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Assessing the impact of essential oil blends as antibiotic alternatives on nutritional and metabolic parameters in non-forage-based diet-fed Nellore steers

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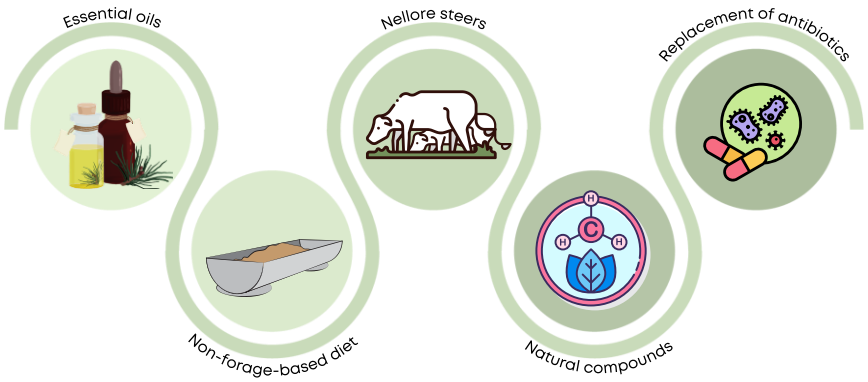
Abstract
Essential oils (EO) are antimicrobials with the potential to replace conventional ones in ruminant diets. Despite the advantage of the EO serving markets that prohibit antibiotics in animal nutrition, the results that prove their effectiveness vary with the type of EO, dose, diet, etc. This study aimed to assess the effects of a blend of essential oil, based on cinnamaldehyde and diallyl disulfide, associated or not with antibiotics on nutritional and metabolic parameters for feedlot Nellore steers fed non-forage-based diet. Five Nellore steers were distributed in a 5×5 simple Latin square design. The treatments assessed were a basal no-roughage diet with the inclusion of monensin (MON), monensin + virginiamycin (MV), monensin + blend of EO (MEO25), monensin + blend of EO (MEO35), and a blend of essential oil (EO). Feed, orts, feces, ruminal fluid, blood, and urine samples were utilized to assess the intake, digestibility, ruminal pH, ruminal NH_3, nitrogen balance, microbial nitrogen synthesis, fecal cortisol metabolites, blood glucose, and D and L-lactate. No difference ($P > 0.05$) has been observed in additives in intake, nutrient digestibility, and ingestive behavior. There was a trend ($P = 0.0553$) of the effect of additives on ruminal pH, and the MV presented the highest value. No difference ($P > 0.05$) for the ruminal NH_3, microbial N synthesis, and nitrogen balance concentration was also verified. The blood glucose and blood lactate concentration were not affected ($P > 0.05$) by additives. The blend of essential oils based on cinnamaldehyde and diallyl disulfide could then be indicated in replacement or association with antibiotics in a non-forage-based diet for Nellore steers.

Keywords: Additives, cinnamaldehyde, diallyl disulfide, monensin, ruminal fermentation, virginiamycin.

Graphical Abstract



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1. Introduction

The growing inclusion of concentrate and improvement in grain processing increased the availability of starch and energy content in Brazilian feedlot diets (Silvestre & Millen, 2021). However, the increased availability of rapidly fermentable substrates can cause an accumulation of organic acids in the rumen, lowering the pH and causing ruminal acidosis (Nagaraja & Titgemeyer, 2007). In this scenario, monensin and virginiamycin are additives used to modulate rumen fermentation in such a way as to prevent the occurrence of acidosis (Goodrich et al., 1984; Coe et al., 1999). On the other hand, public concerns about the use of antibiotics have driven the search for alternative additives to conventional ones.

Essential oils (EO), natural extracts from plants or aromatic herbs, have shown the potential to replace conventional additives due to their antimicrobial activity against a wide variety of microorganisms (Calsamiglia et al., 2007; Cobellis, Trabalza-Marinucci & Yu, 2016). The phytochemicals in EO modulate the microbial population in the rumen by inhibiting sensitive microorganisms, favoring microorganisms resistant to its antimicrobial action (Benchaar et al., 2008). Furthermore, due to the different modes of action of natural antimicrobials, there is an expectation that the combination of two or more EOs may result in beneficial effects in an additive or synergistic way (Calsamiglia et al., 2007).

Looking for efficient products from a nutritional point of view that could replace conventional additives, meet the needs of consumer markets, and contribute to reducing the impact of livestock on climate change, it is necessary to evaluate the effects of these phytochemicals in doses, associations, and breeding systems. In high-concentrate diets, the additive is expected to increase the efficiency of animal energy and protein utilization. Additionally, it mainly helps prevent metabolic disorders without causing adverse effects on the digestibility of nutrients and, consequently, on animal performance.

Given the above, the objective of this work was to evaluate the effects of the inclusion of essential oils (cinnamaldehyde and diallyl disulfide) associated or not with monensin on intake, apparent digestibility of nutrients, ruminal, blood, and hormonal parameters of Nellore steers confined fed non-roughage diets.

2. Materials and Methods

The investigation was conducted at the Universidade Federal de Mato Grosso (UFMT), Cuiabá, Brazil. The Ethical Principles performed in all procedures involving animals in Animal Experimentation were approved by the Ethics Committee on Animal Use (CEUA) of the Universidade Federal de Mato Grosso, protocol number 23108.050556/2020-04.

2.1. Animals, experimental design, and diets

Five Nellore steers (252.6 ± 29.8 kg initial body weight), castrated males, 14 months old, and rumen cannulated, were randomly assigned to five treatments in a simple 5×5 Latin square design. The animals underwent a transition period from a diet with a roughage: concentrate ratio of 60:40 to a diet without roughage, in which the step-up protocol was adopted, with a reduction in roughage by ten percentage points every three days, until its complete replacement. After this stage, the experimental period began with adaptations of 14 days to the treatments, followed by seven collections. One day before each experimental period, the animals were weighed.

Table 1 Ingredients (g kg DM^{-1}), chemical composition (g kg DM^{-1}), and particle size distribution (g kg^{-1}) of the diet fed to Nellore.

Item	
Ingredient, g kg DM^{-1}	
Ground corn	800.0
Cottonseed cake	150.0
Mineral mixture [a]	50.0
Bromatological composition, g kg DM^{-1}	
Dry matter, g kg^{-1}	903.9
Ash	67.1
Organic matter	830.0
Crude protein	138.3
Crude fat	37.4
cNDF [b]	147.3
NDFpe [c]	57.1
Starch	630.1
TDN [d]	691.30
ME [e], Mcal	2.50
Particle size distribution, g kg DM^{-1}	
Particles > 19 mm	0.00
Particles < 19 mm and > 8 mm	76.2
Particles < 8 mm and > 1.18 mm	311.5
Particles < 1.18 mm	612.3

[a] Mineral mixture (assurance levels, g kg^{-1}): Ca, 9 to 12; P, 3.5; Na, 3.0; K, 3.7; S, 1.8; Mg, 1.8; Zn, 0.083; Cr, 0.0003; Mn, 0.029; Cu, 0.020; Co, 0.0014; I, 0.001; Se, 0.00025; Vitamin A, 3.0; Vitamin E, 0.015; [b] neutral detergent fiber corrected for ash; [c] NDFpe neutral detergent fiber physically effective given by multiplying the NDF by the sum of the proportion of particles larger than 1.18 mm; [d] TDN Total digestible nutrients; [e] ME Metabolizable energy.

Treatments consisted of a basal diet without roughage (**Table 1**) containing combinations of additives: ionophore (monensin sodium Bovesin® 200, Phibro, Guarulhos, SP, Brazil), non-ionophore

(virginiamycin Vmax® 2%, Phibro, Guarulhos, SP, Brazil) and phytochemical (an encapsulated blend of essential oils composed of cinnamaldehyde, the main component of cinnamon oil, and diallyl disulfide from garlic oil, Next Enhance® 300, Novus do Brasil Indústria e Comércio, Indaiatuba, SP, Brazil). The basal diet and its mixture with additives were carried out in a feed industry following protocols of good manufacturing practices (Agroquima Produtos Agropecuários, Goiânia, GO, Brazil). The animals were fed twice daily, in the morning and the afternoon, and the amount offered was calculated to allow 5% of leftovers.

The treatments were: MON (monensin sodium at a dose of 30 mg kg DM^{-1}); MV (30 mg kg DM^{-1} of sodium monensin associated with 25 mg kg DM^{-1} of virginiamycin); MEO25 (30 mg kg DM^{-1} of sodium monensin associated with mg kg DM^{-1} of the blend of essential oils); MEO35 (30 mg kg DM^{-1} of sodium monensin associated with 35 mg kg DM^{-1} of the blend of essential oils); EO (35 mg kg DM^{-1} of the essential oil blend). The animals were fed twice, half in the morning and half in the afternoon.

2.2 Intake and digestibility of nutrients

The offered diets and leftovers were weighed daily throughout the experimental period to obtain dry matter and nutrient intakes. For digestibility, from the second to the fourth day of the collection period, fecal samples were collected over 12 hours of observation, directly on the floor of the stalls and immediately after each spontaneous defecation of the animals. Due care was taken to avoid contamination with urine or dirt present on the floor of the stalls. Daily, the collected samples were homogenized, and an aliquot of 200 g of each animal was taken to a circulation and air renewal oven at 55°C for 72 h. After drying, the stool samples were composed by treatment and period and divided into two aliquots for grinding in a knife mill at 1 or 2 mm, depending on the evaluation.

Intakes were obtained from the difference between the number of nutrients offered in the diet and present in the number of leftovers. As these diets have low fiber content, indigestible dry matter (iDM) was used to estimate fecal dry matter excretion and apparent digestibility. For this, samples of diets, leftovers, and feces ground to 2 mm were used, weighed in nonwoven fabric bags (100 $cm^{2\ -1}$), maintaining the ratio of 20 mg DM $cm^{2\ -1}$ (Nocek, 1988), and incubated for 264 h in the rumen of two cannulated cattle (Casali et al., 2008; Valente et al., 2011).

Chemical analysis of dry matter - DM, method 967.06 (AOAC, 1990), ash - CZ, method 942.05 (AOAC, 2002), organic matter - OM, calculated by the difference between 100 and CZ, and crude protein - PB, method 2001.11 (AOAC, 2002) were carried out with modifications proposed by Detmann et al. (2012); factor 6.25 was used to convert the amount of N to CP. For the crude fat (CF), the AOCS Am 05-04 method was followed (AOCS, 2005). Neutral detergent fiber (cNDF) was obtained using the solution described by Van Soest et al. (1991), without the use of sodium sulfite, with the addition of thermostable α-amylase (enzymatic activity 286 KNU-SC/g) and corrected for ash after burning in a muffle furnace. Digestion to obtain NDF took place in an autoclave (0.5 atm for 1 hour) (Pell & Schofield, 1993) and filtration in Gooch crucibles with porous plate number 2. Starch was obtained from the enzymatic method proposed by Zinn (1990) and adapted by Silva et al. (2019). The total digestible nutrients (TDN) of diets were obtained according to the equation proposed by Jayanegara et al. (2019). Digestible energy (DE) was estimated considering that each 1 kg of TDN is equivalent to 4.409 Mcal of digestible energy (DE) and that metabolizable energy (ME) corresponds to 0.82 Mcal of DE (NASEM, 2016).

To characterize the size distribution of the diet particles, a set of three sieves with openings of 19; 8. 1.18 mm and a bottom tray were used. The procedures adopted for sieving were by the recommendations of Lammers, Buckmaster and Heinrichs (1996) and modified by Kononoff, Heinrichs and Buckmaster (2003).

2.3. Ingestive behavior

The ingestive behavior was assessed individually every 10 minutes (Aldrighi et al., 2018) for 12 hours, starting at 7 am. The evaluations were carried out on the first day of each experimental period to avoid changes in the behavior of the animals caused by other collection management. The observed activities were the consumption of dry matter when the animals were in the trough eating feed, rumination, water consumption, and idleness. It was assumed that the activities persisted for 10 minutes. At the end of the evaluations, the time spent on each activity was summed and expressed in minutes day^{-1}.

2.4. Ruminal pH

Ruminal pH was measured various times over 12 hours of observation, always on the fifth day of the collection period. From time zero (feeding), samples of ruminal content were taken every half hour until completing 3 hours of evaluation. After that, they were taken every hour until completing 12 hours, totaling 16 observations per animal and period. To avoid the constant opening of the ruminal cannula, a polyvinyl chloride (PVC) flange (1/2") with a threadable cap to access the rumen was adapted to the cannula cap. This cover was opened during collection times to introduce a flexible hose and a suction pump. Samples were collected from the rumen's caudal, cranial, dorsal, and ventral portions. Ruminal pH measurements occurred after homogenizing the collected content using a digital potentiometer (Tecnal R-TEC-3P-MP, Piracicaba, SP, Brazil). For each treatment and period, the hours that the pH was below 5.8 was observed; 5.5 and 5.2 were indicative of mild acidosis, subclinical acidosis, and clinical acidosis, respectively (Koenig & Beauchemin, 2018; Chibisa et al., 2020).

2.5. Short-chain fatty acids and ruminal ammoniacal nitrogen

Ruminal content samples were obtained at times 0; 2; 4; 6; 9, and 12 hours after the morning feeding on the sixth day of the collection period. The collected content was filtered, and the resulting liquid was centrifuged at 3,000 × *g* for 5 minutes to separate the particles. Approximately 12 mL and 3 mL of the clarified product were stored to analyze ammoniacal nitrogen and short-chain fatty acids (SCFA) and frozen at –20 °C. Ammoniacal nitrogen was quantified by the method of colorimetry of phenol-hypochlorite (Broderick & Kang, 1980). The SCFA, acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate were identified and quantified using a gas chromatograph Agilent 7980A located in Animal Nutrition Laboratory (Universidade Federal de Mato Grosso, Cuiabá, Mato Grosso, Brazil).

2.6. Nitrogen balance and microbial N synthesis

N consumption was obtained in the difference between supplied nutrients and leftovers, and fecal excretion, the amount present feces. For urinary N, spot urine samples were collected from spontaneous urination approximately three hours after the morning feeding on the collection periods of the second, third, and fourth days. Urine was filtered, and 10 mL aliquots were diluted in 40 mL sulfuric acid solution (0.036 N) and frozen at –20 °C (Valadares et al., 1999). Mean daily creatinine excretion was estimated using the equation proposed by Silva et al. (2012). The urinary volume was estimated by dividing the daily creatinine excretion by the creatinine concentration in the spot urine (Silva et al., 2001). For total N excretion, the amount of nitrogen present in feces and urine was added (AOAC, 2002).

Allantoin concentrations in spot urine were analyzed by the colorimetric method of Fujihara et al. (1987), described in detail by Chen and Gomes (1992), and uric acid enzymatic-colorimetric method (Gold Analisa Diagnóstica kit, Belo Horizonte, MG, Brazil). The ruminal synthesis of microbial nitrogen was obtained from the equation by Chen and Gomes (1992), assuming the value of 0.134 for the ratio between N content in microbial N purine derivatives (Valadares et al., 1999).

2.7. Glucose, D- and L-lactate in the blood

Blood samples were collected before and 4 hours after the morning meal, on the seventh day of the collection period, through a puncture with a needle in the middle coccygeal vein to obtain blood glucose. Immediately after collection, respecting a maximum period of 30 seconds (Chenard, 2021), a drop of blood was transferred to a test strip and read on an Accu Check® Active portable glucometer (Roche Diagnóstica Brasil Ltda). The remaining volume was transferred to tubes containing sodium fluoride/EDTA and centrifuged at 3,000 × *g* for 20 minutes. The serum was transferred to Eppendorf tubes and frozen at –20 °C. D-lactate (kit K 002-M) and L-lactate (kit K 044-M) (Elabscience Biotechnology Inc. Houston, Texas, USA) analyses were performed by the colorimetric EIE method at the Hormonal Research Laboratory (Universidade de São Paulo, São Paulo, Brazil).

2.8. Fecal cortisol metabolites

Stool samples were collected for three consecutive days, immediately after each spontaneous defecation, for 12 hours. From each defecation, approximately 20 g were collected, composed per day, and frozen at –20 °C. For the analysis procedure, the feces were thawed, dried at

55 °C for 72 hours, and macerated in a mortar. Next, 0.5 g of dry fecal samples were weighed, adding 3 mL of methanol (MetOH 80% v/v). The tubes were vortexed (1,000 × rpm for 10 minutes) and then centrifuged at 3,000 × rpm for 10 minutes. Two mL of the clarified material were removed, placed in Eppendorf tubes, and kept in an oven at 37 °C until the solvent evaporated. Afterward, 1 mL of methanol PA ACS (MetOH 99.7%) was added to the Eppendorf, the tubes were vortexed again, and the fecal extract was frozen at –20 °C (Gholib et al., 2018; Gholib et al., 2020). The fecal extract was sent to the Hormonal Research Laboratory (University of São Paulo, São Paulo, Brazil), and the technique used was radioimmunoassay (RIA) with a reading by a gamma counter (Wizard, PerkinElmer Inc., Waltham, USA). The values of fecal cortisol metabolites of the animals before the experimental period were used as a reference value when the animals were fed a diet composed of a concentrate: roughage ratio of 70:30.

2.9. Statistical analysis

The variables were analyzed using the Generalized Mixed Linear Model (GLIMMIX) procedure of the SAS® Studio software (University Edition version) according to the statistical model (**Eq. 1**):

$$y_{ijk} = m + l_i + c_j + \tau_k + e_{ijk} \quad (1)$$

Where y_{ijk} refers to the response variable observed in the *i-th* period, *j-th* animal and *k-th* experimental treatment, m is the general mean, and the fixed effect produced by the k-th treatment is represented by τ_k. The *j-th* animal and *i-th* period random effects are represented by l_i and c_j, respectively. The e_{ijk} represents the random experimental error of each of the observations. The experimental units were the animals within the periods.

For the ruminal parameters pH and NH_3 concentrations, and blood glucose, the collection times were included in the model as repeated measures, according to the statistical model (**Eq. 2**):

$$y_{ijkl} = m + l_i + c_j + \tau_k + t_l + (\tau * t)_{kl} + e_{ijkl} \quad (2)$$

Where y_{ijkl} refers to the response variable observed in the *i-th* period, *j-th* animal, *k-th* experimental treatment, and l-th collection time, m is the general mean, and the fixed effect produced by the *k-th* treatment is represented by τ_k. In contrast, t_l represents the fixed model of collection times. $(\tau^*t)_{kl}$ represents the interaction between treatment and collection time. The *j-th* animal and *i-th* period random effects are represented by l_i and c_j, respectively. The e_{ijkl} represents the random experimental error of each of the observations.

Analysis of variance (ANOVA) was performed, and when Tukey's test compared significant means, significance was established as $P < 0.05$. Probabilities between $0.05 \geq P \leq 0.10$ were considered as a trend.

3. Results and Discussion

Intake and apparent digestibility of nutrients did not differ ($P > 0.05$) between additives (**Table 2**), which indicates that supplementation with essential oils did not cause harmful effects on these variables. Even without a statistical difference, the dry matter intake (DMI) was 20 and 50% higher for the EO treatment than the MON and MV treatments. It is recognized that monensin has low acceptability, and this characteristic limit its consumption (Baile et al., 1979). Duffield, Merrill and Bagg (2012), in a meta-analysis of the effects of monensin on feed efficiency, weight gain, and DMI, found that the presence of the ionophore in the diet reduced consumption by 3%.

Silva et al. (2018) found similar results when evaluating the inclusion of additives for feedlot cattle on a high-concentrate diet, with a higher DMI for essential oils compared to the association of monensin with virginiamycin. For the authors, when associating monensin with virginiamycin, the association may modulate rumen fermentation, which results in an improvement in energy efficiency from the increase in the proportion of propionate. Propionate is the liver that stimulates hepatic oxidation, raising the energy status of the animal. However, as a consequence, it inhibits consumption (Allen, 2020). Another possible explanation for the higher consumption of EO is related to their acceptability and attractiveness (Lamag et al., 2021; Renesto et al., 2021).

The effects of supplementation with essential oils on DMI are affected by the type of essential oil (Patra, 2011) and dose used (Khiaosa-Ard & Zebeli, 2013). Yang et al. (2010) observed that the supplementation of increasing doses of cinnamaldehyde to high-grain feedlot cattle affected DMI in a quadratic manner. The lowest dose used by the authors (400 mg day^{-1}) had a negligible effect on DMI and nutrients, while the highest dose (1,600 mg day^{-1}) reduced DMI.

Table 2 Intake (% BW and kg day^{-1}), digestibility (kg DM day^{-1}), and ingestive behavior of non-roughage diets containing essential oils associated or not with antibiotics fed to Nellore.

Variable	Additive					SEM	*P* value
	MON	MV	MEO25	MEO35	EO		
			Intake, % BW				
DM	1.24	1.00	1.45	1.52	1.54	0.23	0.4619
			Intake, kg day^{-1}				
DM	3.29	2.61	3.83	4.11	4.06	0.76	0.5995
OM	2.91	2.37	3.17	3.46	3.34	0.63	0.7525
CP	0.50	0.42	0.54	0.55	0.61	0.10	0.7722
CF	0.16	0.14	0.17	0.19	0.19	0.02	0.4359
cNDF	0.54	0.48	0.58	0.62	0.65	0.11	0.8530
Starch	2.25	1.75	2.49	2.61	2.37	0.47	0.7363
TDN	2.45	1.95	2.64	2.89	2.77	0.51	0.7247
ME (Mcal day^{-1})	8.70	7.06	9.53	10.44	10.02	1.85	0.7225
			Digestibility, g kg DM^{-1}				
DM	635.9	659.3	636.2	670.1	654.2	15.05	0.4471
OM	662.2	690.7	642.8	694.7	656.9	22.80	0.4304
CP	608.3	649.5	622.3	623.3	640.8	18.10	0.5277
CF	880.4	901.1	896.8	890.3	882.2	17.90	0.8799
Starch	812.6	805.8	823.3	827.5	800.0	2.96	0.9550
			Ingestive behavior min/diurnal [a]				
Intake	90	102	106	112	80	22.29	0.8517
Rumination	58	32	84	64	86	16.15	0.2273
Idleness	566	584	530	532	566	24.09	0.4494
Water intake	16	10	10	22	14	5.02	0.4386

MON, monensin; MV, monensin associated with virginiamycin; MEO25, monensin associated with 25 mg kg DM^{-1} of essential oils; MEO35, monensin associated with 35 mg kg DM^{-1} of essential oils; EO, essential oils; [a] Ingestive behavior corresponding to 12 hours of observation; BW, body weight; SEM, standard error of the mean.

In the case of digestibility, studies show that the effects of cinnamaldehyde and garlic oil (diallyl disulfide) are also dose-dependent (Yang et al., 2010; Gholipour et al., 2016). At high doses of garlic oils (10 g kg DM^{-1}) and above 1,600 mg animal day^{-1} of cinnamaldehyde digestibility was impaired, probably due to changes in the ruminal microbiota (Yang et al., 2010; Gholipour et al., 2016). Blanch et al. (2016), when evaluating the same essential oil used in this work, found that at a dose of 400 mg L^{-1} of inoculum, there was a decrease in the total production of SCFA and fermented organic matter, which indicates some inhibition of rumen fermentation.

Even though the diets with EO showed higher intakes, the additives generally did not change the ingestive behavior (**Table 2**). However, the time spent ruminating by the animals that received the OE and MON treatments was 168.75 and 81.25 % higher ($P < 0.05$) than those that received MV, which may be due to the lower DMI presented by the MV treatment (**Table 2**). In addition, with the increase in the inclusion of concentrate, the time spent consuming food and ruminating decreases linearly, while the time spent idling increases (Bürger et al., 2000). In a similar study to this work, Heker Junior et al. (2018) found no difference in the ingestive behavior of cattle fed a diet containing 50% corn silage and 50% concentrate with monensin associated or not with virginiamycin and essential oils. The animals also spent less time with activities related to ingestion and more leisure time.

Table 3 Ruminal pH values of Nellore fed non-roughage diets containing essential oils with or without antibiotics.

Variable	Additive					SEM	*P* value
	MON	MV	MEO25	MEO35	EO		
Ruminal pH	6.11	6.32	5.86	5.98	6.09	0.10	0.0553
			Duration, h/diurnal^{-1}				
pH < 5.8	3.4	3.5	5.3	3.2	2.7	1.73	0.8429
pH < 5.5	1.6	0.8	1.5	2.6	2.0	1.21	0.8741
pH < 5.2	0.4	0.6	0.5	0.9	1.3	0.64	0.8604

MON, monensin; MV, monensin associated with virginiamycin; MEO25, monensin associated with 25 mg kg DM^{-1} of essential oils; MEO5, monensin associated with 35 mg kg DM^{-1} of essential oils; EO, essential oils; [1] h/diurnal corresponding to 12 hours of observation.

The ruminal pH of the MV treatment tended ($P = 0.0553$) to be higher even though the animals spent less time ruminating, which may be associated with the fact that animals in this treatment have lower DMI and, therefore, less substrate for ruminal fermentation (**Table 3**). Rumination stimulates the secretion of saliva, which in turn, in the rumen, acts to neutralize the acids produced by fermentation, which helps maintain ruminal pH (Dijkstra et al., 2012). The main factor determining the ruminal pH is the balance between the production of acids from ruminal fermentation and the secretion of buffers (Allen, 1997). For the diagnosis of ruminal acidosis, it is considered the duration with which the pH remains

below 5.8, 5.6, and 5.2 for 5 to 8 hours (Oetzel, 2000; Alzahal et al., 2007).

In this work, when considering the duration of the pH below the limit values reported in the literature, there is no indication of ruminal acidosis (**Table 3**). The theory would be the adaptation to ruminal fermentation conditions since additives with a modulating effect were associated with the diet. According to Dong et al. (2013), high-concentrate diets supplied for long periods have low pH values and diagnosis of subclinical acidosis only in the first two weeks of supply. However, from the fourth to the eighth week of evaluation, the observed ruminal pH value was above 6.00, indicating the ability of the rumen to adapt. In this work, the animals received the same basal diet, and only the additives and their associations alternated between periods. This shows that regardless of the use of ionophore, non-ionophores, essential oil, or their associations, all of them were able to prevent ruminal acidosis.

In the current study, the additives did not affect ($P > 0.05$) the total and individual concentrations of SCFA (**Table 4**). Consistent with these results, in the *in vivo* experiment by Blanch et al. (2016), no differences were observed in the total concentration and profile of SCFA between the control treatment and the treatment with EO based on cinnamaldehyde and diallyl disulfide. However, the EO treatment tended to present higher total concentrations of SCFA. On the other hand, in the *in vitro* experiments by Blach et al. (2016), the dose of EO affected the concentration of SCFA; at the dose of 300 mg/L, the proportion of propionate was increased without changing the total production of SCFA while at the highest dose tested by the authors (400 mg L^{-1}), the production total SCFA reduced showing a negative effect.

Expected changes in SCFA profiles are associated with the antimicrobial effect of antibiotics or photogenic in favoring the growth of specific microbial populations. However, it is necessary to note that substrate availability for fermentation is essential for producing SCFA. In this work, the low DMI masked the possible effects of different antimicrobials and their associations in changing the SCFA profile.

Table 4 Concentration and molar ratio of SCFA in the ruminal fluid of Nellore fed non-roughage diets containing essential oils with or without antibiotics.

Variable	Additive					SEM	*P* value
	MON	MV	MEO25	MEO35	EO		
			mmol				
Acetate	28.49	26.18	23.93	27.73	30.65	3.96	0.8034
Propionate	12.50	9.33	10.23	15.17	16.82	2.59	0.2389
Butyrate	7.19	5.17	4.15	5.32	5.41	1.13	0.4600
Isobutyrate	0.25	0.29	0.20	0.22	0.20	0.05	0.7182
Valeric	1.14	1.34	1.05	0.99	1.23	0.30	0.9260
Isovaleric	0.88	0.44	0.37	0.44	0.50	0.20	0.4122
Total SCFA	50.45	42.75	39.93	49.87	54.81	6.73	0.5298
			mmol/100 mmol				
Acetate	56.14	60.52	60.03	56.52	55.50	1.62	0.1203
Propionate	25.76	22.28	25.76	28.18	31.40	2.59	0.1907
Butyrate	13.44	12.31	10.25	11.75	9.68	1.45	0.3784
Isobutyrate	0.53	0.65	0.50	0.47	0.37	0.09	0.3064
Valeric	2.58	3.22	2.60	2.30	2.15	0.60	0.7606
Isovaleric	1.56	1.02	0.85	0.78	0.90	0.24	0.1869
Acetate:Propionate	2.24	2.77	2.27	2.12	1.98	0.28	0.3523

MON, monensin; MV, monensin associated with virginiamycin; MEO25, monensin associated with 25 mg kg DM^{-1} of essential oils; MEO35, monensin associated with 35 mg kg DM^{-1} of essential oils; EO, essential oils.

There was no difference ($P > 0.05$) between additives for ruminal NH_3, microbial N synthesis, and nitrogen balance (**Table 5**). The effects of the additives on nitrogen metabolism were similar, which was desired as it indicates that the EO did not cause a harmful effect. It is known that monensin decreases amino acid degradation and ammonia accumulation in the rumen (Ogunade et al., 2018). Reports in the literature suggest that the effects of essential oils (EO) on the nitrogen mechanism are similar to those of monensin, meaning that the phytochemical inhibits certain ammonia-producing bacteria (*Clostridium sticklandii* and *Peptostreptococcus anaerobius*) (McIntosh et al., 2003). Macheboeuf et al. (2008), when testing the activity of EO and its components on ruminal fermentation, found that phytochemicals decreased ruminal NH_3 production and that among the EO tested, cinnamaldehyde had the greatest effect in decreasing molar concentrations of ammoniacal nitrogen. Regarding the nitrogen balance, as in this work, Benchaar, Duynisveld and Charmley (2006) found no difference in monensin or

EO. In contrast, Wanapat, Kang and Polyorach (2013) found that supplementation with EO increased nitrogen balance (N absorption and retention) compared to a diet without additives.

Table 5 Ruminal ammoniacal nitrogen concentrations and microbial N synthesis of Nellore fed non-roughage diets containing essential oils with or without antibiotics.

Variable	Additive					SEM	*P* value
	MON	MV	MEO25	MEO35	EO		
			mg dL^{-1}				
Ruminal NH_3	11.16	9.94	10.58	11.80	11.51	1.45	0.8331
			mmol day^{-1}				
Allantoin	90.91	93.25	112.46	120.56	109.28	16.02	0.6431
Uric acid	7.02	6.37	7.73	9.06	6.73	1.56	0.7606
			g N day^{-1}				
Microbial N	71.20	72.43	87.38	94.24	84.35	12.49	0.6477
Microbial N kg DOM^{-1}	94.42	93.53	117.66	119.27	118.96	15.71	0.5765
			g N day^{-1}				
N intake	80.65	67.56	87.18	87.94	98.06	16.76	0.7718
N excretion							
Feces	32.17	26.42	33.69	32.36	24.37	6.17	0.8915
Urine	8.88	8.87	9.61	13.55	9.98	2.74	0.7427
Total N excretion	41.05	33.52	43.29	45.91	44.73	6.93	0.7328
N digestibility	48.49	41.14	53.49	55.58	63.31	10.95	0.6887
N withheld	39.60	34.05	43.88	42.03	53.33	10.96	0.7961

MON, monensin; MV, monensin associated with virginiamycin; MEO25, monensin associated with 25 mg kg DM^{-1} of essential oils; MEO35, monensin associated with 35 mg kg DM^{-1} of essential oils; EO, essential oils.

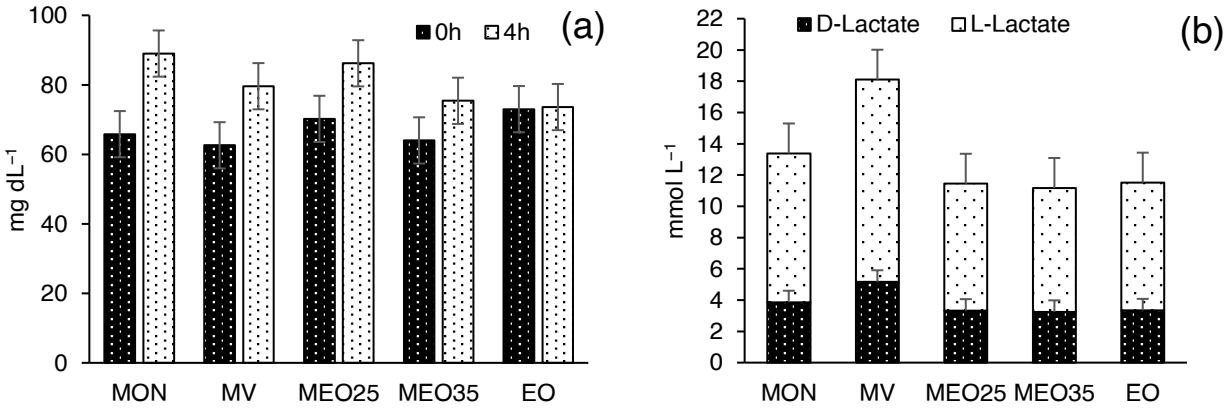

Fig. 1 (a) Blood glucose concentrations at 0 and 4 hours after feeding ($P = 0.8062$), and (b) serum D- and L-lactate ($P = 0.3368$ for D-lactate and $P = 0.3369$ for L-lactate) of Nellore fed non-roughage diets containing essential oils with or without antibiotics.

The lack of effect on protein metabolism between treatments may be related to the absence of a control treatment without the inclusion of additives. However, a non-additive treatment was not considered, given the challenging characteristics of the basal diet, which was characterized by high starch participation and low physically effective fiber.

The blood glucose concentrations found in this work indicate that the energy status was not different ($P > 0.05$) between the additives (**Fig. 1**). Average blood glucose concentrations for ruminants range from 50 to 70 mg dL^{-1} (Gonzáles, 2000). However, when fed a high-concentrate diet, values can exceed 70 mg dL^{-1}. For example, Aferri et al. (2019) found 129.23 mg dL^{-1} of blood glucose in Nellore cattle confined with 800 g kg DM^{-1} of concentrate in the diet. Furthermore, glycemia in cattle is higher in the presence of additives that modulate ruminal fermentation, which increases propionate production, the primary glucose precursor in the liver (Duffield, Merrill & Bagg, 2012). When feeding heifers with high-concentrate diets, Yang et al. (2010) found that different doses of cinnamaldehyde did not affect the animals' blood glucose.

Although there was no statistical difference ($P > 0.05$), the MV treatment showed higher blood D and L-lactate concentrations (**Fig. 1**), which may also help explain the lower DMI found for this treatment since there is a negative correlation between these variables (Foote et al., 2016). Blood D-lactate concentrations greater than 3 mmol L^{-1} indicate

metabolic acidosis (Uribarri, Oh & Carroll, 1998), which in turn is indicative of clinical ruminal acidosis (Nagaraja & Titgemeyer, 2007). The accumulation of lactate in the rumen is caused by the fermentation of rapidly fermentable substrates associated with a decrease in ruminal pH since lactate-consuming bacteria, responsible for removing acid from the medium, are sensitive to pH below 5.5 at the same time as lactate-consuming bacteria. Lactate-producing bacteria are resistant (Nagaraja & Lechtenberg, 2007). The blood lactate concentrations in this study are higher than those reported in the literature in clinical and subclinical ruminal acidosis induction conditions (Muir et al., 1981; Schwaiger, Beauchemin & Penner, 2013; Danscher et al., 2015).

Therefore, considering the nutritional challenge imposed in this study and the absence of ruminal pH values indicating ruminal acidosis, the elevated blood lactate concentrations suggest that the cattle were likely affected by cecal acidosis. Furthermore, the flow of carbohydrates to the intestines increases in diets with a higher proportion of concentrate and less roughage (Gressley, Hall & Armentano, 2011). In this work, the diets consisted of finely ground corn (800 g kg DM^{-1}) and cottonseed cake (150 g kg DM^{-1}), with 650 g kg DM^{-1} of starch and only 57.5 g kg DM^{-1} of NDFfe (**Table 1**). In addition to the large proportion of concentrate, the particle size of corn contributes to a decrease in ruminal starch retention and an increase in the intestinal flow of the nutrient, which results in a greater risk of cecal acidosis (Rémond et al., 2004; Gressley, Hall & Armentano, 2011) Another indication found to support the hypothesis that the animals were affected by cecal acidosis was the presence of mucus in the feces (Gressley, Hall & Armentano, 2011).

Although the concentrations of fecal cortisol metabolites did not differ between the additives ($P > 0.05$), they were higher compared to the reference value obtained when the animals received a diet with a concentrate-to-roughage ratio of 70:30 (**Fig. 2**). Dong et al. (2013) observed that in high-concentrate diets supplied for long periods, despite a transient drop in pH, preceded by an increase to values above 6.00, cortisol concentrations remain high, indicating stress associated with a diet containing a higher proportion of foods concentrated. Even though the ruminal epithelium has adapted to high-concentrate diets (Steele et al., 2011; Dong et al., 2013), the high concentrations of blood lactate and fecal cortisol metabolites indicate that cattle were affected by an associated inflammatory process to cecal acidosis, resulting in nutritional stress. His response was more pronounced in the treatment with the inclusion of MV, which exhibited higher blood lactate concentrations compared to diets with the inclusion of EO.

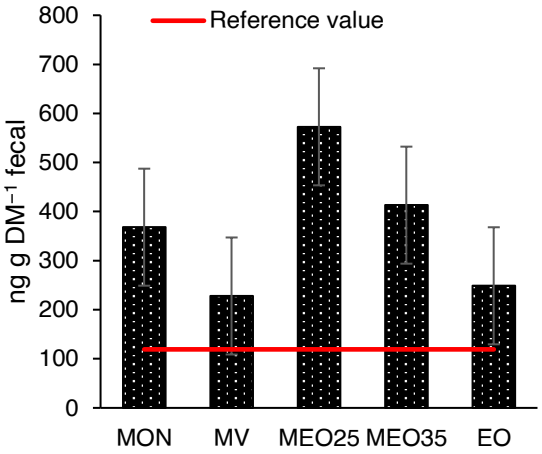

Fig. 2 Fecal cortisol metabolite concentrations ($P = 0.2279$) of Nellore fed non-roughage diets containing essential oils with or without antibiotics.

In summary, supplementation with cinnamaldehyde and diallyl disulfide essential oils in non-forage-based diets for Nellore steers did not have negative effects on nutrient intake and digestibility. These essential oils, particularly cinnamaldehyde, increased dry matter intake compared to MON and MV treatments, indicating improved acceptability. The impact of essential oil supplementation on intake depends on the type and dose of oil, with higher doses potentially reducing intake. Essential oils did not significantly affect ruminal pH, ammonia levels, microbial nitrogen synthesis, nitrogen balance, or short-chain fatty acid concentrations. Blood glucose levels were also unaffected, suggesting no impact on energy status.

However, the MV treatment showed higher blood lactate concentrations, suggesting a potential risk of cecal acidosis compared to essential oil treatments. Elevated lactate and fecal cortisol metabolite levels across all treatments indicate a possible inflammatory response due to cecal acidosis and nutritional stress from the high concentrate content of the diets. In conclusion, cinnamaldehyde and diallyl disulfide essential oils can be considered potential alternatives, along with antibiotics, in non-forage-based diets for Nellore steers, without negatively affecting nutrient utilization or metabolic

parameters. Further research is needed to determine the optimal dosages and effects of different essential oils on rumen fermentation and animal performance.

4. Conclusion

In conclusion, supplementation with essential oils, specifically cinnamaldehyde and diallyl disulfide, in non-forage-based diets for Nellore steers did not have harmful effects on nutrient intake and digestibility. Essential oils, particularly cinnamaldehyde, showed improved acceptability and attractiveness, resulting in higher dry matter intake compared to monensin and monensin + virginiamycin treatments. The effects of essential oil supplementation on intake are influenced by the type and dose of essential oil, with higher doses potentially reducing intake. Essential oils did not significantly affect ruminal pH, ruminal ammonia, microbial nitrogen synthesis, nitrogen balance, and short-chain fatty acid concentrations. Blood glucose concentrations were not affected by essential oil supplementation, indicating no impact on energy status. However, the monensin + virginiamycin treatment showed higher blood lactate concentrations, suggesting a potential risk of cecal acidosis compared to essential oil treatments. The high concentrations of blood lactate and fecal cortisol metabolites in all treatments indicate a possible inflammatory response associated with cecal acidosis and nutritional stress due to the high concentrate content of the diets. Overall, essential oils based on cinnamaldehyde and diallyl disulfide can be considered potential alternatives or in combination with antibiotics in non-forage-based diets for Nellore steers without adversely affecting nutrient utilization or metabolic parameters. Further research is needed to explore the optimal doses and effects of different essential oils on rumen fermentation and animal performance.

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Authors' Contributions

Conceptualization, A. S. R. F., L. G. M., T. S. A., L. S. C., and R. L. G. Investigation, A. S. R. F. and L. G. M. Formal Analysis, M. L. C. A., A. S. R. F., and R. L. G. Supervision, L. S. C., and R. L. G. Writing – Original Draft, A. S. R. F., and R. L. G. All authors read and approved the final manuscript.

Availability of data and materials

Not applicable.

Ethics approval

The Ethical Principles performed in all procedures involving animals in Animal Experimentation approved by the Ethics Committee on Animal Use (CEUA) of the Universidade Federal de Mato Grosso, protocol number 23108.050556/2020-04.

Competing interests

The authors declare that they have no competing interests.

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