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Ruminal fermentation, nutrients digestibility, and nitrogen balance in lambs fed diet containing high concentrate and essential oil concentrations of lemon grass (*Cymbopogon citratus*)

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ABSTRACT. This experiment evaluated the effects of doses of essential oil of lemon grass (*Cymbopogon citratus*) in rumen fermentation, nutrients digestibility and nitrogen balance in lambs. The treatments: negative control/CTL (no additives); positive control/MON (addition of 25 ppm of monensin Kg⁻ of dry matter); and 1.25, 2.5 and 3.75 mL essential oil Kg of diet as feed. Twenty-five crossbreed Dorper x Santa Ines lambs with 45.6 ± 10.7 Kg of body weight and cannulated in the rumen allocated into randomized complete blocks design with five replicates per treatment. It was possible to verify that all the treatments began to alter some parameters of the ruminal fermentation of the diet offering. Essential oil of lemon grass used had similar results to monensin on parameters of ruminal, on day 21 compared with day zero, essential oil presented high concentrations of ammoniacal nitrogen in the rumen, indicating that there was protein degradation greater than monensin. Not verified effect of essential oil in nutrient intake and nitrogen retained, even though there was improvement in the digestibility of dry matter and crude protein, and these effects were similar to monensin. During the 21 days, essential oil evaluated is effective manipulating some parameters of the ruminal fermentation.

Keywords: nutrients digestibility; additive; rumen fermentation, sheep, feed intake.

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Introduction

Some essential oils have a capacity similar to ionophores in selectively acting on the microbial population of the rumen (Calsamiglia et al., 2007), changing the fermentative pattern, reducing the acetate: propionate ratio and methane production, which makes the rumen more energetically efficient. The metabolic pathways of propionate synthesis besides not producing H+, as observed in acetate and butyrate production routes, serve as a hydrogen drain of the ruminal environment (Van Soest, 1994). Thus, maximizing propionate production decreases substrate availability for methanogenic archaebacteria, which reduces methane emissions and improves the animal's energy efficiency.

Ionophores are the most widely used commercial additives in the world to manipulate ruminal fermentation and thus improve the feed efficiency of ruminants. They are effective control in the population of gram-positive bacteria, and there may be little or no activity in gram-negative bacteria. The European Union in 2006 banned the use of antibiotics as growth promoters, including ionophores in this class. As a result, the scientific community has studied antimicrobial properties of secondary compounds of plants that can reproduce the benefits of ionophores.

Cymbopogon citratus, popularly known as lemon grass, is originally from Asia and is internationally called lemon grass from West India. In Brazil it is mainly used by anxiolytic and antihypertensive properties (Lorenzi, 2002). The main component of the essential oil of lemon grass is citral, which is composed of the mixture of neral (β-citral) and geranial (α-citral) isomers (Leal et al., 2003). Geraniol, limonene, citronellal and myrcene are also found in the essential oil (Guerra et al., 2000). Studies prove the antimicrobial action of some essential oils, including evidence that gram-positive bacteria were more sensitive than gram-negative bacteria (Guerra et al., 2000; Zago et al., 2009; Naik et al., 2010).

In this context, we hypothesize that the inclusion essential oil of lemon grass (*Cymbopogon citratus*) has a positive influence on ruminal concentration of short-chain volatile fatty acids (VFA), intake and apparent digestibility of nutrients, and nitrogen balance. Thus, the experiment aimed to evaluate the effects of essential oil of lemon grass on rumen fermentation parameters, nutrients digestibility and nitrogen balance in lambs.

Materials and methods

This study was conducted in the research facilities with sheep from the Sheep and Goat Intensive Production System (SIPOC) of the Department of Animal Science, Escola Superior de Agricultura 'Luiz de Queiroz', Universidade de São Paulo, located in Piracicaba, state of São Paulo, Brazil. All animal procedures followed the guidelines recommended by the Animal Care and Use Committee at the same university (Comitê de Ética no Uso de Animais – CEUA – Number 3045/2013).

Animals and facilities

Twenty-five crossbred (½Dorper x ½ Santa Inês) castrated male (initial body weight 45.6 ± 10.7 kg and approximately 180 days of age) were used. Lambs were vaccinated against clostridia (Sintoxan T, Merial Animal Health, Campinas, SP, Brazil) before weaning and were dewormed with 1.0% moxidectin (Cydectin®, Fort Dodge Animal Health, Campinas, São Paulo, Brazil) at a dosage of 1 mL 50 Kg^{-1} body weight and 5 g of Cloridrate of Levamisol (Ripercol®, Fort Dodge Animal Health, Campinas, São Paulo, Brazil). The lambs were housed in individual pens during the first 15 days for adaptation to diets. Between 15 and 21th days the animals were placed in metabolic cages (1.30 x 0.55 m) provided by feed bunks, waterers and a system to collect feces and urine.

Experimental design

Lambs were blocked by initial body weight and randomly assigned within 10 blocks. The diets were composed by 10% of coast cross hay and 90% of concentrate (Table 1). The experimental diets were: CTL – negative control diet (no inclusion of fed additives); MON – positive control diet by adding 25 mg of monensin (Rumensin® 100, Elanco Brazil, São Paulo, SP, Brazil) Kg^{-1} of DM; and 1.25, 2.5 and 3.75 mL essential oil (EO) of *Cymbopogon citratus* Kg^{-1} of diet as feed.

Table 1. Composition of basal diet with differing feed additives and essential oil doses (mL Kg^{-1} as fed).

Item	Diet [1]				
	CTL	MON	1.25	2.50	3.75
Ingredients (% of dry mater)					
Coastcross hay	10.0	10.0	10.0	10.0	10.0
Soybean meal	16.0	16.0	16.0	16.0	16.0
Corn ground	70.0	70.0	70.0	70.0	70.0
Ammonium chloride	0.35	0.35	0.35	0.35	0.35
Limestone	1.65	1.65	1.65	1.65	1.65
Mineral[2]	2.0	2.0	2.0	2.0	2.0
Essential oil (mL 100 kg^{-1} MS)	0	0	125.0	250.0	375.0
Monensin (ppm)	0	25.0	0	0	0
Chemical composition					
Dry matter	87.5	87.5	87.5	87.5	87.5
Organic matter	95.0	95.0	95.0	95.0	95.0
Crude protein	15.7	15.7	15.7	15.7	15.7
Ether extract	2.83	2.83	2.83	2.83	2.83
NDF[3]	14.5	14.5	14.5	14.5	14.5

[1]CTL = Negative control diet (without additives); MON = positive control diet (with monensin); 1.25, 2.50 or 3.75 mL of essential oil (*Cymbopogon citratus*) per kg as dietary feed. [2]composition: Ca, 13.4%; P, 7.5%; Mg, 1%; S, 7%; Cl, 21.8%; Na, 14.5%; Mn, 1100 mg Kg^{-1}; Fe, 500 mg Kg^{-1}; Zn, 4600 mg Kg^{-1}; Cu, 300 mg Kg^{-1}; Co, 40 mg Kg^{-1}; I, 55 mg Kg^{-1}; Se, 30 mg Kg^{-1}.[3]Fiber insoluble in neutral detergent.

Feed management

Corn and coast cross hay used in the diets were ground using a mill (Nogueira DPM - 4, Itapira, SP, Brazil) with 10 mm sieves. Later, corn and coast cross hay were mixed with soybean meal, limestone, Ammonium chloride and mineral mix using a horizontal mixer with capacity of 500 Kg (Lucato, Limeira, SP, Brazil). On experimental diet MON the sodium monensin was added at the rate of 25 mg Kg^{-1} of dry matter. Essential oil

(EO) was mixed with diets every time the animals were fed. Every day the experimental diets were weight in an electronic scale accurate to 5.0 gram and offered, ad libitum, 07:00 and 19:00 h. The refused feed was collected every day from each animal to calculate the dry matter intake (DMI). The animals were weighed on days 0 and 21st, without fasting to monitoring body weight.

Ruminal sample analysis

On days 0 (before starting the experimental diets), 7, 14 and 21, it was collected ruminal fluid at zero (before feeding), 3, 6 and 9 hours after feeding. Ruminal contents were collected via rumen cannula. Ruminal fluid pH was measured immediately by using a pH meter (DM20, Digimed, São Paulo, SP, Brazil). Two 25 mL aliquots of ruminal fluid were stored at -20°C in plastic containers in each time of collection for future determinations of short-chain volatile fatty acids (VFA) and ammonia-N.

Nutrient digestibility and nitrogen balance

The animals were placed in metabolic cages between 15th and 21st days for total collection of feces, urine and the offered diets of the previous day. The feces were collected using collection bags to avoid urine contamination. The total fecal production of the animals was quantified (Marte, AC 10K, São Paulo, SP, Brazil) at 7:00 a.m., and a sample that was representative (10%) of the daily production was collected and stored at -18°C. The feces collected were used to determine the nutrient digestibility in the total digestive tract.

The apparent digestibility of the nutrients in the total digestive tract (ADTT) and nitrogen balance were calculated according to the following formulas: ADTT % = (Nutrient intake – nutrient excreted in feces) / nutrient intake x 100. Nitrogen retained was calculated: Nretained (g d^{-1}) = Nintake - Nfeces - Nurine.

The urine was collected with plastic recipients containing HCl (6N), to prevent ammonia volatilization, maintaining pH below 3.0. Urine pH was measured twice a day. The total urine production was quantified every day and a sample was collected and stored at -18°C. The urine collected was used to calculate the nitrogen retention.

Sample analysis

The composition of the essential oil of lemon grass (*Cymbopogon citratus*) was determined in the Laboratory of Chemistry and food analysis of the School of Agriculture Luis de Queiroz of University of São Paulo in a chromatograph equipped with RTX 5MS columns with 30 m of length and 0.25 mm thick with and initial temperature of 40°C for 8 minutes (ramp 1) increasing by 3°C per minute to 180°C (ramp 2) and 20°C to 230°C (ramp 3) for a total period of 77.17 minutes (Table 2).

Table 2. Essential oil composition of lemon grass (*Cymbopogon citratus*).

tem	Lemon grass
	% Relative
α pineno	0.64
Canfen	3.42
5 hpeten 6 methyl	4.08
Limonene	0.80
Nonanone	1.52
Rose furan	0.62
Linalool	1.78
α 2 dimethyl	0.77
β citronelal	0.76
Furan oxide	1.16
Isogeranial	1.03
2.6 octadienal 3.7 dimethyl	31.94
Garaniol	0.80
Geranial	41.43
Citroneli acetone	0.86
Eugenol	0.97
α 2 capaeno	0.68
Acetate neril	0.46
β cariofileno	3.31
Cariofileno oxide	1.88
Non identified	1.09

Samples of the offered feed, orts and feces were thawed, composited within animal, 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. DM content was determined by drying the samples at 105°C for 24h, and ash content was obtained by incinerating the samples in an oven at 550°C for 4h (Association of Official Analytical Chemists, 1990). The OM was calculated by the difference between the DM and ash. Total N of offered feed, orts, feces was determined using the Leco FP528 instrument (Leco Corporation, St. Joseph, MI, USA) according to Association of Official Analytical Chemists (1997). The extract ether was determined using the Leco TFE 2000 (Leco Corporation, St. Joseph, MI, USA). 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 N of urine was determined by Micro kjeldahl method (Association of Official Analytical Chemists, 1997).

A profile of short chain volatile fatty acid was determined using Agilent 7890A gas chromatograph equipped with flame ionization detector (7683B) and a fused-silica capillary column (J&W 19091 F-112, Agilent Technologies, Santa Clara, CA, USA), 25 m in length and 320 µm internal diameter. Sample extraction was carried out by withdrawing 1.6 mL of ruminal fluid sample added to 0.4 mL of 3:1 solution of 25% metaphosphoric acid with 98-100% of formic acid and 0.2 mL of internal standard were centrifuged in a Sorvall Superspeed RC2-B, Newton, CT, USA device for 15 minutes at 40 °C. After centrifugation, 1.2 mL of the supernatant from each sample was transferred to chromatographic vials. From the obtained extract 1 µL was injected into the gas chromatograph. The injection was performed automatically, the entrainment gas was H_2, maintained at a flow of 31.35 mL minute^{-1}. The temperature of the injector and detector was 260°C. The total time of the chromatographic run was 16.5 minutes per sample divided into three heating ramps, as follows: 80°C (1 minute), 120 °C (20°C minute^{-1}), 205°C (100°C minute^{-1}).

The NH_3-N concentration was determined with a colorimetric method that was described by Chaney and Marbach (1963), adapted for a microplate reader (EON, BioTek Instruments, Winooske, VT, USA) with a 550 nm absorbance filter.

The methane production was calculated using the equation proposed by Abdl-Rahman (2010) based in stoichiometry of Wolin (1960), as follows:

Fermentative CO_2 = A/2 + P/4 + 1.5*B

Fermentative CH_4 = (A + 2*B) – CO_2

A = mole of acetate

P = mole of propionate

B = mole of butyrate

Statistical analysis

The animal was the experimental unit used to perform all statistical analysis. Statistical analyses were performed using the MIXED procedure of the SAS statistical software program (SAS version 9.0; SAS Inst. Inc., Cary, NC) (Statistical Analysis System Institute, 2002). All data were submitted to the Shapiro-Wilk test to verify the normality of the residuals, the removal of "outliers", and homogeneity of variances using the Levene test. The data that did not comply with those premises was subjected to logarithmic, inverse or square root transformation.

Data for nutrient intake, nutrient digestibility and nitrogen balance were analyzed using the model: $Y_{ij} = \mu + D_i + b_j + e_{ij}$, where µ = overall mean; D_i = fixed effect of diet; b_j = random effect of block; and e_{ij} = random error. Means were obtained by the LSMEANS command. Data such as VFA profile, ruminal pH, and ammonia were analyzed as measurements repeated in the days of collection, and measurements repeated in the hours of the collecting day. The statistical model used was: $Y_{ijk} = \mu + D_i + b_j + e_{ij} + T_k + (DT)_{ik} + (bT)_{jk} + e_{ijk}$, where µ = overall mean; D_i = fixed effect of diet; b_j = random effect of block; e_{ij} = random error A; T_k = fixed effect of the time; $(DT)_{ik}$ = fixed effect of diet × time interaction; $(bT)_{jk}$ = random effect of block × time interaction, and e_{ijk} = random error B. All data was evaluated in an orthogonal counter form: Negative control vs additives; positive control vs oil doses; linear and quadratic effects of oil doses.

All data analyzed as repeated measurement in the day were included as covariance matrices and tested for ‘compound symmetry, heterogeneous compound symmetry, autoregressive (AR), autoregressive heterogeneous, unstructured, banded, variance components, toeplitz and heterogeneous toeplitz’ and defined according to the lowest value obtained for Akaike's Information Criterion. Means were obtained by the LSMEANS control. The effect of diet, week and interaction of diet x week were defined by the test F of analysis of variance and was assessed using the inclusion of SLICE methods. The diet effect was defined by Tukey test and significance was defined as $p < 0.05$ and tendency as $0.05 < p < 0.10$.

Results

Ruminal parameters

The effect of tree doses of essential oil of lemon grass (*Cymbopogon citratus*) compared with negative control treatment (no additives) and positive control treatment (monensin) on the ruminal fermentation parameters with seven, fourteen, twenty-one and all days of offering the experimental diets are summarized in Tables 3, 4, 5 and 6, respectively. There was no effect on day seven for any variables (Table 3).

Table 3. Ruminal parameters in lambs feeding with or without monensin and doses of essential oil of lemon grass (Cymbopogon citratus) along seven days offer the experimental diets.

Item	Treatment[1]					SEM[2]	p value[3]			
	CTL	MON	1.25	2.50	3.75		CTL*Additives	MON*Oil	L	Q
Ruminal pH	5.90	5.75	5.87	5.87	5.88	0.09	0.62	0.25	0.34	0.57
Total VFA, mM	107.99	107.71	109.77	112.18	98.46	5.83	0.88	0.89	0.34	0.19
Acetate, mM	56.01	56.16	56.14	57.77	50.53	3.19	0.82	0.71	0.30	0.27
Propionate, mM	38.01	38.68	40.11	41.10	37.32	4.52	0.79	0.86	0.87	0.55
Butyrate, mM	9.85	8.81	9.23	9.10	6.77	1.68	0.49	0.82	0.42	0.42
Isobutyrate, mM	0.84	0.74	0.84	0.84	0.79	0.05	0.58	0.20	0.56	0.19
Isovalerate, mM	1.40	1.14	1.54	1.43	1.38	0.20	0.63	0.18	0.50	0.26
Valerate, mM	1.77	2.15	1.87	1.91	1.65	0.30	0.71	0.34	0.30	0.97
C_2/C_3	1.72	1.76	1.62	1.78	1.73	0.21	0.98	0.83	0.93	0.82
$N\text{-}NH_3$, mg dL^{-1}	18.20	16.69	17.36	17.87	17.54	0.85	0.38	0.38	0.43	0.52
Methane, mmol	23.11	21.56	22.27	22.77	18.41	2.72	0.54	0.89	0.47	0.36

[1] CTL = Negative control diet (without additives); MON = positive control diet (with monensin); 1.25, 2.50 or 3.75 mL of essential oil (*Cymbopogon citratus*) per kg as dietary feed. [2] SEM = Standard error of mean. [3] p value = means differ significantly ($p < 0.05$). Contrasts of treatments CTL *vs* additives; MON vs Oil doses; Linear effect; Quadratic effect.

There was increase in the concentration of isovalerate on control treatment in relation to additive treatments ($p = 0.05$), trend of quadratic effect ($p = 0.08$) for isovalerate concentration and quadratic effect for the butyrate concentration in the 14th day ($p = 0.05$; Table 4).

Table 4. Ruminal parameters in lambs feeding with monensin or no and doses of essential oil of lemon grass (*Cymbopogon citratus*) along fourteen days offer of experimental diets.

Item	Treatment[1]					SEM[2]	p value[3]			
	CTL	MON	1.25	2.50	3.75		CTL*Additives	MON*Oil	L	Q
Ruminal pH	5.85	5.79	5.81	5.92	5.93	0.09	0.85	0.36	0.20	0.95
Total VFA, mM	112.97	111.98	99.82	115.11	98.80	5.34	0.30	0.25	0.33	0.70
Acetate, mM	59.02	54.61	56.09	60.98	51.70	2.93	0.46	0.98	0.64	0.17
Propionate, mM	42.24	45.79	37.48	41.72	36.57	3.91	0.68	0.13	0.20	0.69
Butyrate, mM	8.27	6.62	8.53	8.62	6.71	0.93	0.55	0.24	0.93	0.05
Isobutyrate, mM	0.80	0.77	0.76	0.90	0.78	0.04	0.89	0.43	0.42	0.21
Isovalerate, mM	1.89	1.10	1.74	1.45	1.31	0.21	0.05	0.12	0.72	0.08
Valerate, mM	1.58	1.84	1.72	1.75	1.77	0.22	0.48	0.73	0.86	0.75
C_2/C_3	1.66	1.46	1.73	1.88	1.74	0.18	0.84	0.12	0.22	0.26
$N\text{-}NH_3$, mg dL^{-1}	16.21	16.00	18.11	17.29	17.58	0.93	0.34	0.15	0.37	0.35
Methane, mmol	23.62	19.04	22.63	25.65	20.51	1.91	0.44	0.10	0.42	0.03

[1] CTL = Negative control diet (without additives); MON = positive control diet (with monensin); 1.25, 2.50 or 3.75 mL of essential oil (*Cymbopogon citratus*) per Kg as dietary feed. [2] SEM = Standard error of mean. [3] p value = means differ significantly ($p < 0.05$). Contrasts of treatments CTL *vs* additives; MON vs Oil doses; Linear effect; Quadratic effect.

On twenty-first day of offering the experimental diets (Table 5), it was observed lower concentration of isobutyrate ($p = 0.03$) for control treatment than in the additive treatments. Quadratic effect was observed for the total VFA mM ($p = 0.01$), quadratic effect for the acetate concentration ($p = 0.03$) and decrease ($p = 0.03$) on nitrogen ammonia in the rumen was observed for monensin treatment compared with tree doses of essential oils of lemon grass (*Cymbopogon citratus*).

In the summarization of all days (zero, seven, fourteen and twenty-one) of ruminal fermentation parameters (Table 6), there was a trend for the increase ($p = 0.06$) in isobutyrate concentration for all essential oil doses in relation to monensin treatment. There was increase ($p = 0.03$) in isovalerate concentration for all essential oils in relation to the monensin treatment, and a trend for increasing linear effect ($p = 0.08$) for the isovalerate concentration as a function of essential oils doses in the diets.

Table 5. Ruminal parameters in lambs feeding with monensin or no and doses of essential oil of lemon grass (*Cymbopogon citratus*) along twenty-one days offer of experimental diets.

Item			Treatment[1]			SEM[2]	p value[3]			
	CTL	MON	1.25	2.50	3.75		CTL*Additives	MON*Oil	L	Q
Ruminal pH	5.85	5.83	5.76	5.92	5.97	0.07	0.84	0.55	0.12	0.44
Total VFA, mM	93.32	92.57	101.95	97.64	81.75	5.02	0.97	0.83	0.12	0.01
Acetate, mM	48.65	48.72	52.25	52.10	44.66	2.43	0.78	0.73	0.27	0.03
Propionate, mM	33.34	30.70	34.37	29.97	24.75	3.49	0.41	0.80	0.17	0.22
Butyrate, mM	7.62	9.13	11.13	11.19	8.31	1.59	0.22	0.56	0.74	0.14
Isobutyrate, mM	0.73	0.81	0.85	0.94	0.84	0.05	0.03	0.25	0.46	0.17
Isovalerate, mM	1.67	1.45	1.63	1.91	1.85	0.24	0.89	0.23	0.19	0.63
Valerate, mM	1.29	1.74	1.69	1.52	1.32	0.26	0.38	0.46	0.25	0.78
C_2/C_3	1.71	1.77	1.69	2.09	2.10	0.21	0.39	0.42	0.14	0.83
N-NH_3, mg dL^{-1}	17.00	16.38	18.87	18.39	18.39	0.82	0.28	0.03	0.15	0.142
Methane, mmol	19.80	21.25	23.10	24.15	20.30	1.89	0.29	0.56	0.83	0.14

[1]CTL = Negative control diet (without additives); MON = positive control diet (with monensin); 1.25, 2.50 or 3.75 mL of essential oil (*Cymbopogon citratus*) per kg as dietary feed. [2]SEM = Standard error of mean. [3]p value = means differ significantly ($p < 0.05$). Contrasts of treatments CTL *vs* additives; MON vs Oil doses; Linear effect; Quadratic effect.

Table 6. Ruminal parameters in lambs feeding with or without monensin and doses of essential oil of lemon grass (*Cymbopogon citratus*) including all days (0; 7; 14 and 21) offer of experimental diets.

Item			Treatment[1]			SEM[2]	p value[3]			
	CTL	MON	1.25	2.50	3.75		CTL*Additives	MON*Oil	L	Q
Ruminal pH	5.73	5.72	5.80	5.75	5.84	0.05	0.49	0.24	0.21	0.89
Total VFA, mM	118.36	114.51	112.03	124.52	109.23	4.14	0.48	0.87	0.85	0.12
Acetate, mM	60.77	58.43	58.43	64.62	56.71	2.32	0.63	0.58	0.92	0.09
Propionate, mM	42.94	43.19	40.94	43.81	39.41	3.22	0.76	0.62	0.55	0.73
Butyrate, mM	10.34	8.73	11.27	11.44	8.74	1.30	0.84	0.26	0.97	0.05
Isobutyrate, mM	0.81	0.78	0.86	0.93	0.83	0.04	0.39	0.06	0.23	0.03
Isovalerate, mM	1.71	1.25	1.68	1.69	1.71	0.17	0.51	0.03	0.08	0.24
Valerate, mM	1.76	2.06	1.84	2.00	1.82	0.19	0.45	0.44	0.51	0.92
C_2/C_3	1.62	1.63	1.69	2.14	1.77	0.20	0.42	0.32	0.35	0.29
N-NH_3, mg dL^{-1}	17.22	17.02	18.77	18.30	17.91	0.72	0.30	0.10	0.47	0.12
Methane, mmol	24.82	22.78	24.62	27.08	22.87	1.91	0.82	0.35	0.75	0.11

[1]CTL = Negative control diet (without additives); MON = positive control diet (with monensin); 1.25, 2.50 or 3.75 mL of essential oil (*Cymbopogon citratus*) per kg as dietary feed. [2]SEM = Standard error of mean. [3]p value = means differ significantly ($p < 0.05$). Contrasts of treatments CTL *vs* additives; MON vs Oil doses; Linear effect; Quadratic effect.

A trend for quadratic effect ($p = 0.09$) was observed for the acetate concentration, quadratic effect for the butyrate concentration ($p = 0.05$) and quadratic effect ($p = 0.03$) for isobutyrate concentration.

Intake and digestibility of nutrients

The effect of different doses of essential oils (1.25, 2.50, e 3.75 mL Kg^{-1} DM) on intake and digestibility of nutrients is shown in Table 7.

Table 7. Nutrient's intake and digestibility on total tract in lambs fed with and without monensin, and levels of essential oils of lemon grass (*Cymbopogon citratus).*

Item[1]			Treatment[2]			SEM[3]	p value[4]			
	CTL	MON	1.25	2.50	3.75		CTL*Additives	MON*Oil	L	Q
DMI, Kg d^{-1}	1.34	1.11	1.25	1.27	1.22	0.090	0.247	0.224	0.433	0.313
DMI, %BW	2.96	2.51	2.85	2.84	2.75	0.240	0.425	0.290	0.519	0.388
$DMI^{0,75}$, g	78.00	63.33	71.67	71.67	70.00	0.005	0.180	0.268	0.458	0.407
OMI, Kg d^{-1}	1.27	1.05	1.19	1.21	1.16	0.080	0.242	0.216	0.425	0.302
CPI, Kg d^{-1}	0.21	0.17	0.19	0.20	0.19	0.014	0.227	0.235	0.493	0.278
EEI, Kg d^{-1}	0.036	0.031	0.035	0.036	0.036	0.002	0.589	0.172	0.185	0.546
NDFI, Kg d^{-1}	0.19	0.16	0.18	0.185	0.176	0.013	0.215	0.207	0.379	0.312
NDFI, % BW	0.44	0.36	0.41	0.41	0.40	0.033	0.239	0.257	0.502	0.360
Digestibilities										
DM, %	79.77	84.79	85.04	82.62	83.41	1.29	0.008	0.471	0.270	0.838
OM, %	84.96	87.63	85.65	81.81	87.65	2.65	0.812	0.494	0.782	0.188
CP, %	76.15	81.34	82.53	79.14	79.22	1.62	0.027	0.650	0.249	0.753
EE, %	74.64	82.07	84.47	81.49	74.39	3.41	0.136	0.628	0.106	0.193
NDF, %	79.98	81.03	80.74	78.00	81.68	1.73	0.848	0.664	0.922	0.267

[1]DMI = dry matter intake; OMI = organic matter intake; CPI = crude protein intake; EEI = ether extract intake; NDFI = neutral detergent fiber intake. [2]CTL = Negative control diet (without additives); MON = positive control diet (with monensin); 1.25, 2.50 or 3.75 mL of essential oil (*Cymbopogon citratus*) per kg as dietary feed. [3]SEM = Standard error of mean. [4] p value = means differ significantly ($p < 0.05$). Contrasts of treatments CTL *vs* additives; MON vs Oil doses; Linear effect; Quadratic effect.

The nutrients intake was not affected by the treatments (p > 0.05). The dry matter digestibility was greater (p = 0.008) for monensin and 1.25, 2.50 and 3.75 mL of essential oil Kg-1 DM. Protein digestibility was greater (p = 0.027) in additives treatments (monensin, 1.25, 2.50 and 3.75 mL of essential oil Kg DM) than in the negative control treatment. There was no effect (p > 0.05) for the other evaluated variables.

Nitrogen balance

Fecal nitrogen excretion (p = 0.017) was higher for the negative control than in the additive treatments (monensin and, 1.25, 2.50, 3.75 mL of essential oil Kg^{-1} of DM) (Table 8).

Table 8. Nitrogen balance in lambs fed with and without monensin, and levels of essential oils of lemon grass (*Cymbopogon citratus).* (*Cymbopogum citratus).*

Item	Treatment[1]					EPM[2]	p value[3]			
	CTL	MON	1.25	2.50	3.75		CTL*Additives	MON*Oil	L	Q
N intake, g	34.00	28.00	31.83	32.33	30.50	0.002	0.230	0.215	0.466	0.253
N feces, g	8.16	4.83	5.66	6.83	6.66	0.0007	0.017	0.085	0.059	0.509
N urine, g	11.00	7.50	9.00	9.33	10.50	0.002	0.509	0.481	0.422	0.948
N retained, g	14.67	15.50	17.00	16.17	13.83	0.003	0.787	0.963	0.682	0.548
Apparent digestibility N AD N, %	76.14	82.79	82.53	79.14	79.22	1.59	0.014	0.190	0.061	0.917

[1]CTL = Negative control diet (without additives); MON = positive control diet (with monensin); 1.25, 2.50 or 3.75 mL of essential oil (*Cymbopogon citratus*) per kg as dietary feed. [2]SEM = Standard error of mean. [3]p value = means differ significantly (p < 0.05). Contrasts of treatments CTL *vs* additives; MON vs Oil doses; Linear effect; Quadratic effect.

In addition, it was observed higher (p = 0.014) apparent digestibility of diet nitrogen on total tract in the additive treatments (monensin and, 1.25, 2.50, 3.75 mL of essential oil Kg^{-1} of DM) in relation to the negative control treatment (no additives). There was a trend (p = 0.085) for the lower fecal nitrogen excretion by monensin treatment in relation to the essential oil treatments. It was observed a trend of linear effect (p = 0.059) for nitrogen fecal excretion and a trend of linear effect (p = 0.061) for apparent digestibility of nitrogen compound sources of diets with additive treatments (monensin and, 1.25, 2.50, 3.75 mL of essential oil Kg^{-1} DM), in which the values were lower when compared with the negative control treatment (no additive). However, there was no effect of treatments on nitrogen retained (p > 0.05).

Discussion

Essential oils are a complex blend of secondary metabolites of plants with high variation in their composition. The expected action of secondary compounds of plants in the rumen is the selective action on rumen microorganisms, especially in Gram-positive rumen bacteria causing an effect similar to the antibiotic additives used in the diets of these animals. In this context, plant metabolites may affect food digestibility due to inhibition of microbial activity in the rumen (Bodas et al., 2012; Cobellis et al., 2016). Therefore, the interest in the investigation of the effect of doses of plant metabolites in inhibition of the activity of Gram-positive ruminal bacteria has gained great importance when referring to mechanisms for manipulation of ruminal fermentation. In the present study, since there was no effect for any of the variables analyzed on day seven (Table 3), we can affirm that the animals were still in the period of adaptation to the experimental diets and this behavior is expected since it is recommended around 15 days for adaptation on any diet change.

In the 14th day of offering the experimental diets (Table 4), there was an increase (p = 0.05) in the concentration of isovalerate on the CTL treatment versus the additives (MON and essential oil ones). This higher concentration of isovalerate corresponds to 28.8% of production, being the control treatment more efficient in acting in the catabolism of valine in the rumen (Van Soest, 1994). The high concentration of butyrate on the treatments CTL, 1.25 and 2.50 mL of essential oil presents the great loss of energy as methane in the rumen. It should be noticed that for these three treatments, as the concentration of butyrate increased, the concentration of acetate also increased, thus intensifying the equivalent balance of hydrogen reduction in the rumen, releasing 4 and 2 hydrogens for each molecule of acetate and butyrate formed, respectively, which in turn favors methanogenesis.

Furthermore, it was verified that the percentage of propionate in relation to the total short chain VFA for all treatments presented an average of 37.0%, not varying between treatments. Effect similar to that of the present study was found by Demirtas et al. (2019), who evaluated three doses of essential oil (0, 187.5, 375

and 750 mg day^{-1}) in an *in vitro* fermentation system. The data showed an increase in butyrate concentration associated to an increase in acetate concentration in treatments zero, 187.5 and 375 mg day^{-1}, evidencing the increase in methanogenesis as acetate and butyrate concentrations (Anassori et al., 2011; Tekippe et al., 2011; Knapp et al., 2014). The tendency towards quadratic effect for isovalerate concentration can be noted. By increasing the dose of oil in the diet, there is a decrease in the concentration of this isoacid in the rumen, indicating a dependent dose effect (Calsamiglia et al., 2007).

In the 21th day of offering the experimental diets (Table 5), there was a higher concentration (15.0%) of isobutyrate found for treatments that received additives (monensin and essential oil doses) than in the control treatment, confirming that the additives, depending on the dosage in the diet, have high deamination capacity in the rumen, especially with regard to the catabolism of the valine in the case of isobutyrate formation (Van Soest, 1994) since most of the essential oils tested were effective in increasing this isoacid in the rumen (Tekippe et al., 2013).

There was reduction of 12.0% in ammoniacal nitrogen concentration in the rumen for MON vs essential oil doses. This is one of the main effects of monensin, causing a reduction in the digestibility of protein in the rumen and, consequently, there is a reduction in ammoniacal nitrogen concentration caused by inhibition of the activity of microorganisms producing ruminal ammonia (Russell, 2002). Several studies evaluating doses of inclusion of essential oils on ruminal fermentation parameters found a reduction in ammoniacal nitrogen concentration in the rumen (Anassori et al., 2011), which was not observed in the present study, where the essential oil of lemon grass (*Cymbopogon citratus*) increased the concentration of ammoniacal nitrogen, mg dL^{-1}, in relation to the MON treatment, but not differing from the CTL, corroborating with several authors (Wu et al., 1994; Cardozo et al., 2004; Fraser et al., 2007; Demirtas et al., 2019).

The quadratic effect for total VFA concentration indicates that as essential oil was increased, the concentration of total VFA decreased (93.32, 101.95, 97.64, 81.75 mM for treatments CTL, 1.25, 2.50 and 3.75 mL of essential oil, respectively), confirming that for many variables the effect is a dose dependent (Cardozo et al., 2004). The reduction in the concentration of total VFA was accompanied mainly by the numerical reduction in propionate concentration, i.e., as the dosage of essential oil in the diet increased, the concentration of propionate numerically decreased (33.34, 34.37, 29.97 and 24.75 mM for treatments CTL, 1.25, 2.50 and 3.75 mL of essential oil, respectively), and these values correspond to a reduction of 13.0 and 28.0% propionate for treatments 2.50 and 3.75 mL in relation to CTL and 1.25 mL treatments, respectively. Thus, it can be inferred that as the dosage of this essential oil increases, an inhibiting effect occurs in the activity of Gram-negative bacteria, main producers of propionate (Russell, 2002).

Some other studies found a reduction in the concentration of total VFA and propionate as the doses of essential oil in the diet was increased (Demirtas et al., 2019) confirming the dose-dependent effect of essential oils on some parameters of ruminal fermentation. The quadratic effect observed for acetate concentration, as the dosage of essential oil in the diet increased, it confirms the dose-dependent effect for this essential oil in the present study. The results were 48.65, 52.25, 52.10 and 44.66 mM of acetate for the treatments CTL, 1.25, 2.50 and 3.75 mL of essential oil in the dry matter of the diet. In this context it is clear the effect of this essential oil with the doses of 2.50 and 3.75 mL on the inhibition of the activity of both Gram-positive bacteria and gram negative, which confirms the reduction in the concentration of total VFA for the doses 2.50 and 3.75 mL Kg^{-1} of dry matter.

A behavior similar to that of acetate was observed for butyrate concentration and it can be stated that even the intermediate dose (2.50 mL Kg^{-1} of dry matter in the diet) presented high concentration of total VFA, and this treatment also increased the concentration of butyrate and the acetate/propionate ratio, making the rumen less energy efficient (Russell, 2002). The strong trend of high isobutyrate concentration for the essential oil doses used in the present study in relation to MON treatment confirms the hypothesis that until day 21 the essential oils were efficient in increasing the concentration of this isoacid which is a product of valine catabolism that is mainly required for the growth of cellulolytic bacteria (Van Soest, 1994). The quadratic effect observed for this isoacid was also a dose dependent (Cardozo et al., 2004), i.e., higher concentration in intermediate dose (2.50 mL Kg^{-1} of dry matter of the diet) and lower concentrations for doses 1.25 and 3.75 mL, respectively.

By evaluating all days together (0, 7, 14 and 21; Table 6), there was a trend of quadratic effect in the acetate concentration (60.77, 58.43, 58.43, 64.62 and 56.71 mM, for CTL, MON, 1.25, 2.50, 3.75 mL of essential oil per Kg of dry matter, respectively). This data shows that MON treatment, lower essential oil dose (1.25 mL Kg^{-1}

dry matter) and higher essential oil dose (3.75 mL Kg^{-1} dry matter) were more efficient in reducing acetate concentration over 21 days and this reduction was on average 4.0% less for MON and 1.25 mL essential oil treatments, respectively, and 6.7% for the treatment 3.75 mL of essential oil compared to CTL treatment and intermediate dose of essential oil (2.50 mL).

This effect on reducing acetate concentration for MON, 1.25 and 3.75 mL of essential oil confirms that such treatments were more effective in acting on the Gram-positive rumen population by decreasing their activity (Lana & Russell, 2001). The effect on butyrate concentration shows that the MON treatment and 3.75 mL of essential oil Kg^{-1} of dry matter of the diet were more energetically efficient than the other treatments, in which there was a reduction of 21.0% in the concentration of butyrate in relation to the others. It can be confirmed by the lower concentrations of acetate accompanied by lower concentration of butyrate for these two treatments.

Tendency towards linear effect was observed for isovalerate, in which there was a higher concentration of this isoacid for the essential oil doses, decreasing the concentration as the dose of essential oil was increased. Isovalerate is a product of leucine catabolism that also has the same importance as isobutyrate for cellulolytic bacteria. In this context, the data of the present study for these isoacids are in agreement with several authors (Tekippe et al., 2011; Demirtas et al., 2019).

There was an interaction of treatment (1.25 and 2.50 mL of essential oil) vs. day for total VFA concentration. It was verified in this interaction that on day 14 there was a lower concentration of 110.20 mM of total VFA, for the treatment 1.25 mL vs 153.10 mM for the treatment 2.50 mL of essential oil, and 28.0% less total VFA for the treatment 1.25 mL compared to the 2.50 mL treatment of essential oil.

The results related to nutrient intake and digestibility are shown in Table 7. Even without statistical effect, the MON treatment was efficient in reducing DMI in kg/day by 13.0% in relation to the other treatments, an effect that is a classic for monensin in high concentrate diets for animals in feedlot (Lana & Russell, 2001). The absence of effect on variables consumption-related can be explained in such a way that the doses of the essential oil of lemon grass used in the present study did not affect the intake of nutrients, namely, the main variable that should be evaluated together with the digestibility of nutrients in the diet of ruminants which is one of the main objectives of the present study (Firkins et al., 1998).

The MON treatment and the three doses of essential oil were efficient in increasing the digestibility of dry matter and crude protein from the diet in relation to the CTL treatment. These improvements in dry matter and protein digestibility with the inclusion of additives (MON or essential oil doses) were on average 5.0% higher than the CTL treatment. According to Hart et al. (2008), the main effect of essential oils on the rumen is the decrease in protein and amino acid digestibility. The present study contradicts this hypothesis, given that the concentration of ammoniacal nitrogen mg/dL was higher for the three doses of essential oil used in the present study which indicates that there was an increase in protein degradation in the rumen. In addition, it contradicts the hypothesis of Hart et al. (2008), since in the present study there was an increase in the degradation of valine and leucine when the essential oil of lemon grass was included in the diet, which can be confirmed by the increase in the isoacids isobutyrate and isovalerate concentrations (Van Soest, 1994).

Several other studies indicate that essential oils have little or no effect on nutrient intake and digestibility (Fraser et al., 2007; Benchaar et al., 2007; Malecky et al., 2009; Meyer et al., 2009; Santos et al., 2010; Tager & Krause, 2011) reinforcing the data of the present study, since no effect was observed for any of the variables related to the consumption, indicating that these effects are also dosage and type of oil dependent, among other factors such as concentrate content in the diet.

Reinforcing the improvement in the digestibility of crude protein in the diet, there was a decrease in nitrogen excretion in feces (g day^{-1}) for treatments with additives (MON and 1.25, 2.50 and 3.75 mL of essential oil). This was in the order of 26.0% less nitrogen excreted in feces compared to CTL treatment, and 14.0% less nitrogen excreted in feces for MON versus the essential oil doses used in the present study (Table 8). The linear effect for nitrogen excretion in feces as the dose of essential oil is increased in the diet. It may be related to the formation of some barrier that prevents the enzymatic and/or microbial attack on the protein molecule.

Even with linear effect of increased oil dose, the essential oil decreased fecal excretion in 22.0% compared with the CTL, accompanied by lower excretion of nitrogen in feces for additives in relation to CTL treatment. Consequently, there was higher digestibility of dietary nitrogen for additives (6.0%) than the CTL. Therefore, 6.0% better nitrogen digestibility and 5.0% better digestibility of the protein for the treatments with additives confirm better use of protein than the CTL treatment.

Conclusion

In the present study, it was possible to verify that all the treatments began to alter some parameters of the ruminal fermentation in the fourteenth day of the diet offering. The doses of essential oil of lemon grass (*Cymbopogon citratus*) used in the present study had similar results to monensin on the parameters of ruminal fermentation, except on day 21 compared with day zero, in which the doses of essential oil presented high concentrations of ammoniacal nitrogen in the rumen, indicating that in that compartment there was protein degradation greater than monensin. It was not verified effect of essential oil in nutrient intake and nitrogen retained, even though there was improvement in the digestibility of dry matter and crude protein of the diet compared with control, and these effects were similar to monensin. During the 21 days of evaluation, the essential oil of lemon grass is effective in positively manipulating some parameters of the ruminal fermentation.

Data availability

The data in this article are part of a doctoral thesis entitled "Impacts of essential oils from Brazilian plants on ruminal fermentation parameters, digestibility and nitrogen balance in sheep," which is available at http://www.teses.usp.br/teses/disponiveis/10/10135/tde-14092015-105231/

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