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Effect of Aroeira (*schinus terebinthifolius*) leaf essential oils on rumen fermentation, nutrient digestibility, and nitrogen balance in lambs fed with high grain diet

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ABSTRACT. Evaluate the effect of Aroeira (*Schinus terebinthifolius*) leaf essential oils on rumen fermentation parameters, nutrient digestibility, and nitrogen balance in lambs. Treatments were a negative control (CTL - no additives), a positive control (MON - addition of 25 mg of monensin kg^{-1} of dry matter), doses of 1.25, 2.5, and 3.75 mL of essential oil kg^{-1} of diet as feed. Thirty crossbred Dorper x Santa Ines lambs with rumen cannulas and weighing 53.08 ± 11.04 kg were assigned to randomized blocks with five replicates per treatment. Across the entire observation period 0, 7, 14, and 21 days the feed additives proved effective in reducing butyrate and increasing ammonia. Linear increase in essential oils on isovalerate. Essential oils exhibited a quadratic effect on acetate, butyrate, C2/C3, and methane ($p < 0.05$), lower values in treatments at 1.25 and 2.5 mL kg^{-1}. Treatments did not impact with nutrient intake and digestibility. The additives affected the reduction of nitrogen (N) intake and N retained ($p < 0.05$), Essential oils displayed an effect compared to monensin when decreasing N intake and N retained, with a linear decrease due to the essential oils level when decreasing N intake ($p < 0.05$). Doses of 1.25, 2.50 mL kg^{-1} reduced methane production and positively influenced the VFAs.

Keywords: Digestibility; rumen parameters; sheep; VFA; methane.

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Introduction

Several essential oils have exhibited a spectrum of beneficial properties, including antimicrobial, antifungal, antiviral, antiparasitic, insecticidal, antiprotozoal, and antioxidant activities, as documented in various studies (Burt, 2004; Cowan, 1999). In this context, the aroeira plant has shown antimicrobial activity in both aqueous and 30% ethanol extracts (Lima et al., 2004; Martínez et al., 1996). Essential oils have proven effective in influencing ruminal fermentation in vitro, as verified in studies involving *Schinus terebinthifolius Raddi*, a tree species indigenous to regions spanning from northeast to south Brazil. This tree is characterized by dense, dark green foliage and clusters of red fruits, commonly known by the names red aroeira and pepper-aroeira (Lorenzi, 2002). These essential oils are derived from leaves, fruits, and trunk, containing a composition of compounds such as α-pinene, sabinene, β-pinene, α-phellandrene, Δ-3-carene, β–phellandrene, terpinen-4-ol, α-copaene, germacrene-D, bicyclogermacrene, β-caryophyllene, δ-cadinene, and α-cadinol (Santos et al., 2010).

The potential of these essential oils in manipulating ruminal fermentation parameters has been a subject of interest. That aroeira leaves in an *in vitro* system revealed similarities in the effects of these essential oils and monensin. There was an increase in C3 concentration compared to the control treatment (without additive), an increase in C4 and isoacids, and a reduction in the C2/C3 ratio, indicating the capability of these essential oils to influence specific aspects of ruminal fermentation. This study aimed to assess the effect of essential oils of Aroeira leaves (*Schinus terebinthifolius*) on ruminal fermentation parameters, nutrient intake, digestibility, and nitrogen balance in lambs.

Materials and methods

This study was carried out at research facilities of Sheep and Goat Intensive Production System (SIPOC) of the Department of Animal Science, "Escola Superior de Agricultura Luiz de Queiroz – ESALQ", University of 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 (Comite de Ética no Uso de Animais – CEUA – Number 3045/2013).

Animals and facilities

Thirty crossbred (½ Dorper x ½ Santa Inês) castrated males, initial body weight of 53.08 ± 11.40 kg and approximately 210 days old 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} BW and 5 g of Levamisole hydrochloride (Ripercol®, Fort Dodge Animal Health, Campinas, São Paulo, Brazil). The animals were housed in individual pen during the first 15 days for adaptation to diets. Between 15- and 21-days 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

The lambs were blocked by initial body weight and randomly assigned within 10 blocks. Diets were composed by 10% of coast cross hay and 90% of concentrate. Experimental diets were: CTL – negative control diet (no inclusion of fed additives); MON – positive control diet adding of 25 mg of monensin (Rumensin® 100, Elanco Brazil, São Paulo, SP, Brazil)/kg of DM; and inclusion of 1.25, 2.50 and 3.75 mL of essential oils of *Schinus terebinthifolius* leaf/kg of as feed.

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. Afterwards, 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) (Table 1).

Table 1. Composition of basal diet with different feed additives and essential oils doses (mL kg^{-1} as fed).

Item	Diet [1]				
	CTL	MON	1.25	2.50	3.75
Ingredients, % of dry mater					
Coast cross hay	10.00	10.00	10.00	10.00	10.00
Soybean meal	16.00	16.00	16.00	16.00	16.00
Corn ground	70.00	70.00	70.00	70.00	70.00
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]	2.00	2.00	2.00	2.00	2.00
Essential oils (mL 100 kg^{-1} MS)	0.00	0.00	109.0	218.0	327.0
Monensin (ppm)	0.00	25.00	0.00	0.00	0.00
Chemical composition					
Dry matter	87.50	87.50	87.50	87.50	87.50
Organic matter	95.60	95.60	95.60	95.60	95.60
Crude protein	15.00	15.00	15.00	15.00	15.00
Ether extract	2.60	2.60	2.60	2.60	2.60
NDF[3]	12.50	12.50	12.50	12.50	12.50

[1] CTL = Negative control diet (without additives); MON = positive control diet (with monensin); 1.25, 2.50 or 3.75 mL of essential oils of Aroeira leaf (*Schinus terebinthifolius*) per kg of 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.

On experimental diet MON, the sodium monensin was added at the rate of 25 mg kg^{-1} of DM. Essential oils were mixed with diets every time the animals were fed. Daily experimental diets were weight on an electronic scale accurate to 5.0 gram and offered ad libitum, at 07:00am and 7pm. Refused feed was collected every day from each animal to calculate dry matter intake.

The animals were weighted on day 0 and day 21, without fasted, to monitoring body weight.

Ruminal sample analysis

On days zero (before starting offering experimental diets), 7, 14 and 21, ruminal fluid was collected 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

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

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

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

Sample analysis

Oil of Aroeira leaf (*Schinus terebinthifolius*) composition was determined at Chemistry and Food Analysis (School of Agricultura Luis de Queiroz of University of São Paulo, Brazil) 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 to 230°C (ramp 3) for a total period of 77.17 minutes (Table 2).

Table 2. Composition of essential oils of Aroeira leaf (*Schinus terebinthifolius*).

	Aroeira leaves
Component	% Relative
A pineno	40.16
β pineno	11.26
1,6 octadieno	2.83
α felandreno	3.95
Δ- 3- Careno	0.81
Cimeno	10.38
α-Terpineno	0.42
P-cimeno	16.15
Limoneno	0.54
Terpinoleno	0.51
α copaeno	0.27
Muroleno gama	6.94
Cariofileno	0.54
Germacreno- D	0.59
Cadineno Gama	2.64
α- elemol	0.28
Viridiflorol	0.44
Non identified	1.29

Samples of the offered feed, orts and feces were thawed, composited within animal, dried in a forced-air oven at 55 °C for 72 h 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 24 h, and ash content was obtained by incinerating samples in an oven at 550 °C for 4 h (Association of Official Analytical Chemists [AOAC], 1990). The OM was calculated by the difference between DM and ash. Total N of offered feed, orts and feces was determined using Leco FP528 instrument (Leco Corporation, St. Joseph, MI, USA) according to AOAC (1997). Extract ether was determined using 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 Microkjeldahl method (AOAC, 1997).

Short chain fatty acid profile 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 with 0.4 mL of 3: 1 solution of 25% metaphosphoric acid with 98-100% formic acid and 0.2 mL of (internal standard) were centrifuged in apparatus (Sorvall Superspeed RC2-B, Newton, CT, USA) 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 gas chromatograph. Injection was performed automatically, the entrainment gas was H_2, maintained at a flow of 31.35 mL minute^{-1}. Temperature of the injector and detector was 260°C. Total time of 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 min^{-1}).

The NH_3-N 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 550 nm absorbance filter.

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

Fermentative $CO2 = A/2 + \frac{P}{4} + 1.5 * B$

Fermentative $_{CH4 = (A+2*B) - CO2}$

A = mole of acetate.

P = mole of propionate.

B = mole of butyrate.

Statistical analysis

Animal was the experimental unit for all statistical analysis. Statistical analyses were performed using the MIXED procedure of the SAS statistical software program (version 9.0; SAS Inst. Inc., Cary, NC). 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. Data that did not respect 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. The means were obtained by the LSMEANS command. For the data analyzed as repeated for days only, measures over time as AGCC profile, ruminal pH, and ammonia, 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 the form of orthogonal counter: Negative control vs additives; positive control vs oil doses; linear effect and quadratic effect.

All data that were analyzed as repeated for day measures were put on 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 LSMEANS command. Effect of diet, week and interaction of diet x week were defined by F test of variance analysis and was evaluated using the inclusion of SLICE methods. 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

Effects of three doses of essential oils of Aroeira leaf compared with negative control treatment (no additives) and positive control treatment (monensin) on ruminal fermentation parameters with seven, fourteen, twenty-one and all days of offering experimental diets are summarized in Tables 3, 4, 5 and 6, respectively.

On day 7 of supplementation, it was observed a linear effect on methane production ($p = 0.04$; Table 3).

Table 3. Ruminal parameters in lambs feeding with monensin and doses of essential oil of Aroeira leaf (*Schinus Terebinthifolius*) compared with a control treatment in the 7th day of offering the experimental diets.

Item	Treatment[1]						*P* value[3]			
	CTL	MON	1.25	2.50	3.75	EPM[2]	CTL*Additives	MON*Oil	L	Q
Ruminal pH	5.55	5.60	5.52	5.53	5.44	0.08	0.82	0.31	0.24	0.93
Total VFA, mM	106.69	107.89	113.08	106.61	116.71	5.36	0.48	0.49	0.42	0.65
Acetate, mM	48.56	51.92	51.84	50.41	55.11	2.28	0.21	0.84	0.44	0.30
Propionate, mM	37.25	42.58	42.83	40.90	39.63	3.00	0.23	0.67	0.43	0.80
Butyrate, mM	15.85	8.89	12.60	9.74	15.98	2.08	0.10	0.11	0.06	0.55
Isobutyrate, mM	0.95	0.83	0.97	0.86	0.96	0.08	0.66	0.32	0.48	0.80
Isovalerate, mM	1.68	1.58	1.48	1.87	2.28	0.32	0.74	0.43	0.10	0.43
Valerate, mM	1.64	1.74	2.75	2.61	2.46	0.44	0.17	0.10	0.31	0.21
C_2/C_3	1.45	1.31	1.32	1.28	1.55	0.10	0.49	0.55	0.16	0.23
$N\text{-}NH_3$, mg dL^{-1}	19.94	20.47	22.45	22.10	21.05	1.09	0.20	0.28	0.78	0.18
Methane, mmol	23.09	19.75	21.51	19.85	25.63	1.72	0.49	0.20	0.04	0.25

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

In the 14th day of experimental diets, there was a tendency ($p = 0.08$) of effect for ruminal pH in the contrast CTL vs additives (Table 4).

Table 4. Ruminal parameters in lambs feeding with monensin and doses of essential oil of Aroeira leaf (*Schinus Terebinthifolius*) compared with a control treatment in the 14th day of offering the experimental diets.

Item	Treatment[1]						*p* value[3]			
	CTL	MON	1.25	2.50	3.75	EPM[2]	CTL*Additives	MON*Oil	L	Q
Ruminal pH	5.67	5.64	5.48	5.42	5.47	0.09	0.10	0.08	0.16	0.24
Total VFA, mM	113.04	109.76	108.03	108.34	109.29	4.77	0.43	0.82	0.95	0.77
Acetate, mM	52.79	52.77	51.88	50.44	55.17	2.38	0.93	0.92	0.59	0.24
Propionate, mM	40.95	41.10	41.83	43.32	35.10	3.16	0.86	0.78	0.24	0.16
Butyrate, mM	12.97	10.85	8.54	8.49	12.85	1.55	0.11	0.62	0.39	0.03
Isobutyrate, mM	0.97	0.92	0.84	0.86	0.98	0.08	0.47	0.80	0.55	0.22
Isovalerate, mM	1.44	1.75	1.32	1.99	2.13	0.36	0.38	0.88	0.27	0.44
Valerate, mM	1.86	2.21	2.31	2.59	2.29	0.43	0.31	0.67	0.75	0.61
C_2/C_3	1.39	1.43	1.28	1.18	1.67	0.10	0.97	0.68	0.16	0.002
$N\text{-}NH_3$, mg dL^{-1}	16.87	19.33	19.88	21.43	21.14	1.46	0.03	0.38	0.29	0.77
Methane, mmol	22.64	21.54	19.75	18.64	25.23	1.63	0.46	0.86	0.17	0.01

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

Quadratic effect on butyrate concentration ($p = 0.03$), quadratic effect on acetate/propionate rate ($p = 0.002$) and effect on $N\text{-}NH_3$ concentration ($p = 0.03$) on CTL vs additives contrast were observed. There was also quadratic effect on methane production ($p = 0.01$). In the 21st day of experimental diets, feed additives increased $N\text{-}NH_3$ concentration ($p = 0.01$; Table 5) compared to control. Quadratic effect was observed on acetate ($p = 0.03$), propionate ($p = 0.04$), butyrate ($p = 0.02$), isobutyrate ($p = 0.04$), isovalerate ($p = 0.004$) and acetate/propionate rate ($p = 0.0001$).

Table 5. Ruminal parameters in lambs feeding with monensin and doses of essential oil of Aroeira leaf (*Schinus Terebinthifolius*) compared with a control treatment in the 21st day of offering the experimental diets.

Item	Treatment[1]						*P* value[3]			
	CTL	MON	1.25	2.50	3.75	EPM[2]	CTL*Additives	MON*Oil	L	Q
Ruminal pH	5.74	5.59	5.45	5.58	5.63	0.09	0.10	0.71	0.57	0.32
Total VFA, mM	106.55	111.68	109.49	101.50	106.58	4.81	0.88	0.30	0.28	0.45
Acetate, mM	51.64	55.67	52.20	50.03	56.02	2.20	0.46	0.25	0.91	0.03
Propionate, mM	35.56	39.48	42.92	40.45	33.52	2.50	0.21	0.86	0.07	0.04
Butyrate, mM	13.87	12.23	9.02	8.10	12.28	1.61	0.06	0.19	0.91	0.02
Isobutyrate, mM	0.98	0.90	0.75	0.80	0.85	0.04	0.007	0.08	0.66	0.04
Isovalerate, mM	1.68	1.62	1.10	1.18	1.88	0.20	0.31	0.32	0.35	0.004
Valerate, mM	1.49	1.75	1.72	2.10	1.99	0.18	0.05	0.32	0.13	0.82
C_2/C_3	1.60	1.53	1.27	1.29	1.75	0.08	0.17	0.37	0.09	<0.0001
$N\text{-}NH_3$, mg dL^{-1}	15.61	17.92	19.69	20.94	19.88	1.40	0.01	0.17	0.26	0.32
Methane, mmol	23.87	24.08	19.88	18.95	25.77	1.55	0.33	0.16	0.55	<0.0001

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

When all means were analyzed together (days, 0; 7; 14 and 21 days), feed additives decreased butyrate concentration (P = 0.02) and increased N-NH_3 concentration (p = 0.005) compared to control (Table 6). There were no differences between monensin and oil. Linear effect was observed for N-NH_3 concentration (p = 0.03). Oil dosage induced a quadratic effect in acetate (p = 0.01), butyrate (p = 0.01) and acetate/propionate rate (p = 0.003). There was also quadratic effect on methane production (p < 0.0001).

Table 6. Ruminal parameters in lambs feeding with monensin and doses of essential oil of Aroeira leaf (*Schinus Terebinthifolius*) compared with a control treatment along all days (0, 7, 14 and 21) of offering the experimental diets.

Item	Treatment[1]						*p* value[3]			
	CTL	MON	1.25	2.50	3.75	EPM[2]	CTL*Additives	MON*Oil	L	Q
Ruminal pH	5.66	5.60	5.48	5.52	5.56	0.05	0.08	0.23	0.76	0.16
Total VFA, mM	112.43	111.56	108.80	106.40	116.59	3.71	0.70	0.82	0.44	0.08
Acetate, mM	53.00	54.16	50.75	50.56	56.16	1.78	0.96	0.40	0.45	0.01
Propionate, mM	38.25	40.20	41.11	41.47	38.31	2.11	0.40	0.96	0.57	0.33
Butyrate, mM	15.97	12.25	11.45	9.59	15.93	1.37	0.02	0.96	0.14	0.01
Isobutyrate, mM	1.04	0.91	0.87	0.86	1.04	0.05	0.08	0.83	0.16	0.07
Isovalerate, mM	1.84	1.83	1.41	1.94	2.46	0.25	0.80	0.71	0.03	0.06
Valerate, mM	1.79	2.00	2.60	2.45	2.21	0.29	0.11	0.19	0.69	0.13
C_2/C_3	1.52	1.46	1.33	1.27	1.59	0.09	0.19	0.51	0.29	0.003
N-NH_3, mg dL^{-1}	18.12	20.57	21.02	22.16	20.96	0.99	0.005	0.46	0.59	0.39
Methane, mmol	24.92	23.15	20.82	19.71	26.46	1.24	0.09	0.56	0.11	<0.0001

[1] CTL = Negative control diet (without additives); MON = positive control diet (with monensin); 1.25, 2.50 or 3.75 mL of essential oils of Aroeira leaf (*Schinus terebinthifolius*) per kg^{-1} of dietary feed; [2] EPM = Standard error; [3] *p* value = means differ significantly (p < 0.05). Contrasts of treatments CTL *vs* additives; MON vs Oil doses; Linear effect; Quadratic effect.

Intake and digestibility of nutrients

There was no effect (p > 0.05) for any variables related to intake and digestibility of nutrients (Table 7).

Table 7. Nutrients digestibility on total tract and intake in lambs receiving monensin and levels of essential oils of Aroeira leaf (*Schinus Terebinthifolius)* compared with a control treatment.

Item[1]	Treatment[2]						*P* value[4]			
	CTL	MON	1.25	2.50	3.75	EPM[3]	CTL*Additives	MON*Oil	L	Q
DMI, kg d^{-1}	1.46	1.49	1.35	1.55	1.31	0,09	0.755	0.442	0.441	0.592
DMI, %BW	2.15	2.30	2.01	2.31	2.00	0.13	0.943	0.226	0.331	0.934
$DMI^{0,75}$, g	61.41	65.06	57.41	65.99	56.91	3.88	0.986	0.255	0.344	0.846
OMI, kg d^{-1}	1.39	1.42	1.29	1.48	1.25	0.09	0.741	0.456	0.452	0.582
CPI, kg d^{-1}	0.21	0.22	0.20	0.23	0.19	0.014	0.781	0.434	0.409	0.536
EEI, kg d^{-1}	0.038	0.036	0.036	0.040	0.035	0.002	0.683	0.860	0.891	0.365
NDFI, kg d^{-1}	0.18	0.18	0.17	0.19	0.16	0.01	0.850	0.572	0.532	0.485
NDFI, % BW	0.26	0.28	0.25	0.29	0.25	0.26	0.792	0.314	0.385	0.769
Digestibilities										
DM, %	84.22	85.43	84.46	84.54	86.50	1.42	0.509	0.866	0.591	0.289
OM, %	87.45	86.57	87.24	86.77	88.60	1.30	0.909	0.504	0.319	0.641
CP, %	79.04	80.93	80.59	81.01	83.07	2.03	0.284	0.781	0.434	0.536
EE, %	87.72	87.23	86.95	87.11	87.92	0.83	0.660	0.922	0.557	0.521
NDF, %	82.81	80.50	78.73	80.66	82.83	1.50	0.246	0.897	0.226	0.231

[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 of Aroeira leaf (*Schinus terebinthifolius*) per kg^{-1} of dietary feed; [3] EPM = Standard error; [4] *p* value = means differ significantly (p < 0.05). Contrasts of treatments CTL *vs* additives; MON vs Oil doses; Linear effect; Quadratic effect.

Nitrogen balance

Feed additives decreased nitrogen intake (p = 0.0009) and retained nitrogen (p = 0.005) for the CTL vs Additives contrast (Table 8). In addition, Aroeira leaf oil decreased nitrogen intake (p = 0.01) and retained nitrogen (p = 0.03) compared with monensin.

Discussion

In our study, Aroeira leaf essential oil at varying doses displayed significant impacts on rumen fermentation. Feed additives, mainly doses of 1.25 and 2.50 mL of Aroeira leaf essential oil were able to decrease butyrate concentrations, This trend persisted consistently across comparative evaluation days (Tables 4, 5, and 6),

underscoring superior efficiency of 1.25 and 2.50mL doses in energy production within rumen. The link between butyrate production and energy utilization was evident, considering expenditure of 148 kcal of energy for every mol of butyrate formation (Resende et al., 2011). Moreover, higher methane production was observed for CTL and 3.75 mL oil treatments, showing 10.5% and 19.4% more methane compared to the 1.25- and 2.50-mL doses, reinforcing the association between higher butyrate concentrations and lower methane production for doses.

Table 8. Nitrogen balance in lambs receiving monensin and levels of essential oil of Aroeira leaf (*Schinus Terebinthifolius)* compared to a control treatment.

Item	Treatment[1]						*P* value[3]			
	CTL	MON	1.25	2.50	3.75	EPM[2]	CTL*Additives	MON*Oil	L	Q
N intake, g	43.33	38.00	31.83	28.83	30.50	0.0020	0.0009	0.01	0.035	0.13
N feces, g	7.00	7.33	5.16	7.00	6.00	0.0009	0.56	0.25	0.61	0.54
N urine, g	15.83	13.83	15.67	14.33	14.00	0.0026	0.64	0.78	0.94	0.68
N retained, g	20.50	16.67	10.67	8.92	10.67	0.2330	0.005	0.03	0.09	0.15
Apparent digestibility N AD N, %	84.21	80.66	83.64	75.62	80.49	1.8759	0.06	0.73	0.32	0.61

[1] CTL = Negative control diet (without additives); MON = positive control diet (with monensin); 1.25, 2.50 or 3.75 mL of essential oil of Aroeira leaf (*Schinus terebinthifolius*) per kg^{-1} of dietary feed; [2] EPM = Standard error; [3] *p* value = means differ significantly ($p < 0.05$). Contrasts of treatments CTL *vs* additives; MON vs Oil doses; Linear effect; Quadratic effect.

Our findings contrast with some previous studies. For instance, Demirtas et al. (2019) found elevated butyrate concentrations with different aldehyde doses, leading to increased methane yields. Similarly, Cobellis et al. (2016) reported higher butyrate levels with essential oils treatments, despite observing reduced methane production. Studies such as Macheboeuf et al. (2008) and Patra and Yu (2012), also pointed out substantial reductions in methane production with several essential oils. At the core of our observations lies a complex interplay between essential oils composition, diet type, and ruminal microbial interactions. Notably, the 1.25 and 2.50 mL Aroeira leaf essential oils doses consistently revealed lower methane production suggesting their potential as more effective additives.

Furthermore, we observed a tendency towards decreased ammonia concentrations in the rumen with the MON treatment and Aroeira leaf oil doses, contradicting general expectations regarding impact of essential oils on hyperammonia-producing bacteria. The multifaceted nature of these observations underlines the dose-dependent effects of essential oils on rumen fermentation and its subsequent implications for energy production and methane emission in cattle.

No effect was observed for any of variables related to nutrient intake and digestibility. These results are in agreement with several authors (Benchaar et al., 2007; 2008; Malecky et al., 2009; Santos et al., 2010) who also did not find effects on nutrient intake and digestibility variables.

In our study, we observed a decreased in nitrogen intake between the control (CTL) and additive treatments, with CTL displaying a 25.5% higher nitrogen intake compared to additives. The MON treatment also exhibited a 20.0% increase in nitrogen intake in comparison to the various doses of Aroeira leaf essential oils (Table 8). Upon calculating the ratio of nitrogen to ammonia (N/N-NH3) intake in ruminal fluid, it became evident that nitrogen utilization efficiency (N retained g d^{-1}) was substantially higher for the CTL treatment (51.0%) in comparison to the additives and even higher (51.0%) for MON treatment compared to three doses of Aroeira leaf essential oils utilized. This demonstrates better nitrogen utilization and retention with CTL and MON treatments, underscoring their efficiency in using dietary nitrogen in contrast to other treatments.

Furthermore, higher nitrogen digestibility (DAP %) was noted in the CTL compared to additive treatment, aligning with expectations due to higher consumption in CTL. Similarly, urinary and fecal excretion of nitrogen in treatments with additives (MON and essential oils doses), result in increased nitrogen retention. It suggests superior nitrogen utilization from diet for CTL treatment compared to other treatments.

Despite these observations, essential oils did not exhibit significant efficacy in reducing concentration of N in ruminal fluid, indicating a possible increase in hyper ammonia-producing bacteria activity. This outcome was consistent on days 21 and across the comparison of multiple days, implying a potential influence on bacterial activity despite the lack of notable impact on nitrogen concentration.

The effects found in the reduction of enteric methane were only observed from day 14 (Tables 4 and 5), of the inclusion of essential oils in the animals' diet. Most efficient dosages in reducing enteric methane production were 1.25 and 2.5 ml of essential oils from Aroeira leaf. This effect can be confirmed because the essential oils used in the present study contains limonene and pinene in the composition, as expressed by

Araújo (2010). It can improve the animals energetic efficiency and reduce the environmental impact of methane emissions into the atmosphere.

In general, for all evaluation days (Table 6), dosages of 1.25 and 2.5 ml of essential oils from Aroeira leaf were able to reduce methane production by 16.4% and 21% in relation a control treatment. Furthermore, the dosages above mentioned reduced methane production by 10% and 14.8% respectively, in relation to treatment with monensin,The data from the present study are in agreement with Klevenhusen et al. (2011) when evaluating inclusion of garlic essential oils in diet of sheep that achieved a 10% reduction in methane production, in relation to control treatment.

Conclusion

The doses of 1.25 and 2.50 mL kg^{-1} reduced methane production and positively influenced the VFAs. However, these treatments were less efficient in terms of N utilization compared to the control treatment.

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