FULL ARTICLE

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Brazilian red pepper fruit essential oil (*Schinus terebinthifolius*) may replace monensin in high concentrate diets for feedlot lambs

Óleo essencial dos frutos de aroeira (Schinus terebinthifolius) pode substituir a monensina em dietas com elevado teor de concentrado para cordeiros confinados

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ABSTRACT
Essential oil (EO) from Brazilian red pepper fruit contains compounds with antimicrobial activity, and could be possible substitutes for the antibiotics commonly used in ruminant nutrition. The objectives of the present study were to evaluate the effects of the Brazilian red pepper fruit EO (*Schinus terebinthifolius*) as a substitute for monensin on performance, carcass characteristics and meat of lambs fed high concentrate diets. Forty-eight lambs were used, 24 males (20 ½ Dorper × ½ Santa Inês and 4 Santa Inês) and 24 females (24 ½ Dorper × ½ Santa Inês), with 21.54 ± 0.88 kg of initial body weight (BW) and 78 ± 2.4 days of age, in a randomized complete block design. The experiment lasted 56 days, divided into 2 periods of 28 days each. The treatments were defined by the inclusion in diets of 8 ppm of monensin (MON), and the doses 0.14% (14EO), 0.28% (28EO) and 0.42% (42EO) of red pepper fruit EO. The additives were included in a base diet with a 10:90 of forage to concentrate ratio. At the end of 56 days, 32 animals were slaughtered for the measurement of carcass parameters and meat composition. There was no interaction among treatments and periods for average daily gain ($P = 0.08$), DM intake ($P = 0.36$), feed efficiency ($P = 0.24$) and oocyst of *Eimeria* ssp. in feces ($P = 0.46$). The treatments did not affect ($P > 0.05$) the average daily gain (ADG), dry matter intake (DMI) and feed efficiency. Lambs fed diets containing monensin had less ($P < 0.01$) oocyst/g compared with the diet 14EO. There was no effect of diets on carcass characteristics. The treatments with higher doses of the Brazilian red pepper fruit EO had reduced mineral content of meat compared to monensin. The red pepper fruit EO demonstrated the potential to replace monensin in feedlot lambs fed high concentrate diets, maintaining performance and carcass characteristics. However, the monensin has greater capacity to control coccidiosis in feedlot lambs.

Keywords: Additive. Vegetal extracts. Performance. Carcass characteristic.

RESUMO
Os óleos essenciais (OE) dos frutos de aroeira possuem compostos com atividade antimicrobiana, sendo possíveis substitutos aos antibióticos comumente utilizados na nutrição de ruminantes. Os objetivos do presente estudo foram avaliar os efeitos da inclusão do óleo essencial de aroeira fruta (*Schinus terebinthifolius*) como substituto da monensina sobre o desempenho, características de carcaça e da carne de cordeiros alimentados com dietas contendo elevado teor de concentrado. Foram utilizados 48 cordeiros, 24 machos (20 ½ Dorper × ½ Santa Inês e 4 Santa Inês) e 24 fêmeas (24 ½ Dorper × ½ Santa Inês), com peso inicial de 21,54 ± 0,88 kg e 78 ± 2,4 dias de idade, em delineamento de blocos completos casualizados. O experimento teve duração de 56 dias, divididos em 2 períodos de 28 dias cada. Os tratamentos foram definidos pela inclusão na dieta de 8 ppm de monensina sódica (MON) e as doses de 0,14% (14EO), 0,28% (28EO) e 0,42% (42EO) de óleo essencial dos frutos da aroeira. As dietas experimentais foram compostas por 10% de volumoso e 90% de concentrado. Ao final dos 56 dias, 32 animais foram abatidos para a mensuração dos parâmetros de carcaça e análise química da carne. Não houve interação entre tratamento e período para o ganho médio diário ($P = 0,08$), consumo de MS ($P = 0,36$), eficiência alimentar ($P = 0,24$) e contagem de oocistos de *Eimeria* ssp. ($P = 0,46$). Não houve efeito ($P > 0,05$) dos tratamentos no ganho de peso médio diário (GMD), consumo de matéria seca (CMS) e eficiência alimentar (EA). Cordeiros alimentados com dietas contendo monensina tiveram menor ($P < 0,01$) contagem de oocistos/g de fezes comparado com a dieta 14OE. Não houve efeito das dietas sobre as características de carcaça.

A inclusão de 0,28 e 0,42% de OE de aroeira fruto reduziram a concentração de matéria mineral da carne dos cordeiros comparados ao tratamento MON. O OE dos frutos da aroeira demonstrou capacidade de substituir a monensina, apresentando resultados similares com relação ao desempenho e características de carcaça. Entretanto, a monensina apresentou maior capacidade no controle de coccidiose.

Palavras-chave: Aditivos. Extratos vegetais. Desempenho. Características de carcaça.

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Introduction

The adequacy of diets for the nutritional requirements of food-production animals and the use of feed additives that modify the ruminal environment are common methods used to increase energy retention (Perna et al., 2017) and protein utilization by ruminants (Del Valle et al., 2017). The most used feed additives in animal nutrition are ionophores antibiotics, non-ionophores antibiotics and probiotics (Nicodemo, 2001).

In recent years, public concern about the continued use of antibiotics in animal nutrition has increased due to the possible emergence of pathogenic microorganisms resistant to antibiotics or to active principles, which may present a risk to human health (Benchaar et al., 2006). In this context, there has been increased interest in evaluating alternatives to modulate ruminal fermentation, including the use of organic acids, plant extracts and antibodies (Calsamiglia et al., 2006). The essential oils (EO) may be an alternative feed additive in ruminant nutrition since they have antimicrobial, antifungal, antiviral, antiparasitic, insecticidal, antiprotozoal and antioxidant effects (Cowan, 1999).

Volatile chemical compounds present in EO are limonene, α-pinene, β-carophylenne, p-cymene, α-humulene, and others (Burt, 2004). The Brazilian red pepper (*Schinus terebinthifolius* Raddi, Anacardiaceae) exhibits antifungal properties (Johann et al., 2010), antimicrobial activity (Lima et al., 2006) and the ability to change fermentative parameters *in vitro* and *in vivo*, having the α-pinene as the main constituent (Araújo, 2010; Faleiro Neto, 2015).

Therefore, it has been hypothesized that Brazilian red pepper fruit EO could replace the monensin in feedlot lambs as a feed additive. The objectives of the present study were to evaluate the effects of Brazilian red pepper fruit EO as a substitute for monensin on performance, occurrence of coccidiosis by *Eimeria* ssp., carcass characteristics and meat of lambs fed high concentrate diets.

Materials and Methods

Forty-eight lambs were used, 24 males (20 ½ Dorper × ½ Santa Inês and 4 Santa Inês) and 24 females (24 ½ Dorper × ½ Santa Inês), with 21.54 ± 0.88 kg of initial body weight (BW) and 78 ± 2.4 days of age.

The experiment consisted of a 56-d period, divided into two 28-d periods. The animals were weighed after 14 h of solid fasting period on d 0, 28 and 56 of the trial period. The experiment design used was randomized complete block. Each block was defined by sex, breed and initial BW. The lambs were kept indoors, in an individual tie-stall system, with a slatted floor, feed bunk, and waterer. On d 28 and 56, feces samples were collected to evaluate the coccidiosis oocyst *Eimeria* ssp. (COC), using the method described by Gordon & Whitlock (1939).

The red pepper fruit EO composition was performed by gas chromatography coupled to mass spectrometry provided with an automatic injector, using a capillary column with a 30 meters length and 25 mm internal diameter. The total chromatographic run time was 60 min, divided into five heating cycles, as follows: 50 °C (30 min), 200 °C (4 °C/min), 240 °C (10 °C/min), 280 °C (10 °C/min), and 290 °C (5°C/min).

The experimental diets were defined by the addition of monensin (Rumensin 200, Elanco Animal Health, São Paulo, SP, Brazil), or doses of red pepper fruit EO (Lazlo Aromaterapia, Belo Horizonte, MG, Brazil). The experimental diets were as follows: inclusion of 8 mg of monensin/kg of DM (M); diets with 0.14% (14EO), 0.28% (28EO) and 0.42% of red pepper fruit EO (42EO). The additives were included in a basal diet with a 10:90 of forage to concentrate ratio (Table 1). The experimental diets were formulated

Table 1 – Proportion of the ingredients and chemical composition of the experimental diets containing monensin or red pepper fruit EO (Piracicaba, 2019)

Item	Diets[1]			
	MON	14EO	28EO	42EO
Ingredient, % DM				
Coastcross hay	10.00	10.00	10.00	10.00
Ground corn	72.00	71.86	71.72	71.58
Soybean meal	14.00	14.00	14.00	14.00
Urea	0.50	0.50	0.50	0.50
Mineral premix	1.50	1.50	1.50	1.50
Ammonium chloride	0.50	0.50	0.50	0.50
Limestone	1.50	1.50	1.50	1.50
Red pepper fruit EO	0.00	0.14	0.28	0.42
Monensin, ppm	8.00	0.00	0.00	0.00
Chemical composition[2]				
DM, as-fed basis	88.19	87.93	87.92	87.82
Ash	6.33	6.49	6.24	6.53
CP	19.02	19.26	19.32	19.25
NDF	18.10	18.39	18.17	18.75
ADF	7.03	6.97	6.93	7.15
EE	4.26	4.30	4.32	4.31
NFC	52.29	51.56	51.95	51.16
TDN[3]	80.60	78.15	79.49	79.46

[1]MON: diet containing 8 mg of monensin/kg of DM; 14EO: inclusion of 0.14% of red pepper fruit EO (DM basis); 28EO: inclusion of 0.28% of red pepper fruit EO; 42EO: inclusion of 0.42% of red pepper fruit EO. [2]DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; EE: Ether extract; NFC: non-fiber carbohydrate; [3]TDN: total digestible nutrient (Weiss et al., 1992).

according to National Research Council (National Research Council, 2007).

The total mixed diets were offered once a day at 08:00 a.m. and animals were allowed *ad libitum* access to feed. The experimental diets were prepared weekly to avoid possible changes in the compounds present in the EO and, at each mixture, a sample was collected and stored at -18 °C for further analysis. Orts were recorded daily to determine the dry matter intake (DMI), and the refused feed did not exceed 5% of daily offer. In each experimental period (28d), average daily gain (ADG) and feed efficiency (FE) were calculated.

The DM and ash content were determined according to Association of the Official Analytical Chemists (1990). Sequential detergent fiber analyses were determined according to Van Soest et al. (1991) using an Ankon 2000 fiber analyzer (Ankom Tech. Corp., Fairport, NY, USA). Total N was determined using the Leco TruMac N (Leco Corp., St. Joseph, MI, USA) according to the Association of the Official Analytical Chemists (1990). The ether extract of the diets was determined according to the Association of the Official Analytical Chemists (1990). Non-fiber carbohydrates of the diets were estimated according to the following equation: NFC (%) = 100% - (% NDF + %CP + % fat + % ash). The total digestible nutrient (TDN) content was calculated according to Weiss et al. (1992).

At the end of the 56 days, 32 lambs (16 male and 16 female) were slaughtered, following the norms described in the Regulation of the Industrial and Sanitary Inspection of Products of Animal Origin - RIISPOA. The lambs were weighed after 14 h fasting period to obtain the slaughter weight (SW). Carcass characteristics evaluated were hot carcass weight (HCW) and hot carcass yield (HCY), obtained at the time of slaughter. After 24 h of chilling (4°C), the chilled carcass weight (CCW), chilled carcass yield (CCY), subcutaneous fat thickness over the 12^{th} rib (SFT), body wall thickness (BWT), and Longissimus muscle area (LM area) were obtained. Approximately 15 cm samples of the *Longissimus dorsi* muscle from the right half carcass of each animal were collected and stored at -18 °C for further determination of the chemical composition of the meat. The DM, ash, total N and ether extract content of the *Longissimus dorsi* meat were determined according to Association of the Official Analytical Chemists (1990).

Statistical analyses were performed using the MIXED procedure of the SAS (SAS version 9.0; SAS Inst. Inc., Cary, NC, USA). All data were submitted to the Levene test to verify the homogeneity of variances, the Shapiro-Wilk test to check the normality of the residuals and the removal of "outliers". The animals were considered as the experimental unit to perform statistical analyses. The treatment means were obtained by the LSMEANS command. The treatment effect was defined by Tukey test. The period effect and treatment x period interaction were defined by the *F* test of ANOVA. Statistical significance was declared at $P \leq 0.05$, with trends noted at $P > 0.05$ to $P < 0.10$.

Results

The main components found in the red pepper fruit EO used in the present study were α-pinene (40.4%), α-limonene (13.1%), Δ-3-carene (8.3%), β-pinene (7.0%), *p*-cymene (7.0%), myrcene (5.6%), α-phellandrene (3.5%). In addition, the compounds sabinene, Δ-cadinene, β-phellandrene, β-caryophyllene, germacrene D, terpinen-4-ol, α-copaene, α-terpinolene, δ-terpinene, camphene and α-terpinene were identified in the red pepper fruit EO in concentrations less than 2.5%.

There was no interaction among treatments and period for ADG, DMI, FE and COC (Table 2). In addition, the treatments did not affect the lambs´ performance. Lambs fed diets containing monensin had less (P < 0.01) oocyst/g compared with 14EO, while the 28EO and 42EO treatments presented intermediate values.

There was period effect for ADG (P < 0.01), DMI (P < 0.01), FE (P < 0.01) and COC (P < 0.01). The DMI increased (P1: 904.37 ± 42.13; P2: 1077.88 ± 42.13 g/day) and ADG decreased (P1: 278.8 ± 18.81; and P2: 258.9 ± 18.81 g/day) during the experimental periods; consequently, the FE was greater in the first period compared with second (P1: 0.30 ± 0.01; P2: 0.23 ± 0.01). The oocyst count increased between the periods (P1: 1.50 ± 0.42; and P2: 3.44 ± 0.66 oocysts/g).

The treatments did not affect the carcass characteristic (Table 3). In addition, there was no effect on DM, CP and EE in *Longissimus dorsi* meat. However, lambs fed diets containing monensin had a greater ash content in lamb meat when compared with 28EO and 42EO.

Table 2 – Performance and oocyst counts of feedlot lambs fed high-concentrate diets containing monensin or red pepper fruit EO. (Piracicaba, 2019)

Item[4]	Diets[1]				SEM[2]	P-value[3]		
	MON	14EO	28EO	42EO		Diets	P	D × P
BW, kg								
Initial	21.66	21.28	21.71	21.49	0.88	0.88	-	-
28d	29.47	29.22	29.59	29.49	1.15	0.97	-	-
56d	36.67	36.97	35.16	35.72	1.58	0.40	-	-
ADG, g	273.51	271.06	260.86	269.94	22.96	0.95	0.02	0.08
DMI, g/dia	1018.86	988.03	987.25	970.36	46.63	0.59	<0.01	0.36
FE, gain:feed	0.268	0.274	0.264	0.278	0.02	0.88	<0.01	0.24
Oocyst/g	0.92^{b}	4.73^{a}	2.30^{ab}	2.17^{ab}	0.86	0.02	<0.01	0.46

[1]MON: diet containing 8 mg of monensin/kg of DM; 14EO: inclusion of 0.14% of red pepper fruit EO (DM basis); 28EO: inclusion of 0.28% of red pepper fruit EO; 42EO: inclusion of 0.42% of red pepper fruit EO. [2]SEM: standard error of the means; [3]P: period effect; D × P: diets and periods interaction; [4]ADG: average daily gain; DMI: dry matter intake; FE: feed efficiency. $^{a-b}$ means in the same row with different superscripts differ (P ≤ 0.05). $^{A-B}$ means in the same row with different superscripts tented to differ (P > 0.05 to P < 0.10).

Table 3 – Carcass characteristics and meat composition of feedlot lambs fed high-concentrate diets containing monensin or red pepper fruit EO. (Piracicaba, 2019)

Item[3]	Diets[1]				SEM[2]	P-value
	MON	14EO	28EO	42EO		
Slaughter weight, kg	38.80	36.85	38.02	37.84	1.94	0.76
Carcass characteristics						
HCW, kg	19.30	18.87	18.67	18.64	0.97	0.84
HCY, %	49.77	51.48	49.03	49.50	0.80	0.90
CCW, kg	18.77	18.31	18.12	18.12	0.95	0.82
CCY, %	48.41	49.69	47.66	48.05	0.79	0.88
BWT, mm	12.48	13.78	13.35	14.41	1.06	0.60
SFT, mm	1.71	1.64	1.52	1.65	0.21	0.78
LM area, cm^2	13.40	13.93	13.69	12.80	0.79	0.67
Meat composition, %						
DM	25.71	25.80	25.59	25.65	0.23	0.91
CP	23.64	23.60	23.60	23.42	0.24	0.90
Ash	1.31^{a}	1.30^{ab}	1.23^{c}	1.24^{bc}	0.02	<0.01
EE	2.26	2.13	2.36	2.47	0.19	0.63

[1]MON: diet containing 8 mg of monensin/kg of DM; 14EO: inclusion of 0.14% of red pepper fruit EO (DM basis); 28EO: inclusion of 0.28% of red pepper fruit EO; 42EO: inclusion of 0.42% of red pepper fruit EO. [2]SEM: standard error of the means. [3]HCW: hot carcass weight; HCY: hot carcass yield; CCW: chilled carcass weight; CCY: chilled carcass yield; BWT: body wall thickness; SFT: subcutaneous fat thickness; LM area: Longissimus muscle area; DM: dry matter; CP: crude protein; EE: ether extract. $^{a-b}$ means in the same row with different superscripts differ (P ≤ 0.05). $^{A-B}$ means in the same row with different superscripts tented to differ (P > 0.05 to P < 0.10).

Discussion

Similar results observed in this study have been reported in other studies with different EOs sources (Anassori et al., 2011; Anassori et al., 2017). As for ionophores, the major effects of plant extracts on the ruminant metabolism were related to modulation of rumen fermentation. The limonene and pinene were able to inhibit methane production by altering the fermentation process (Cattani et al., 2016; Cobellis et al., 2016). Essential oils can also increase dietary energy efficiency and concentration of ruminal propionate, besides reducing the ruminal deamination (McIntosh et al., 2003).

Araújo (2010) conducted an *in vitro* study evaluating the use of essential oils from plants native to Brazil with potential for manipulation of ruminal fermentation and found that the red pepper (leaves and fruit) and lemon grass EO inclusion resulted in greater energy efficiency during the fermentation process. Additionally, the author observed that the inclusion of Brazilian red pepper EO increased the propionate concentration, reduced the acetate:propionate ratio and decreased methane production.

The extraction method may influence the chemical composition of the EO (Burt, 2007). The environment in which the plant develops and the type of crop can also cause variations in composition (Dorman & Deans, 2000). In this study, the compounds with the highest concentration were α-pinene and α-limonene. However, authors who also used red pepper fruit EO founded differences in the chemical composition of EO. Araújo (2010) related that α-pinene (25.0%), Δ-3-carene (22.4%), α-felandren (18.5%), silvestrene (15.9%) and p-cymene (11.0%), were the main components found in the red pepper fruit EO, whereas Faleiro Neto (2015) reported α-pinene (21.4%), silvestrene (18.2%), Δ-3-carene (17.4%), p-cymene (16.2%) and α-felandren (15.1%) as main components. This variation can be attributed to the issue intrinsic to the material used for the extraction of EO, since the technique of extraction and analysis of composites was the same in all the experiments described. However, in all experiments, the main compound identified in red pepper fruit OE was the α-pinene.

It is important to emphasize that some EO may decrease the ration acceptability reducing DMI (Calsamiglia et al., 2007). Estell et al. (1998) consider that the inclusion of high doses of α-pinene may decrease DMI. In addition, of the 23 volatile compounds examined, only four were related to intake when tested individually, camphor, α-pinene, camphene, and caryophyllene oxide (Estell et al., 2008). However, although red pepper fruit EO presented a characteristic and marked odor, the doses used in the present study did not affect DMI when compared to the diet containing monensin.

In previous studies, the α-pinene was the main compound that has shown positive results in animal performance (Silva et al., 2010). In a study with juniper oil (*Juniperus communis*), which contained 35% α-pinene, an increase of 17% on ADG in lambs was observed (Chaves et al., 2008) although it did not change the molar ratio and the total concentration of short chain fatty acids (SCFA). In addition, the essential oils containing pinene increased the feed efficiency of finishing steers (Meyer et al., 2009). In the present study, the increased doses of Brazilian red pepper fruit EO did not affect the ADG and FE compared with monensin.

The lambs presented higher ADG in the first period of the experiment. This is due to the allometric characteristics causing the tissues to have different growth rates, which change in distinct phases of the animals' life (Petrovic et al., 2015). As the animal reaches physiological maturity, muscle growth decreases and fat tissue deposition increases, leading to lower weight gain. Furthermore, there is an increase in DMI so the animal can meet higher nutritional requirements. Thus, the ADG, DMI and FE are also affected by the growth stages of the animal.

Although it is of great importance, the studies that evaluate the use of EO in the performance of ruminants do not evaluate the data on the contamination by *Eimeria* ssp., which can be controlled with the addition of antibiotics such as monensin in the diet (Amarante, 2015). In this experiment, the animals that received MON treatment presented lower infestation by *Eimeria* ssp., when compared to the animals that received red pepper fruit EO. Calhoun et al. (1979) performed an experiment using four doses of monensin (5.5, 11, 22 and 33 mg/kg) in diet of lambs, and observed monensin decreased the number of coccidian oocysts at all fed levels, demonstrating that even the dose of 5.5 mg/ kg monensin was effective in the control of coccidiosis. Among the diets containing red pepper fruit EO, the lambs that received the 14EO diet showed a greater number of oocysts, inferring that low doses of EO may be ineffective against coccidiosis. In addition, the infestation by the animals was higher in the second period of the experiment. According to Amarante (2015), the multiplication of parasites in the host may cause clinical coccidiosis; however, this was not observed in the present study.

The lack of effect on carcass characteristics can be justified by the similar performance among treatments during the experimental period, which caused the same slaughter weight among treatments. The slaughter weight of lambs in the present study was higher than the slaughter weight commonly observed in the Brazilian market, which normally ranges from 28 to 35 kg of BW. This weight range avoids unsatisfactory conditions of muscular development and finishing, allied with the slaughter of young lambs (Müller, 1991). However, the lamb intensive system allows the slaughter of lambs less than 5 months old and weighing more than 40 kg.

According to Osório & Osório (2001), in order to avoid shortening of muscle fibers by exposure to cold, there is an adequate fat thickness that the carcasses must reach. The percentage of fat in the carcass is usually affected by age, genotype, sex, breed, nutrition and feeding time. In the present study, the mean fat thickness was 1.63 mm, which was similar to Cartaxo et al. (2017) that reported SFT values for crossbreed Dorper × Santa Inês lambs with a mean of 1.25 mm, whereas ¾ Dorper × ¼ Santa Inês lambs had SFT 27% higher than the ½ Dorper × ½ Santa Inês animals. However, these values were lower in relation to the recommendation of 2.0 to 5.0 proposed by Osório & Osório (2001). The protein content of the diet may also influence fat deposition in the carcass, since the protein levels ingested by the animals may change the proportion of fat in the carcass.

The centesimal composition of meat can be influenced by several aspects such as puberty, breed, age and weight at slaughter, nutrition, management strategy and others (Guerrero et al., 2013). According to Spears (1990), numerous studies indicate that ionophores affect the apparent absorption of minerals, but mechanisms by which ionophores influence mineral absorption have not been elucidated. Significant increases in magnesium absorption were observed in steers and lambs fed high-concentrate diets with ionophores (Starnes et al., 1984; Greene et al., 1988). The apparent absorption of phosphorus, calcium (Spears et al., 1989) and potassium was increased by the monensin (Kirk et al., 1985; Greene et al., 1988; Spears et al., 1989). Starnes et al. (1984) reported that monensin increased apparent sodium uptake in steers fed a high-concentrate diet. Compared to the animals that received the control diet, lambs fed monensin showed greater apparent absorption of phosphorus, magnesium and potassium (Droke et al., 1989). This increase in minerals absorption caused by the inclusion of monensin in the diets may explain the increase in the ash content in the meat of lambs fed with the ionophore. In addition, the 14EO presented similar values to MON, suggesting that the inclusion of low doses of Brazilian red pepper fruit EO may change the mineral metabolism in the feedlot lambs.

Conclusion

The Brazilian red pepper fruit EO can be included in high concentrate diets in feedlot lambs, since they presented performance and carcass characteristics similar to animals fed with MON. However, it is important to consider whether the environment has high contamination of coccidia, since monensin was more efficient in controlling the number of oocysts in the feces. As a suggestion for new research, it is necessary to study the efficiency of use of red pepper fruit essential oil for feedlot lambs in relation to diets without additives.

Conflict of Interest

We have no conflict of interest to declare.

Ethics Statement

The ethics committee approval certificate is attached (CEUA nº 5468211016).

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