rumen parameters, nutrient digestibility, and nitrogen balance in wethers fed high concentrate diets

[*Efeito do óleo essencial de tomilho sobre os parâmetros ruminais, a digestibilidade dos nutrientes e o balanço de nitrogênio em borregos alimentados com elevado teor de concentrado*]

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ABSTRACT

This trial aimed to evaluate the effects of thyme essential oils (EO) on rumen parameters, nutrient digestibility and nitrogen balance in wethers fed with high-concentrate diet. Twenty rumen-cannulated wethers were blocked according to body weight (BW= 64.0±2.1kg), and received one of the following treatments: 25mg of monensin/kg of dry matter (DM; MON) or doses of thyme EO (1.25, 2.50 or 3.75g/kg of DM). The diet was composed of 90% concentrate. Thyme EO was composed mainly by thymol (46.6% of DM) and p-cymene (38.9% of DM). The nutrient intake and apparent digestibility were similar among treatments. The inclusion of 3.75g of thyme EO tended (P= 0.07) to increase butyrate compared to MON and 1.25OE and wethers fed with 1.25g of thyme EO tended (P= 0.07) to decrease ruminal pH on the 14th day compared to MON. The treatments did not affect acetate:propionate ratio, total short chain fatty acids (SCFA) and nitrogen retention. Results from this study suggest that adding thyme EO to high-concentrate diets may be used as an alternative to monensin as feed additive in feedlot lambs.

Keywords: monensin, p-cymene, ruminal pH, thymol

RESUMO

O objetivo do estudo foi avaliar os efeitos do óleo essencial (OE) de tomilho nos parâmetros ruminais, na digestibilidade e no balanço de nitrogênio em borregos alimentados com elevado teor de concentrado. Vinte borregos providos de cânulas ruminais foram blocados de acordo com o peso corporal (PC=64,0±2,1kg) e receberam um dos tratamentos: 25mg de monensina/kg de matéria seca (MS; MON) ou doses de OE de tomilho (1,25; 2,50 ou 3,75g/kg de MS). A dieta foi composta por 90% de concentrado. A composição do OE de tomilho foi principalmente timol (46,6% da MS) e p-cimeno (38,9% da MS). A ingestão e a digestibilidade dos nutrientes foram semelhantes entre os tratamentos. A inclusão de 3,75g de OE de tomilho tendeu (P=0,07) a aumentar o butirato em relação aos tratamentos MON e 1,25OE. Os borregos alimentados com 1,25g de OE tenderam (P=0,07) a apresentar menor pH ruminal no 14º dia comparado a MON. No entanto, os tratamentos não afetaram a relação acetato:propionato, concentração total de ácidos graxos de cadeia curta e retenção de nitrogênio. Os resultados sugerem que a adição de OE de tomilho em dietas com elevado teor de concentrado pode ser uma alternativa à monensina como aditivo alimentar para cordeiros em confinamento.

Palavras-chave: monensina, p-cimeno, pH ruminal, timol

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INTRODUCTION

The feed additives used in ruminant nutrition aim to increase energy efficiency in ruminal fermentation, resulting in an increase in animal performance. In the last years, ionophores have been the main feed additive used as growth-promoters in Brazilian feedlot (Oliveira and Millen, 2014). However, monensin was banned in the European Union (Comission…, 2003), and it is necessary to find other compounds as an alternative to monensin as feed additive. Thus, it is known that compounds present in thyme essential oils (EO) have antimicrobial properties, which may allow its use as an alternative to ionophores (Lis-Balchin and Deans, 1997; Cristani *et al*., 2007).

Based on these characteristics, the potential of thyme EO as a rumen manipulator has been extensively studied *in vitro* (Castillejos *et al*., 2008; Chaves *et al*., 2008). However, there are few *in vivo* studies evaluating thyme EO as feed additive (Khorrami *et al*., 2015). It is described that thyme EO decreased the molar proportion of acetate and ratio of acetate to propionate, and increase the molar proportion of propionate (Vakili *et al*., 2013). Similarly, monensin increases the molar proportion of propionate and reduces the acetate (Ellis *et al*., 2012).

High propionate concentration increases energetic gain from rumen fermentation, which may improve animal performance (Wolin, 1960; Ellis *et al*., 2012). On the other hand, an EO blend (thyme, clove and cinnamon) did not affect apparent nutrient digestion, rumen fermentation and rumen microbial populations in sheep fed with 50% of hay (Khateri *et al*., 2017). Thus, this trial aimed to determine the effects of using three doses of thyme EO on ruminal parameters, nutrient intake, nutrient digestibility and nitrogen balance in wethers fed with a high-concentrate diet.

MATERIALS AND METHODS

The experiment was carried out at sheep facilities of the Department of Animal Science, "Luiz de Queiroz" College of Agriculture, University of São Paulo, São Paulo State, Brazil. Both experiments were approved by the Animal Care and Use Committee from the College of Veterinary and Animal Science (FMVZ), University of São Paulo (#4214031014).

Twenty rumen-cannulated crossbreed (½ Dorper x ½ Santa Inês) wethers were blocked by initial body weight (BW= 64.0±2.1kg) to receive one of the treatments: a positive control (MON) with inclusion of 25mg of monensin (Rumensin® 100, Elanco Brazil, São Paulo, SP, Brazil)/kg of dry matter (DM); inclusion of 1.25 (1.25EO), 2.50 (2.50EO) or 3.75 (3.75EO)g of thyme EO/kg of DM, whose EO composition is shown in Table 1. The wethers were housed in individual pens, with feed bunks, mineral boxes and waterers. At the beginning of the experiment, wethers were dewormed with 1.0% moxidectin (Cydectin, Fort Dodge Animal Health, Campinas, São Paulo, Brazil) at a dosage of 1mL/50kg BW and received ADE vitamin supplements.

Table 1. Chemical composition of thyme essential oil used in the experiment

Item	% of dry matter
Thymol	46.60
p-cymene	38.90
Linalool	3.30
α-pinene	1.00
Myrcene	0.60
1,8-cineole	1.70
Camphene	0.80
Limonene	1.20
β-pinene	0.30
γ-terpinene	3.80
Others	1.80

The experiment lasted 23 days, where the first 18 days were used for diet adaptation and the last 5 days for sampling. The diets, composed of 10% coastcross hay and 90% concentrate, were formulated according to NRC (Nutrient…, 2007; Table 2). Corn and coastcross hay were coarsely ground and mixed with soybean meal, limestone, mineral mix and ammonium chloride using a horizontal mixer. The feed additives were mixed every day before feeding, avoiding losses by thyme EO volatilization. The diets were weighed daily with an electronic scale accurate to 1g and offered *ad libitum* in the morning and afternoon. The refusal was weighed daily to obtain the dry matter intake (DMI) per animal.

The feces were obtained using collection bags to avoid urine contamination, which was quantified daily. Urine was collected with recipients

containing HCl (6N), maintaining pH below 3.0 to prevent ammonia volatilization. Feces and urine were sampled (10% daily) and stored at -18°C. The samples were analyzed to access the nutrient digestibility in the total digestive tract.

Table 2. Proportions of ingredients and chemical composition of the experimental diets

Item	Diets[1]			
	MON	1.25EO	2.50EO	3.75EO
Ingredients (g/kg of DM)				
Coastcross hay	100.0	100.0	100.0	100.0
Ground corn	700.0	698.7	697.5	696.2
Soybean meal	162.0	162.0	162.0	162.0
Limestone	16.0	16.0	16.0	16.0
Ammonium chloride	04.0	04.0	04.0	04.0
Mineral mix[2]	18.0	18.0	18.0	18.0
Thyme essential oil	0.00	1.25	2.50	3.75
Chemical composition (g/kg of DM)				
Organic matter	939.0	937.0	940.0	936.0
Crude protein	148.0	148.0	149.0	151.0
Neutral detergent fiber	222.0	228.0	224.0	229.0

[1]Diets: MON= 25mg/kg of dry matter of monensin; 1.25EO= 1.25g of thyme EO/kg of DM; 2.50EO= 2.50g of thyme EO/kg of DM; 3.75EO= 3.75g of thyme EO/kg of DM. [2] Composition: 7.5% P; 13.4% Ca; 1.0% Mg; 7% S; 14.5% Na; 500ppm Fe; 300ppm Cu; 4600ppm Zn; 15ppm Se.

Ruminal fluid was collected at 0 (before feeding), 3, 6, 9 and 12 hours after feed supply on the 7th, 14th and 23rd days of the experiment. The ruminal fluid collected was filtered using nylon cloth, and then ruminal pH was measured with a digital potentiometer (DIGIMED DM20, Piracicaba, SP, Brazil). Ruminal fluid collected at each hour was homogenized with same day samples, resulting in a sample per animal per day. These collections aimed to evaluate the effect of the treatments during the adaptation period. The aliquots were stored in plastic vials, and frozen at -18°C for subsequent SCFA and ammoniacal nitrogen analysis, which was determined according to Ferreira *et al*. (2016).

The thyme EO composition was determined using a Chromatograph equipped with RTX 5MS column with 30m length, and 25mm thick with an initial temperature of 40°C for 8 minutes (ramp 1) increasing by 30°C per minute to 180°C (ramp 2) and 20°C to 230°C (ramp 3) for a total period of 77.17 minutes.

The feed offers, orts and feces samples were thawed, compounded by wethers, dried in a forced-air oven at 55°C for 72h and ground with a Wiley mill (Marconi, Piracicaba, SP, Brazil) to pass a 1-mm screen. The DM content was determined by drying the samples at 105°C for 24 hours, and ash content was obtained by incinerating the samples in an oven at 550°C for 4 hours (Official…, 1990). The OM was calculated by the difference between the DM and ash. The NDF was determined according to Van Soest *et al*. (1991) using α-amylase and sodium sulfite in a 2000 Ankom system (Ankom Tech. Corp., Fairport, NY, USA). Total nitrogen of offered feed, orts, feces and urine were determined using the Leco FP528 instrument (Leco Corporation, St. Joseph, MI, USA) according to AOAC (Official…, 1990).

The ammoniacal nitrogen concentration was determined with a colorimetric method that was described by Chaney and Marbach (1962), adapted for a microplate reader (EON, BioTek Instruments, Winooske, VT, USA) with a 550nm absorbance filter.

Wethers were considered as an experimental unit for all statistical analyses. The nutrient intake, nutrient digestibility and nitrogen balance were analyzed using MIXED procedure of the SAS statistical software program (SAS version 9.0; SAS Inst. Inc., Cary, NC). All data were submitted to the Shapiro-Wilk and Levene test to verify the normality of the residuals and homogeneity of variances respectively. The removal of outliers (studentized residual >3 or <-3) was also done. Nutrient intake and digestibilities, variables related to N, and SCFA

profile were analyzed using the model $y_{ij=} \mu + D_i + b_j + e_{ij}$, in which μ= overall mean, D_i= fixed effect of diet, b_j= random effect of block, and e_{ij}= random error.

The ruminal pH was analyzed as repeated measures over time, which was determined by covariance matrices and tested for "compound symmetry, heterogeneous compound symmetry, autoregressive, autoregressive heterogeneous, unstructured, banded, variance components, toeplitz and heterogeneous toeplitz" and defined according to the lowest value obtained for Akaike's Information Criterion. The statistical model used was $y_{ijk}= \mu + D_i + b_j + e_{ij} + T_k + D_iT_k + b_jT_k + e_{ijk}$, in which μ= overall mean, D_i= fixed effect of diets, b_j= random block effect, e_{ij}= random error A, T_k= fixed effect of time, D_iT_k= fixed effect of diet × time interaction, b_jT_k= random effect of block × time interaction, and e_{ijk}= random error B. The effect of diet, day and diet x day interaction were defined by the variance analysis F test. The means were obtained by the LSMEANS command. The diet effect was defined by Tukey test and significance was defined as P< 0.05.

RESULTS

The main compounds in thyme EO used in this experiment were thymol (46.6% of DM) and p-cymene (38.9% of DM; Table 1). Experimental diets did not affect DM, OM, CP and NDF intake (Table 3). Similarly, the digestibility of DM, OM, CP, and NDF were not affected by treatments either. In addition, nitrogen intake, fecal, urinary and retention were similar among treatments (Table 4).

Table 3. Intake and digestibility of the diets with thyme essential oil or monensin

Item	Diets[1]				SEM[2]	*P*-value
	MON	1.25EO	2.50EO	3.75EO		
Intake (kg/d)						
Dry matter	1.92	1.93	1.75	1.79	0.12	0.11
Organic matter	1.81	1.80	1.64	1.68	0.11	0.11
Crude protein	0.29	0.29	0.25	0.27	0.02	0.11
Neutral detergent fiber	0.43	0.44	0.40	0.41	0.03	0.53
Digestibility (%)						
Dry matter	85.33	88.30	85.13	83.79	1.61	0.25
Organic matter	87.21	90.13	87.53	86.41	1.51	0.34
Crude protein	83.85	87.84	83.52	81.99	1.97	0.21
Neutral detergent fiber	68.89	76.75	70.65	67.38	3.43	0.33

[1]Diets: MON= 25mg/kg of dry matter of monensin; 1.25EO= 1.25g of thyme EO/kg of DM; 2.50EO= 2.50g of thyme EO/kg of DM; 3.75EO= 3.75g of thyme EO/kg of DM. [2] Standard error of the mean.

Table 4. Nitrogen metabolism in wethers fed high-concentrate diets with thyme essential oil or monensin

Item (g/d)	Diets[1]				SEM[2]	*P*-value
	MON	1.25EO	2.50EO	3.75EO		
N intake	45.22	45.16	41.28	42.21	2.53	0.16
Fecal N	7.35	5.53	6.28	7.56	0.84	0.36
Urinary N	9.64	12.49	10.94	10.58	1.62	0.56
N retention	28.22	26.93	23.45	24.06	2.67	0.20

[1]Diets: MON= 25mg/kg of dry matter of monensin; 1.25EO= 1.25g of thyme EO/kg of DM; 2.50EO= 2.50g of thyme EO/kg of DM; 3.75EO= 3.75g of thyme EO/kg of DM. [2] Standard error of the mean.

The experimental diets did not affect molar proportion of acetate, propionate, isobutyrate, isovalerate and valerate (Table 5). The inclusion of 3.75g of thyme EO/kg of DM tended (P= 0.07) to increase butyrate compared to MON and 1.25OE. Feed additives did not affect acetate:propionate ratio, total SCFA, ammonia nitrogen and ruminal pH.

There was a day effect on valerate (P= 0.02) and ammonia nitrogen (P= 0.05), and a tendency (P= 0.09) on butyrate molar proportion. Ruminal molar proportion of valerate decreased during the experiment, where the smallest concentration was on the 23rd day (1.26mM/100mM). However, ammonia nitrogen increased throughout the experiment, where the highest

concentration was on the 23rd day (23.13mg/dL). Furthermore, molar proportion of butyrate tended to increase on the 23rd day (12.43mM/100mM).

There was a tendency (P= 0.07) for interaction between diet and day for ruminal pH (Figure 1). Analyzing this interaction, there was no effect on ruminal pH on the 7th and 23rd days. However, wethers fed diets containing 1.25g of thyme EO had a smaller ruminal pH than MON on the 14th day.

Table 5. Ruminal parameters of wethers fed high-concentrate diets with thyme essential oil or monensin

Item	Diets[1]				SEM[2]	*P*-value
	MON	1.25EO	2.50EO	3.75EO		
SCFA[3] (mM/100mM)						
Acetate (Ac)	54.22	51.84	51.38	55.76	2.15	0.39
Propionate (Prop)	31.33	33.84	34.12	26.01	3.23	0.23
Isobutyrate	1.01	0.83	0.90	0.97	0.09	0.46
Butyrate	9.82	9.19	10.18	13.51	1.34	0.07
Isovalerate	2.44	1.85	1.72	2.08	0.46	0.69
Valerate	1.28	1.52	1.46	1.33	0.11	0.43
Ac:Prop ratio	2.02	1.85	1.70	2.32	0.28	0.28
Total SCFA (mM)	86.63	97.68	96.68	102.29	7.06	0.42
Ammonia (mg/dL)	19.28	19.87	22.57	22.37	2.33	0.54
pH	5.89	5.76	5.76	5.86	0.07	0.25

[1]Diets: MON= 25mg/kg of dry matter of monensin; 1.25EO= 1.25g of thyme EO/kg of DM; 2.50EO= 2.50g of thyme EO/kg of DM; 3.75EO= 3.75g of thyme EO/kg of DM. [2] Standard error of the mean. [3] Short chain fatty acids.

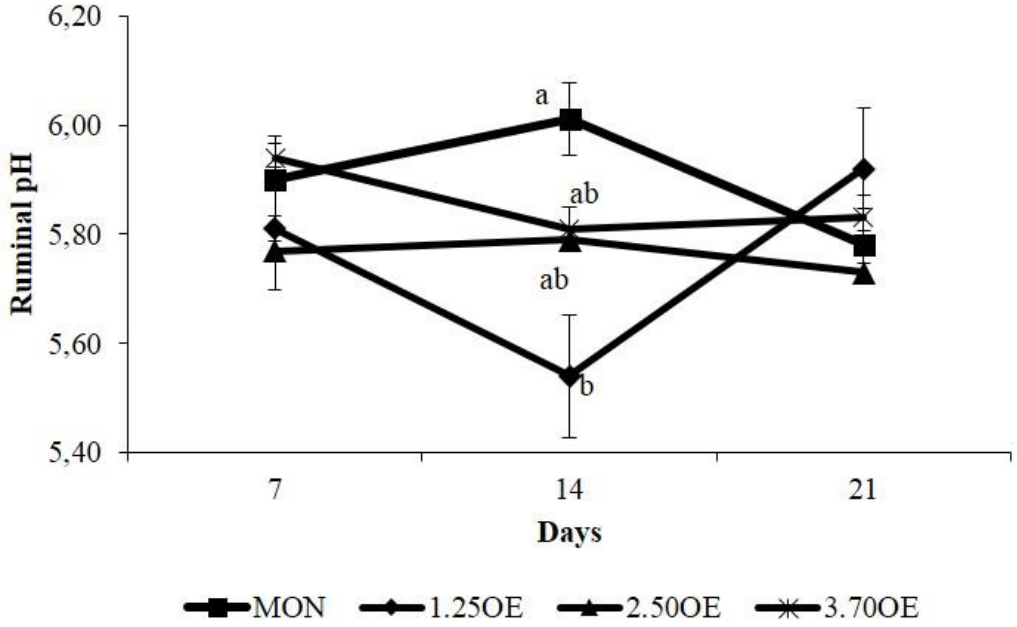

Figure 1. Rumen pH on days 7, 14 and 21 of experiment in wethers fed high-concentrate diet. There was a tendency (P= 0.07) for interaction between diet and day in ruminal pH. Wethers fed 1.25 (1.25EO) g thyme essential oil (EO)/kg DM tended to have lower pH on day 14.

DISCUSSION

Using additive in feed may improve products of ruminal fermentation by changes in ruminal microbial population, which induce an increase in ruminant's energy efficiency. The main result reported in this study was the similarity of results among thyme EO doses and monensin. This is a promising result because it is well known that monensin is an efficient additive used in ruminant nutrition (Duffield *et al*., 2012; Ellis *et al*., 2012). Normally, monensin increases the molar proportion of propionate, and reduces the acetate and butyrate (Ellis *et al*., 2012). The propionate is the main glucose precursor in ruminants, which results in energetic gain when the molar proportion of propionate is increased. Decreasing molar concentration of acetate and butyrate, there is a hydrogen liberation reduction, which is the main methane precursor (Wolin, 1960). The similar results between thyme EO doses and monensin make the thyme EO use as a possible monensin substitute.

The main compounds in thyme EO (*Thymus vulgares*) used in this study were p-cymene and thymol, which is characterized with a phenolic compound (Cobellis *et al*., 2016) able to decrease enzymatic activity and disrupt membrane integrity due to changes in protein reactions, inhibiting gram-positive and gram-negative bacteria (Lis-Balchin and Deans, 1997). Helander *et al*. (1998) showed that thymol from thyme oil disrupts the cell membrane thereby decreasing the intracellular ATP pool and increasing the extracellular ATP pool. The p-cymene compound is a monoterpene hydrocarbon able to diffuse through the plasma membrane to the bacterial cytoplasm (Cristani *et al*., 2007). Furthermore, antimicrobial effects of p-cymene were also attributed to the accumulation in the bacterium plasma membrane, causing the expansion of this cellular structure, allowing the passage of ions (Ultee *et al*., 2002). High amounts of thymol and p-cymene compounds in thyme EO should be highlighted due to antimicrobial properties already cited.

Several authors reported that using EO in diets may decrease DMI (Benchaar *et al*., 2006, 2007) probably because EO are able to confer odors and flavors on feed. The similar DMI among treatments shown when adding EO in the diet that the processing as material encapsulation is not necessary. The addition of thyme EO did not change the apparent digestibility of nutrients and the total concentration of total SCFA. Thus, it can be inferred that the use of higher doses, such as 3.75g of thyme EO/kg of DM, would be unnecessary since the same results were obtained with the lower doses.

The effects of monensin on ruminal fermentation parameters are well known and described in literature, one of the most discussed results is the molar ratio of propionate increase, which normally decreases the acetate:propionate ratio (Duffield *et al*., 2012; Ellis *et al*., 2012). Similar to monensin, it described that thyme EO decreased the molar proportion of acetate and ratio of acetate to propionate, and increased the molar proportion of propionate (Vakili *et al*., 2013). The use of thyme EO in this study did not differ from the treatment that used monensin as additive. Thus, we may infer that the effects caused by both additives were equivalent.

The diets used in this study aimed to maximize wether performance, which was composed of 90% grain, mainly corn. According to NRC (Nutrient…, 2016), corn is composed by approximately 71.3% of starch, which represented diets with fast and extensive fermentation, resulting in high SCFA production, decreasing ruminal pH. In many cases, the non-use of feed additives leads to a drop in ruminal pH, resulting in nutritional disorders, of which acidosis is the most common (Owens *et al*., 1998). Despite the characteristics of diets, the ruminal pH observed in this experiment was not harmful, confirming the capacity of thyme EO in preventing ruminal pH from reaching critical levels. In addition, the 2.50 and 3.75g doses of thyme EO were able to keep the ruminal pH similarly to monensin level along the experiment.

Ruminants have low efficiency in nitrogen utilization compared to non-ruminants. However, the nitrogen utilization efficiency may be improved by monensin use because this additive may control processes such as nitrogen capture in the rumen and protein degradation (Calsamiglia *et al*., 2010). In addition, the doses of thyme EO used in this study showed similar nitrogen balance compared to monensin, which may be considered a positive effect.

Furthermore, several studies have shown that thyme EO decreased the ammonia concentration in *in vitro* systems (Castillejos *et al*., 2008). Castillejos *et al*. (2007) report that EO has the ability to interact with the bacterial cell membrane, inhibiting the growth of some strains, resulting in ammonia concentration decrease.

CONCLUSION

The use of thyme EO containing a high concentration of thymol and p-cymene had similar effects on ruminal fermentation nutrient digestibility and nitrogen balance when compared to monensin, indicating that thyme EO may be used as an alternative to monensin.

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