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Blend of cinnamaldehyde and diallyl disulfide associated or not to antibiotics on ruminal fermentation, cortisol and blood metabolites of feedlot steers fed no-forage diet

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ABSTRACT: The objective of this study was to evaluate the effects of an essential oil blend (EO), based on cinnamaldehyde and diallyl disulfide, associated or not with antibiotics on intake and nutrient digestibility, ingestive behavior, rumen fermentation, ruminal microbial synthesis, and blood metabolites of feedlot cattle fed a no-forage diet. The study was carried out as a Latin Square with five treatments consisting of a blend of essential oil (EO), monensin (MON) and virginiamycin (VM), both separately and combined as follows: CON (monensin at 30 mg kg^{-1} DM), VM (virginiamycin at 25 mg kg^{-1} DM and monensin at 30 mg kg^{-1} DM), MEO25 (monensin at 30 mg kg^{-1} DM and EO at 25 mg kg^{-1} DM), MEO35 (monensin at 30 mg kg^{-1} DM and EO at 35 mg kg^{-1} DM) and EO35 (blend of EO at 35 mg kg^{-1} DM). There were no effects from additives and their combinations on the intake and apparent digestibility of nutrients or ingestive behavior. Furthermore, treatments did not modify the ruminal pH, nor the concentration of short-chain fatty acids and ammonia, nor the microbial protein synthesis. Blood glucose concentration was higher 4 h after morning feeding for all treatments. There was a significant contrast between the VM and EO for the blood concentration of *D*-Lactate and *L*-Lactate. There was no difference between the additives in the concentration of cortisol metabolites in the feces. The blend of essential oil studied, containing cinnamaldehyde and diallyl disulfide, associated or not with antibiotics, does not change the nutritional parameters nor the metabolism of feedlot cattle fed a no-forage diet.

Keywords: cinnamaldehyde, diallyl disulfide, feedlot, starch

Introduction

The increase in grain levels in feedlot cattle diets allows for the increase in energy concentration needed to meet the requirements of high-performance animals. Previous studies have evaluated the effects of feedlot diets without roughage on ruminant performance (Faleiro et al., 2011) by increasing feed efficiency and ruminal fermentation (Iraira et al., 2013; Chibisa et al., 2020), which has been attributed to increases in the proportion of propionate in the rumen. However, for this type of diet to become possible, feed additives are required to decrease the incidence of metabolic disorders such as ruminal acidosis.

Monensin (MON) and virginiamycin (VM) are antimicrobial feed additives used to modulate ruminal fermentation in feedlot cattle which inhibit the growth of lactic acid-producing bacteria, thereby limiting the accumulation of lactate, one of the main contributors to rumen acidosis (Coe et al., 1999; Marques and Cooke, 2021). Thus, the including of these additives in grain-based diets can prevent ruminal acidosis. However, alternatives for substituting traditional antimicrobials, such as essential oils, must be studied to reduce the risk of developing antibiotic-resistant bacteria.

Essential oils are aromatic compounds extracted from certain types of plants that reportedly control the growth and activity of ruminal microorganisms associated with methanogenesis and starch fermentation, which present potential for being used to manipulate rumen fermentation. The cinnamaldehyde and diallyl disulfide mix in a dairy cattle diet increases the molar proportion of propionate and decreases ruminal ammonia production (Blanch et al., 2016). Cinnamaldehyde and diallyl disulfide have shown potential for increasing ruminal pH (Fraser et al., 2007; Ahmed et al., 2021). Furthermore, in a more acidic ruminal environment, the effect of cinnamaldehyde is more pronounced on ruminal fermentation, which implies that those compounds would be more effective in feedlot cattle fed high grain diets (Cardozo et al., 2005).

However, information needs to be included about the effects of the mix of these two essential oils on a diet with a high concentration of starch, especially in the case of no-forage based diets. Given this context, we hypothesized that feeding steers with a mix of essential oils in this diet could change ruminal fermentation and might prevent ruminal acidosis.

Thus, this study aimed to evaluate the effects of an essential oil blend containing cinnamaldehyde and diallyl disulfide, associated with or without monensin, on intake and apparent digestibility, ingestive behavior, ruminal fermentation, microbial protein, and blood metabolites of beef cattle fed a no-forage diet.

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Materials and Methods

The experiment was conducted in Cuiabá, MT, Brazil (15°51′05.9″ S 56°04′14.5″ W, altitude 141 m). The experimental protocols were reviewed and approved by the Ethics Committee on Animal Use of the Universidade Federal do Mato Grosso (23108.050556/2020-04).

Animals and treatments

Five rumen fitted castrated Nellore × Senepol crossbred steers (331.4 ± 20.32 kg) approximately 14 months old were used. The experimental design was a 5 × 5 Latin square. Each experimental period lasted 21 days, with 14 days for diet adaptation to the treatment and seven days for data collection. The animals were kept in separate concrete stalls (14 m^2) equipped with a feed bunk and water fountain.

Before the study, to increase the inclusion of concentrate in diets progressively, animals were fed a diet composed of 40:60 forage:concentrate, which had 10 % of its forage (elephant grass silage) proportion reduced every four days until 100 % of a no-forage diet was achieved. After that, animals were fed the experimental diet, consisting of ground corn, cottonseed cake, mineral premix, and tested additives (Table 1). The steers were fed twice a day at 08h00 and 16h00, allowing 5 to 10 % orts.

There were five treatments consisting of an essential oil blend containing cinnamaldehyde and diallyl disulfide (NE300®, Novus International Inc.) and commercial additives in the following combination: CON (monensin at 30 mg kg^{-1} DM); VM (virginiamycin at 25 mg kg^{-1} DM and monensin at 30 mg kg^{-1} DM); MEO25 (monensin at 30 mg kg^{-1} DM and blend of essential oil at 25 mg kg^{-1} DM); MEO35 (monensin at 30 mg kg^{-1} DM and blend of essential oil at 35 mg kg^{-1} DM); and EO35 (blend of essential oil at 35 mg kg^{-1} DM). The dose used for the blend of essential oil followed the manufacturer's recommendation.

Table 1 – Ingredient and chemical composition and particle size distribution of the diet.

Item	Dieta
Ingredients, (% of DM)	
Ground corn	82.0
Cottonseed cake	15.0
Urea	0.1
Mineral and vitamin premix[1]	2.9
Chemical composition[2], (% of DM)	
DM, % as feed	90.37
Organic Matter	93.29
Crude protein	12.80
Crude fat	1.75
NDF	15.26
aNDF	13.76
TDN	76.40
Starch	60.09
Metabolizable energy (Mcal kg^{-1} MS)[3]	2.75
Particle size (mm)	
> 19.0	0.13
8.0 – 19.0	4.82
4.0 – 8.0	8.30
< 4.0	86.63
peNDF (%)[4]	1.82

[1]Premix: Ca = 12 g kg^{-1}; P = 3.5 g kg^{-1}; S = 1.8 g kg^{-1}; K = 3.7 g kg^{-1}; Na = 3 g kg^{-1}; Mg = 1,800 mg kg^{-1}; Zn = 83 mg kg^{-1}; Cr = 0.3 mg kg^{-1}; Mn = 29 mg kg^{-1}; Cu = 20 mg kg^{-1}; Co = 1.4 mg kg^{-1}; I = 1 mg kg^{-1}; Se = 0.25 mg kg^{-1}; Met = 250 mg kg^{-1}; Vit A = 3000 mg kg^{-1}; Vit E = 15 mg kg^{-1}. [2]DM = dry matter; NDF = neutral detergent fiber; aNDF = neutral detergent fiber corrected for ash; TDN = total digestible nutrients.[3]Estimated with equation proposed by NASEM (2016). [4]Physically effective neutral detergent fiber (peNDF) calculated by multiplying the percentage of weight from the sieves above 4 mm by the percentage of NDF of the diet.

Feeding behavior, feed, and fecal sampling and analysis

On day 15 of each experimental period, feeding behavior was measured every 10 min for 12 h from the morning feed. Time eating and drinking were computed when every animal was at the feed bunk and water fountain. In contrast, when steers did chewing movements unrelated to eating activities, rumination time was considered. Idle time lying down, and standing was computed when steers were resting. Time spent on other activities included activities unrelated to ingestion.

Feed intake was measured over the seven days of the sampling period by measuring diet delivery and respective orts daily. On 16, 17, and 18 days of each experimental period, samples of diet, orts, and feces were collected and frozen at –20 °C, ort samples being collected before the morning feed, whereas fecal samples were collected daily every time the steer defecated at 12 h intervals starting after the morning feed, making four samples daily. After defecation, fecal samples were collected from the upper part of the feces that did not have contact with the concrete floor.

After thawing, samples of diet, orts, and feces were pre-dried in an air circulation oven at 55 °C for 72 h and ground in a Willey mill using 1- and 2-mm sieves, which were used for chemical analysis and indigestible neutral detergent fiber determination, respectively. After this, 1-mm samples were analyzed to dry matter (DM, method 967.06) (AOAC, 1990), ash (MM, method 942.05) (AOAC, 2002), and crude protein (CP, method 2001.11) (AOAC, 2002). Crude fat was obtained using the Ankom XT15 extraction system (ANKOM Technology Corporation). Neutral detergent fiber (NDF) was obtained without sodium sulfite, with the addition of thermostable amylase (Van Soest et al., 1991), and filtered in crucibles with porous plate number 2. The indigestible neutral detergent fiber (iNDF) was determined by incubation of 2 g of samples (ingredients, fecal and orts) into the rumen for 240 h using non-textile fabric bags (100 g m^{-2}) according to Valente et al. (2011). To estimate fecal

excretion (FE), iNDF was used as an internal indicator by the following equation: FE (kg d^{-1}) = (iNDF in diet – iNDF in orts) / (iNDF in feces).

Total digestible nutrients (TDN) in the diet were estimated using the equation TDN = 0.323 NDF + 0.883 NFC + 1.829 EE + 0.885 CP proposed by Jayanegara et al. (2019). Starch analysis was performed following the method proposed by Zinn (1990) with modifications suggested by Silva et al. (2019). The metabolizable energy (ME) of the diet was estimated using the equations proposed by the NASEM (2016), in which 1 kg TDN = 4.4 Mcal of digestible energy (DE) and ME = 0.82 × DE.

During the first three days of each sampling period, the particle size of the feed was evaluated by collecting samples, and then the particle size was obtained using the Penn State Particle Separator (Kononoff et al., 2003), where a 4 mm sieve replaced the 1.18 mm sieve.

For fecal cortisol metabolite analysis 20 g of feces were separated, placed in plastic bags, and kept under refrigeration. Next, they were composed for each period and frozen at –20 °C. For analysis, fecal samples were thawed, dried at 55 °C for 72 h, composited by animal and period, ground and macerated in a mortar. Half a g of dry fecal samples was weighed, and 3 mL of methanol (MetOH 80 % v v^{-1}) was added. The tubes were vortexed (104.72 rad s^{-1} for 10 min) and then centrifuged at 314.16 rad s^{-1} for 10 min (Megafuge 40, Thermo Scientific) (Gholib et al., 2021). After that, 2 mL of the clarified material was collected, placed in microtubes, and kept in an oven at 37 °C until the solvent evaporated. Subsequently, 1 mL of methanol (MetOH 99.7 %) was added into the microtubes, which were vortexed again, and the fecal extract frozen at –20 °C. A radioimmunoassay (RIA) assay was used with a commercial diagnostic kit (MP Biomedicals), marked with I125 for fecal cortisol metabolites measurement. Samples were read in a Wisard model (PerkinElmer Inc.) gamma counter. The reference value of cortisol metabolite was used as an indicator of stress, and was obtained from fecal samples collected during the period that steers were fed the diet containing 40 % roughage and 60 % concentrate, following the same methodology mentioned above (Gholib et al., 2021).

Urine, ruminal fluid, and blood sampling and analysis

Spot urine samples were collected once a day, 4 h after the morning feed (Meschiatti et al., 2019), over three days during each sampling period in plastic tubes. The samples from each day were categorized by the animal within each period and collected when spontaneous urination occurred. When voluntary urination did not occur, the foreskin was stimulated. Approximately 200 mL of urine from each animal was collected and filtered, and 10 mL of urine was added to 40 mL of sulfuric acid (H_2SO_4) (0.036 N) and stored at –20 °C.

After thawing at room temperature, allantoin concentration was analyzed by the colorimetric method (Chen and Gomes, 1992), on a wavelength of 522 nm using a UV/visible spectrophotometer (Libra S32, Biochrom) and creatinine using a commercial kit (Weiner Lab) on a wavelength of 510 nm. Uric acid was obtained using a commercial kit (Kit n.451, Gold Analisa), and the reading was taken on a wavelength of 505 nm (Libra S32).

Daily urinary excretion was calculated between the daily creatinine excretion as a function of body weight and the creatinine concentration observed in the urine. The equation to calculate daily creatinine excretion in relation to body weight was obtained from Silva et al., 2012, where UCE = 0.0345 ($EBW^{0.9491}$), and UCE = urinary creatinine excretion (g d^{-1}) and EBW = empty body weight (kg). The excretion and absorption of purine derivatives and the microbial nitrogen synthesis were calculated according to Chen and Gomes (1992).

On day 19, ruminal fluid was collected at 0, 2, 4, 6, 9, and 12 h after the morning feed. To collect the rumen fluid, a manual suction pump coupled to a flexible hose was used, which was introduced into the rumen through a ½″ flange coupled to the cannula cover. Approximately 200 mL of ruminal fluid was collected, filtered, and separated into 12 mL aliquots for ammonia N (NH_3) analysis. Two mL for short-chain fatty acids (SCFA) analyses was obtained, and the samples were frozen at –20 °C.

Ruminal fluid samples were thawed, and 1 mL was transferred to microtubes for NH_3 analysis. Next, 100 µL trichloroacetic acid (100 g L^{-1}) was added, and the samples were centrifuged at 1,000 × g for 10 min at 4 °C. The supernatant was collected and analyzed in spectrometry (Epoch, BioTek Instruments) on a wavelength of 620 nm, according to Chaney and Marbach (1962). The samples separated for SCFA analyses were composited by day and centrifuged at 11,269 × g for 12 min at 4 °C, and the supernatant was collected for analysis by gas chromatography (Goestch and Galyean, 1983). On day 20, the rumen fluid was sampled for pH evaluation over 12 h. Approximately 50 mL of ruminal fluid was collected every 30 min until 3 h after feeding and every 1 h until 12 h after feeding, and the pH was read immediately using a digital pH meter (R- TEC-3P-MP, TECNAL®).

On day 21, to obtain blood glucose, approximately 10 mL of blood samples were collected before and 4 h after the morning feed through a puncture with a needle in the middle coccygeal vein. Immediately after collection, respecting a maximum period of 30 s, a drop of blood was transferred to a test strip and read on an Accu Check® Active portable glucometer (Roche Diagnóstica Brasil). The remaining volume was transferred to tubes containing sodium fluoride/EDTA, and centrifuged at 3,000 × g for 20 min. The serum was transferred to microtubes and frozen at –20 °C. *D*-lactate (kit K 002-M Elabscience Biotechnology Inc.) and *L*-lactate (kit K 044-M, Elabscience Biotechnology Inc.) analyses were carried out using the colorimetric enzyme-immunoassay method (EIE).

Statistical analysis

Data were analyzed following the generalized mixed linear model procedure (GLIMMIX), using the SAS® Studio software (SAS® OnDemmand) according to the statistical model $Y_{ijk} = \mu + p_i + a_j + \alpha_k + e_{ijk}$, where Y_{ijk} refers to the response variable observed in period *i*, *j* the animal and *k the* experimental treatment. The general average was represented by μ, the period random effect by p_i, the random effect of animal by a_j and the fixed effect produced by the treatment was represented by α_k. The term e_{ijk} denoted the random experimental error of each of the observations. The animals in each period were considered as a single experimental unit.

The ruminal pH and NH_3 and blood glucose data were analyzed by adding the collection times in the model as repeated measures, according to the statistical model $Y_{ijkl} = \mu + p_i + a_j + \alpha_k + t_l + (\alpha t)_{kl} + e_{ijkl}$, where Y*ijk* referred to the response variable observed in the period *i*, *j* the animal, *k* the treatment and *l* the collection time. The general average was represented by μ; the period random effect, by p_i; the random effect of the animal, by a_j the fixed effect produced by the treatment was represented by α_k, and t_l the fixed effect of collection times. The interaction between treatments and collection times was represented by $(\alpha t)_{kl}$, and the term e_{ijkl} by the random experimental error of each observation.

Analysis of variance was performed, and when significant, means were compared using Tukey's test, with significance established as $p < 0.05$. Probabilities between $0.05 \geq p \leq 0.10$ were considered as a trend. Planned contrast was performed between isolated essential oil (EO35) and treatments containing monensin (CON; VM; MEO25 and MEO35) and between virginiamycin associated with monensin (VM) and treatments containing essential oil (MEO25; MEO35 and EO35).

Results

The treatments did not influence ($p > 0.05$) any ingestive behavior variable (Table 2). However, there was a trend ($p = 0.087$) towards lying idle between the EO35 and the treatments with the addition of monensin.

There was no effect ($p > 0.05$) of additives and their combinations on dry matter intake, nutrient intake (Table 3) or ME intake ($p > 0.05$). The additives also had no effect on the apparent digestibility of nutrients ($p > 0.05$), except for the apparent digestibility of CP, where higher digestibility values ($p = 0.048$) were observed for treatments with essential oil (798.9 g kg^{-1}) compared to VM (712.3 g kg^{-1}).

There was no difference between the additives ($p = 0.766$) in the concentration of cortisol metabolites in the feces, with an average of 357.61 ng g^{-1} fecal DM (Figure 1). In the adaptation period, when the animals were consumed a diet with 40 % corn silage and 60 % concentrate, the mean value of cortisol metabolites (reference value) observed in the feces was 28.90 ng g^{-1} fecal DM.

No effects of interaction among treatments and sampling time were observed on ruminal pH ($p = 0.839$). There was no effect of treatments ($p > 0.05$; Figure 2) on ruminal pH (average of 5.97). However, a linear reduction in ruminal pH was observed as a function of the time after the morning feed ($p < 0.001$; Figure 2), in which a reduction in ruminal pH of 0.026 units was observed every hour from 08h00 to 20h00.

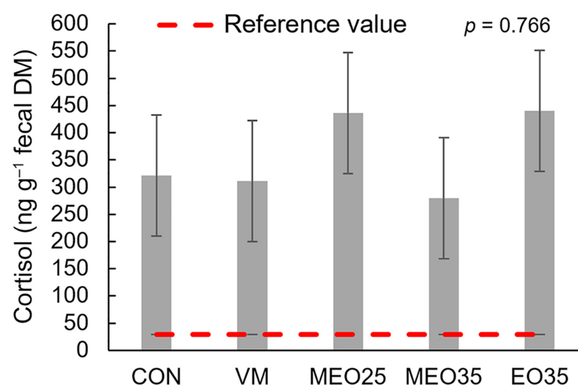

Figure 1 – Cortisol metabolites on fecals of beef steers fed a no-forage diet with blend of essential oil, monensin and virginiamycin. Reference value = 28.90 ng g^{-1} fecal DM. CON = monensin at 30 mg kg^{-1} DM; VM = virginiamycin at 25 mg kg^{-1} DM and monensin at 30 mg kg^{-1} DM; MEO25 = monensin at 30 mg kg^{-1} DM and blend of essential oil at 25 mg kg^{-1} DM; MEO35 = monensin at 30 mg kg^{-1} DM and blend of essential oil at 35 mg kg^{-1} DM; EO35 = blend of essential oil at 35 mg kg^{-1} DM.

Table 2 – Ingestive behavior of beef steers fed a no-forage diet with blend of essential oil associate or not to antibiotics (determined for 12 h).

Activity (h)	Treatments[1]					SEM	*p*-value	Planned contrasts (*p*-value)[2]	
	CON	VM	MEO25	MEO35	EO35			EO35 *vs* MON	VM *vs* EO
Feed bunk	1.73	2.10	1.80	1.83	2.16	0.325	0.841	0.421	0.662
Drinking water	0.39	0.43	0.50	0.43	0.40	0.158	0.991	0.822	0.948
Rumination	0.63	0.96	0.66	0.96	0.76	0.294	0.878	0.899	0.630
Idle stand	4.16	3.80	4.00	3.26	4.90	0.653	0.523	0.151	0.738
Idle lay down	2.76	2.80	3.40	3.50	2.26	0.422	0.259	0.087	0.605
Other activities	2.43	2.03	1.80	2.16	1.66	0.471	0.798	0.411	0.776

[1]Treatments: CON = monensin at 30 mg kg^{-1} DM; VM = virginiamycin at 25 mg kg^{-1} DM and monensin at 30 mg kg^{-1} DM; MEO25 = monensin at 30 mg kg^{-1} DM and blend of essential oil at 25 mg kg^{-1} DM; MEO35 = monensin at 30 mg kg^{-1} DM and blend of essential oil at 35 mg kg^{-1} DM; EO35 = blend of essential oil at 35 mg kg^{-1} DM. [2]Planned contrasts: EO35 *vs* MON (CON; VM; MEO25 and MEO35); VM *vs* EO (MEO25; MEO35 and EO35).

Table 3 – Intake and nutrient digestibility of beef steers fed a no-forage diet with blend of essential oil associate or not to antibiotics.

	Treatments[1]					SEM	*p*-value	Planned contrasts (*p*-value)[2]	
	CON	VM	MEO25	MEO35	EO35			EO35 *vs* MON	VM *vs* EO
Intake[3]									
DM, kg d^{-1}	4.18	3.19	4.81	3.94	5.05	0.800	0.512	0.269	0.143
DM, % BW	1.24	0.94	1.38	1.17	1.40	0.216	0.576	0.372	0.148
OM, kg d^{-1}	3.99	2.83	4.58	3.84	4.55	0.850	0.601	0.445	0.144
CP, kg d^{-1}	0.542	0.343	0.560	0.478	0.605	0.121	0.597	0.369	0.159
CF, kg d^{-1}	0.069	0.056	0.071	0.072	0.064	0.017	0.961	0.874	0.517
NDF, kg d^{-1}	0.627	0.515	0.671	0.643	0.722	0.133	0.854	0.477	0.298
Starch, kg d^{-1}	2.58	1.87	3.03	2.46	2.95	0.554	0.601	0.461	0.156
ME (Mcal d^{-1})	11.73	8.27	13.45	11.75	13.34	2.552	0.614	0.452	0.152
Apparent digestibility (g kg^{-1})									
DM	870.2	865.3	857.7	880.5	842.5	2.48	0.864	0.383	0.884
OM	880.3	823.0	858.5	880.1	852.3	2.58	0.515	0.780	0.187
CP	816.1	712.3	784.6	833.0	779.3	3.67	0.200	0.757	0.048
CF	914.8	903.0	939.4	928.9	901.0	2.55	0.845	0.569	0.586
NDF	659.9	606.5	569.1	639.5	590.9	8.32	0.936	0.762	0.948
Starch	910.5	911.0	901.2	921.7	892.2	2.03	0.874	0.415	0.800

[1]Treatments: CON = monensin at 30 mg kg^{-1} DM; VM = virginiamycin at 25 mg kg^{-1} DM and monensin at 30 mg kg^{-1} DM; MEO25 = monensin at 30 mg kg^{-1} DM and blend of essential oil at 25 mg kg^{-1} DM; MEO35 = monensin at 30 mg kg^{-1} DM and blend of essential oil at 35 mg kg^{-1} DM; EO35 = blend of essential oil at 35 mg kg^{-1} DM. [2]Planned contrasts: EO35 *vs* MON (CON; VM; MEO25 and MEO35); VM *vs* EO (MEO25; MEO35 and EO35). [3]DM = dry matter; OM = organic matter; CP = crude protein; CF = crude fat; NDF = neutral detergent fiber; ME = metabolizable energy.

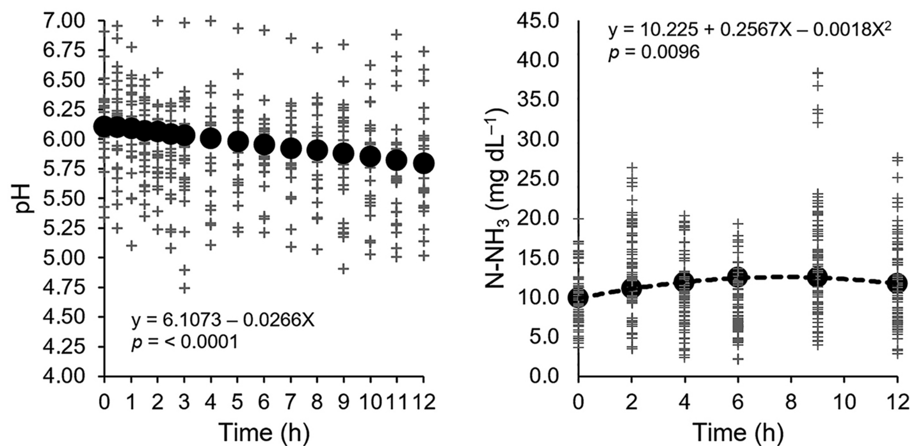

Figure 2 – Ruminal pH and NH_3 during 12 h of beef steers fed a no-forage diet with blend of essential oil, monensin and virginiamycin.

No effects of interaction between treatments and sampling time were observed on ruminal NH_3 (p = 0.765). The additives did not influence the concentration of ruminal NH_3 ($p > 0.05$), whose average was 11.56 mg dL^{-1}, but a quadratic effect of sampling time was observed (Figure 2), where the highest concentration of NH_3 (12.46 mg dL^{-1}) occurred at 9 h after the morning feed.

No effect of additives was observed on the total concentration or the proportion of the SCFA profile (Table 4), except for the proportion of isovalerate, which was observed by a difference between treatments ($p < 0.05$) and contrast between treatments with essential oil and the control treatment ($p = 0.002$).

There was no difference between treatments ($p > 0.05$) regarding the excretion of purine derivatives in urine, microbial nitrogen flow and microbial efficiency (Table 5). Mean values observed for uric acid, allantoin and total purine excretion were 6.78, 90.42 and 97.20 mmol d^{-1}, respectively, whereas the mean of microbial nitrogen flow into the small intestine was 70.67 g N d^{-1} (Table 5).

There was a contrast ($p = 0.040$) between VM treatment and those with the addition of EO for blood *D*-Lactate and *L*-Lactate concentration (Table 5), in which a higher concentration of *D*-Lactate and *L*-Lactate was observed in the blood of animals fed with VM treatment compared with those who had EO.

The blood glucose concentration was higher ($p = 0.002$) at 4 h after the morning feed in all treatments (Figure 3), with an average of 65.93 mg dL^{-1} before feeding and 77.79 mg dL^{-1} 4 h after feeding; however, no effect ($p > 0.05$) attributable to the treatments was observed.

Table 4 – Rumen short-chain fatty acid (SCFA) concentration of beef steers fed a no-forage diet with blend of essential oil associate or not to antibiotics.

	Treatments[1]					SEM	*p*-value	Planned contrasts (*p*-value)[2]	
	CON	VM	MEO25	MEO35	EO35			EO35 *vs* MON	VM *vs* EO
SCFA total (mM)	48.66	43.09	36.26	44.00	48.10	5.910	0.594	0.449	0.399
Acetate, mol 100 mol^{-1}	64.38	64.47	64.78	59.23	59.44	2.672	0.365	0.221	0.308
Propionate, mol 100 mol^{-1}	23.71	21.93	23.16	21.92	27.47	3.135	0.716	0.187	0.897
Butyrate, mol 100 mol^{-1}	9.68	9.88	9.81	15.06	9.89	1.850	0.214	0.565	0.383
Isobutyrate, mol 100 mol^{-1}	0.15	0.34	0.15	0.30	0.38	0.123	0.549	0.295	0.390
Valerate, mol 100 mol^{-1}	1.79	3.03	1.86	3.01	2.16	0.913	0.774	0.797	0.608
Isovalerate, mol 100 mol^{-1}	0.27 b	0.32 ab	0.23 b	0.45 ab	0.63 a	0.081	0.014	0.002	0.082
Acetate to propionate	3.01	3.01	2.88	2.88	2.67	0.472	0.985	0.604	0.721

Means followed by the same letter in each row do not differ statistically by the Tukey test ($p < 0.05$). [1]Treatments: CON = monensin at 30 mg kg^{-1} DM; VM = virginiamycin at 25 mg kg^{-1} DM and monensin at 30 mg kg^{-1} DM; MEO25 = monensin at 30 mg kg^{-1} DM and blend of essential oil at 25 mg kg^{-1} DM; MEO35 = monensin at 30 mg kg^{-1} DM and blend of essential oil at 35 mg kg^{-1} DM; EO35 = blend of essential oil at 35 mg kg^{-1} DM. [2]Planned contrasts: EO35 *vs* MON (CON; VM; MEO25 and MEO35); VM *vs* EO (MEO25; MEO35 and EO35).

Table 5 – Urine purine derivatives, microbial nitrogen flow and blood lactate concentration of beef steers fed a no-forage diet with blend of essential oil associate or not to antibiotics.

	Treatments[1]					SEM	*p*-value	Planned contrasts (*p*-value)[2]	
	CON	VM	MEO25	MEO35	EO35			EO35 *vs* MON	VM *vs* EO
Uric acid (mmol d^{-1})	6.63	5.37	8.51	6.95	6.47	1.63	0.749	0.831	0.316
Allantoin (mmol d^{-1})	89.68	83.62	114.62	84.97	79.21	22.97	0.825	0.591	0.729
Total PD (mmol d^{-1})[3]	96.31	88.99	123.13	91.92	85.68	24.31	0.819	0.602	0.693
Microbial N flow (g N d^{-1})	70.02	64.70	89.52	66.82	62.29	17.67	0.819	0.602	0.693
D-Lactate, (mmol L^{-1})	3.86	5.17	3.32	3.25	3.34	0.740	0.337	0.504	0.040
L-Lactate, (mmol L^{-1})	9.52	12.93	8.12	7.92	8.17	1.910	0.337	0.506	0.040

[1]Treatments: CON = monensin at 30 mg kg^{-1} DM; VM = virginiamycin at 25 mg kg^{-1} DM and monensin at 30 mg kg^{-1} DM; MEO25 = monensin at 30 mg kg^{-1} DM and blend of essential oil at 25 mg kg^{-1} DM; MEO35 = monensin at 30 mg kg^{-1} DM and blend of essential oil at 35 mg kg^{-1} DM; EO35 = blend of essential oil at 35 mg kg^{-1} DM. [2]Planned contrasts: EO35 *vs* MON (CON; VM; MEO25 and MEO35); VM *vs* EO (MEO25; MEO35 and EO35). [3]Total PD = Total purine derivatives.

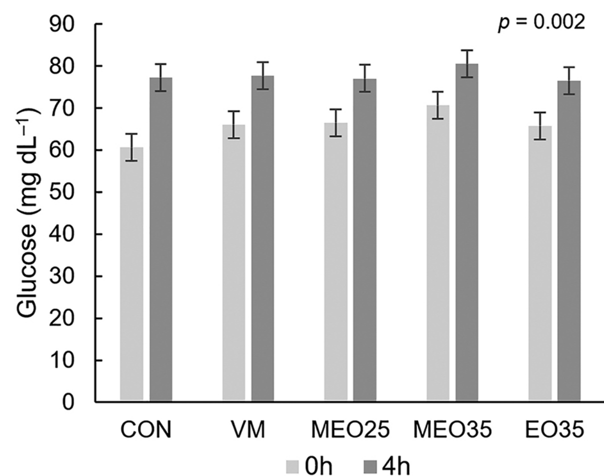

Figure 3 – Blood glucose concentration of beef steers feeding a no-forage diet with blend of essential oil, monensin and virginiamycin. CON = monensin at 30 mg kg^{-1} DM; VM = virginiamycin at 25 mg kg^{-1} DM and monensin at 30 mg kg^{-1} DM; MEO25 = monensin at 30 mg kg^{-1}DM and blend of essential oil at 25 mg kg^{-1} DM; MEO35 = monensin at 30 mg kg–1 DM and blend of essential oil at 35 mg kg^{-1} DM; EO35 = blend of essential oil at 35 mg kg^{-1} DM.

Discussion

The beef cattle industry in Brazil has changed rapidly over the last two decades, with a growing number of cattle finished in feedlots and a rapid increase in the inclusion of grains in the diets. These aspects have demanded more training and technologies to prevent and control digestive disturbances such as rumen acidosis. The use of antibiotics is one of the most important technologies.

Due to the high concentration of rapid fermentation carbohydrates in the experimental diet and its consequent rumen acidification, the steers accessed the feeding bunk less frequently. None of the additive evaluated was able to change feeding behavior which was basically determined by diet characteristics that included chemical and physical aspects.

Since rumination is a critical factor affecting rumen pH, influenced by diet characteristics, specifically its content of physically effective neutral detergent fiber (peNDF), it has been frequently monitored in behavioral studies when animals are fed with grain-based diets. Thus, in this study, the low rumination time observed in the animals (< 1 h) can be explained by the low peNDF amount in the diets, since 86 % of the diet had a particle size of less than 4 mm (Table 1). In connection with this,

Faleiro et al. (2011) observed a reduction in rumination time in heifers fed a diet without barley silage compared to heifers fed a diet with silage.

In a study evaluating the effect of clove and cinnamon essential oil in a diet with a roughage:concentrate ratio of 10:90, Ornaghi et al. (2017) observed that the animals, on average, spent 6 h on food in a 24 h observation period.

Because of the short time spent feeding or ruminating in which the steers of this study passed the time during 12 h of monitoring, the significant activities observed were those related to idle time and other activities not related to eating or drinking, which has been reported in the literature (Iraira et al., 2013).

The addition of a blend of essential oil (cinnamaldehyde and diallyl disulfide) and the combinations with monensin in the diet did not cause changes in dry matter intake, even in animals fed with monensin, which is reported to modulate and to have control over dry matter intake (Duffield et al., 2012). However, monensin was the control treatment in this study since it is being compared to EO. Thus, considering that VM and many products containing EO are not expected to affect dry matter intake negatively, it could be expected that in cases where steers were fed those treatments without monensin the dry matter intake (DMI) would be higher. This was not observed in this study.

The relatively low DMI observed in this study (1.22 % of BW) may be explained differently. Firstly, it could be inferred that animals eat to meet their energy requirements, and as they were fed a high density energy diet, they needed to eat only a small amount of the diet daily. Secondly, because of the low rumen pH, the animals tried to self-regulate the rumen pH by eating less. Thirdly, high starch diets tend to be fermented to a high amount of propionate which, it is suggested, to regulate DMI (Allen et al., 2009).

Furthermore, the additives had no influence on the dry matter digestibility of the diet, which has been corroborated by other studies, whereby the effects of adding essential oils to the diet did not cause changes in the digestibility of organic matter and dry matter (Latack et al., 2021; Meyer et al., 2009; Meschiatti et al., 2019; Ornaghi et al., 2017). Although treatments do not affect starch digestibility, it should be highlighted that the digestibility coefficients observed are in line with the NASEM (2016), which considers starch digestibility to be above 90 %.

In the present study, a combination of virginiamycin and monensin reduced CP digestibility compared to treatments based on EO (p = 0.048), which might be explained by known effects of virginiamycin and monensin in controlling the growth of rumen proteolytic organisms (van Nevel and Demeyer, 1990; Callaway et al., 1997). Given this context, it may have caused a drop in protein degradability in the rumen. This hypothesis was also confirmed by Montano et al. (2015), as they observed a reduction in ruminal protein digestibility when virginiamycin was added to the diet. In contrast, Ives et al. (2002) observed that virginiamycin promoted a protein escape effect from ruminal fermentation and reduced ruminal deaminase activity.

Although it could be expected that a lower ruminal degradability of protein caused by antibiotics could be compensated by a higher intestinal digestibility, which would not tend to cause any differences in protein digestibility in the total gastrointestinal tract. However, considering the rumen is the primary digestive compartment in the ruminants (Van Soest, 1994), protein degradability in the total tract can be depressed significantly, as the intestinal digestibility is unable to compensate for it. This is what seemed to have occurred in this study.

Despite the lower CP digestibility in animals fed a combination of virginiamycin and monensin, attributable to the lower rumen degradability of CP, the ruminal NH_3 concentration did not differ between treatments, which can be considered adequate in the support of microbial growth according to Satter and Slytter (1974).

The concentration of cortisol metabolites in feces has been used to indicate stress in animals. Although there was no difference between treatments, the average fecal cortisol concentration observed in this study (357.61 ng g^{-1} fecal DM) indicated that the animals might have suffered stress due to the metabolic challenge imposed by a no-forage diet. However, in feedlot cattle classified as high and low efficiency animals, fecal cortisol metabolite levels of 32.2 ng g^{-1} and 19.2 ng g^{-1} were observed, respectively, suggesting no stress in either group (Montanholi et al., 2013).

The lack of effect of essential oils on the production of ruminal NH_3 when compared to other antimicrobial additives has been commonly observed (Khorrami et al., 2015; Benchaar, 2016; Meschiatti et al., 2019), although it has been reported that EO can affect the growth of hyper ammonia producing bacteria (*Clostridium sticklandii* and *Peptostreptococcus anaerobius*) and other proteolytic bacteria (*Prevotella ruminicola* and *Ruminobacter amylophilus*) in the rumen (Wallace, 2004). Thus, they may also cause a reduction in proteolysis in the rumen (Wallace, 2004) in the same way as monensin and virginiamycin, which helps to explain the similar results related to NH_3 concentration in rumen fluid among the additives evaluated.

No effect of the evaluated additives was observed on rumen pH, whereas there was a linear drop after morning feeding. The absence of forage in the diet associated with the large amount of rapidly fermentable carbohydrates available in the rumen promoted the linear effect of reducing the pH in the ruminal content, which is mainly related to the low rumination time and saliva production due to the low peNDF that results in low amount of buffer flow into the rumen (Chibisa et al., 2020).

Ruminal pH typically varies over a 24-h period, being influenced by the intake of fermentable carbohydrates and the animal's ability to promote buffering, which includes the absorption of SCFA by the ruminal epithelium, the action of bicarbonate and phosphate ions in saliva, as well as the passage of SCFA through the reticulo-omasal orifice (Nagaraja and Titgemeyer, 2007). Furthermore, the researchers did not observe any differences in the ruminal pH of the cows compared to the control (monensin) when evaluating the same essential oil blend used in the diet of lactating cows (Blanch et al., 2016).

Due to the low intake of nutrients, a low concentration of SCFA was observed in the rumen of the steers in this study, which was not affected by the additives and was below that observed by Chibisa et al. (2020) in beef heifers fed no-forage diets. This low concentration of SCFA can be explained by relatively low DMI, which affects the amount of organic matter to be fermented in the rumen, resulting in the high ruminal pH values observed despite too much starch concentration in the diets (Nagaraja and Titgemeyer, 2007). Low DMI was probably how animals were found to cope with high fermentability in their diets to prevent ruminal acidosis.

Furthermore, the additives evaluated did not affect the SCFA profile, except for isovalerate, which was higher in the rumen of animals fed with EO35 than those fed with monensin or monensin associated with virginiamycin. Isovalerate is produced in the rumen by fermentation of branched-chain amino acid isoleucine (Erfle et al., 1982). Thus, a lower proportion of this SCFA in the rumen fluid of animals fed antibiotics suggests a lower degradation of CP in the rumen than in animals fed EO (Broderick, 2018) since those antibiotics are reported to be effective in controlling the growth of obligate amino acid fermenters (*Clostridium sticklandii* and *Peptostreptococcus anaerobius*) (Wallace, 2004).

The flow of microbial protein to the duodenum depends on the energy and nitrogen availability in the rumen, the presence of minerals and other growth factors (branched-chain fatty acids), as well as adequate chemical conditions (pH, osmolality, etc) (Owens and Basalam, 2016). Thus, considering that urine excretion, microbial nitrogen flow, and microbial efficiency were similar among diets, it could be inferred that the additives evaluated presented similar effects in rumen microbial growth.

Microorganisms in the rumen degrade part of the dietary protein to be used in the metabolism of their own proteins and, consequently, contribute to 50 to 90 % of the metabolizable protein of beef cattle (NASEM, 2016). Thus, the additives used in the diets must maintain adequate levels of microbial protein production so that they do not affect the animal's protein intake. Results regarding differences in microbial protein synthesis between essential oil blends and ionophore and non-ionophore additives had not been observed in studies conducted by Latack et al. (2021) and Meschiatti et al. (2019).

The diet used in the experiment consisted of ground corn with 60 % starch, a non-fibrous carbohydrate that is rapidly degraded in the rumen and converted to microbial cells and SCFA, the major sources of amino acids and energy for ruminants. From SCFA absorbed by the rumen wall, acetate and butyrate are used by animal tissues as an energy source. At the same time, propionate is the liver's primary precursor for glucose synthesis (Elliot, 1980).

The starch escaping from the rumen is digested by enzymes secreted by the animal in the small intestine, resulting in free glucose, which is absorbed by enterocytes and transported to the bloodstream (Huntington, 1997). Thus, after the diet intake, the supplying of starch in the animal's gastrointestinal tract is increased, both in the rumen and the small intestine. This explains the higher concentration of glucose in the blood 4 h after the morning feed. The same blood glucose concentration in dairy cows fed the same essential oil blend was not observed by Blanch et al. (2016).

The higher concentration of *D* and *L*-Lactate in the blood of animals fed VM treatment indicates a higher concentration of lactate in the rumen, which indicates that this treatment was less effective in terms of controlling the growth of lactate-producers in the rumen. However, these additives are known to be able to do this in such types of organisms. In an acidic ruminal environment, the proportion of SCFA found in the protonated form increases and tends to increase its absorption through the rumen epithelium (Shen et al., 2019). Metabolic acidosis by *D*-Lactate in the blood is considered above a concentration of 3 mmol L^{-1} (Uribarri et al., 1998). Thus, it was possible to verify the occurrence of metabolic acidosis by *D*-Lactate, considering that all treatments presented levels above 3 mmol L^{-1} and above those found by Schwaiger et al. (2013). In this case, it could be inferred that none of the additives evaluated were able to prevent ruminal acidosis when a no-forage diet was fed. This indicates that rumen acidosis is a digestive disturbance that should not be considered only by low rumen pH (Hernández et al., 2014).

The energetic concentration of diet with the increase in the inclusion of starch associated with the removal of forage from the diet of feedlot cattle is a feeding strategy that should be implemented with the use of additives that modulate ruminal fermentation associated with good adaptation protocol and bunk management. Although the additives and their combinations promoted ruminal pH control to avoid acute and subacute acidosis, the low dry matter intake observed is a factor that would negatively affect animal performance under practical conditions.

Thus, several factors must be considered when adopting this diet, such as the period and method of adaptation, the additives that modulate the rumen fermentation, and the amount of rapidly degraded carbohydrates in the rumen. The blend of essential oil studied containing cinamaldehyde and dialil disulfide

associated or not with monensin, does not alter the nutritional parameters and the metabolism of beef steers fed a diet without roughage compared to antibiotics testes. More studies are needed to evaluate the effects of the essential oil blend in higher doses for this type of diet.

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Conceptualization: Mota LG, Andrade TS, Cabral LS, Galati RL. **Data curation:** Mota LG, Fonseca ASR, Andrade TS, Cabral LS, Galati RL. **Formal analysis:** Mota LG, Fonseca ASR, Cabral LS, Galati RL. **Funding acquisition:** Andrade TS, Cabral LS, Galati RL. **Investigation:** Mota LG, Fonseca ASR, Cabral LS, Galati RL. **Methodology:** Mota LG, Andrade TS, Cabral LS, Galati RL. **Project administration:** Mota LG, Cabral LS, Galati RL. **Resources:** Cabral LS, Galati RL. **Supervision:** Cabral LS, Galati RL. **Writing-original draft:** Mota LG, Cabral LS, Galati RL. **Writing-review & editing:** Mota LG, Fonseca ASR, Andrade TS, Duff G, Galati RL, Cabral LS.

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