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The Fermentative and Nutritional Effects of Limonene and a Cinnamaldehyde–Carvacrol Blend on Total Mixed Ration Silages

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Abstract

This study evaluated the effects of different doses of limonene essential oil (LEO) and a blend of cinnamaldehyde and carvacrol (BCC) on the fermentative quality and chemical–bromatological composition of total mixed ration (TMR) silages. Two independent trials were conducted, each focused on one additive, using a completely randomized design with four treatments (0, 200, 400, and 600 mg/kg of dry matter), replicated across two seasons (summer and autumn), with five replicates per treatment per season. The silages were assessed for their chemical composition, fermentation profile, aerobic stability (AS), and storage losses. In the LEO trial, the dry matter (DM) content increased significantly by 0.047% for each mg/kg added. Dry matter recovery (DMR) peaked at 97.9% at 473 mg/kg ($p < 0.01$), while lactic acid (LA) production reached 5.87% DM at 456 mg/kg. Ethanol concentrations decreased to 0.13% DM at 392 mg/kg ($p = 0.04$). The highest AS value (114 h) was observed at 203.7 mg/kg, but AS declined slightly at the highest LEO dose (600 mg/kg). No significant effects were observed for the pH, neutral detergent fiber (NDF), acid detergent fiber (ADF), crude protein (CP), or non-fiber carbohydrates (NFCs). In the BCC trial, DMR reached 98.2% at 548 mg/kg ($p < 0.001$), and effluent losses decreased by approximately 20 kg/ton DM. LA production peaked at 6.41% DM at 412 mg/kg ($p < 0.001$), and AS reached 131 h at 359 mg/kg. BCC increased NDF (from 23.27% to 27.73%) and ADF (from 35.13% to 41.20%) linearly, while NFCs and the total digestible nutrients (TDN) decreased by 0.0007% and 0.039% per mg of BCC, respectively. In conclusion, both additives improved the fermentation efficiency by increasing LA and reducing losses. LEO was more effective for DM retention and ethanol reduction, while BCC improved DMR and AS, with distinct effects on fiber and energy fractions.

Keywords: additives; lactic acid fermentation; phytogenic

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1. Introduction

Total mixed ration (TMR) silage is a technique used to preserve feed while providing balanced diets, requiring less labor and allowing for the inclusion of wetter and less-palatable ingredients [1]. However, during the storage process, the silage quality can be compromised due to the presence of undesirable microorganisms or inadequate fermentation [2]. Therefore, adopting methods that ensure proper fermentation and maintain nutritional quality, such as the use of additives, is essential to ensure consistent herd productivity [3].

These additives can enhance the nutritional value, preserve silage nutrients, reduce fermentation losses, improve aerobic stability, and help ensure the feed maintains animal health [2,4]. The use of essential oils has shown promising results in controlling undesirable microorganisms during ensiling, in addition to improving the fermentation quality [5]. However, research findings often report both reductions in undesirable and desirable fermentations when using essential oils. Ref. [6] observed that, in sugarcane silages, the addition of lemongrass essential oil reduced yeast growth and ethanol production. However, lactic acid production and the population of lactic acid bacteria also decreased as the dose of essential oil increased.

Ref. [5] highlighted that the moisture content of the ensiled material significantly influences the action of limonene essential oil and, consequently, the fermentation profile. In their study, higher doses of the essential oil (600 mg/kg of dry matter) in wetter silages (30% dry matter), with an average of 4.95% of dry matter, significantly impaired lactic fermentation. In contrast, in less moist silages (40% dry matter), the dose of 300 mg/kg DM of limonene essential oil resulted in the most effective lactic fermentation, with an average of 8.1% of dry matter.

These findings demonstrate that, in addition to the type, concentration, and dose of essential oils, the chemical composition and moisture content of the material also influence the success of using these oils as fermentation modulators in silages. Therefore, although there are studies on the application of essential oils as silage additives, further research is needed to understand the optimal doses and the impacts of these oils on the fermentation quality and nutritional parameters across different types of silage.

In this context, our hypothesis is that essential oils can act as fermentation modulators in silages by improving lactic acid production and aerobic stability without significantly altering nutritional value. The objective of this experiment was to evaluate the effects of different doses of limonene essential oil (LEO) and a blend of essential oils containing cinnamaldehyde and carvacrol (BCC) on the fermentation process and nutritional quality of TMR silages.

2. Materials and Methods

2.1. A Description of the Study Site and the Experimental Trials

The research was conducted at Embrapa Western Agriculture (22°16′44″ S, 54°49′10″ W), located in the municipality of Dourados, MS, Brazil. The region is classified as having a Cwa climate (humid mesothermal with rainy summers), according to the Köppen classification [7].

The experiment consisted of two separate trials, in which each additive was individually evaluated for its effects on the fermentative and nutritional characteristics of TMRs based on BRS Capiaçu grass. The first experimental period took place in February 2024 (summer) and the second in May 2024 (autumn) for both experimental trials.

The tested doses were 0, 200, 400, and 600 mg/kg of DM for both additives (LEO and BCC). The doses were selected based on recommendations and precedents established in the scientific literature. Previous studies indicate that these concentration ranges are effective in modulating fermentation and improving silage quality without compromising the microbial development necessary for the proper preservation of the material.

Each treatment had five replicates, totaling 20 experimental silos per trial in each evaluation period. The essential oils used were the following commercial products: Activo

Liquid® (Limonene) and Blend Activo Liquid® (75% cinnamaldehyde and 25% carvacrol), both supplied by GRASP, a company located in Paraná state, Brazil.

2.2. TMR Preparation and Filling of Experimental Silos

The TMR was formulated to meet the nutritional requirements of lactating dairy cows producing 20 kg of milk/day, with a body weight of 450 kg and an average DM of 14 kg/day [8]. The forage-to-concentrate ratio was 33:67, with the forage being composed of BRS Capiaçu grass (90 days old) and the concentrate being formulated using ground corn, soybean meal, dicalcium phosphate, and calcitic limestone. The ingredient proportions and chemical composition of the diet are detailed in Table 1.

Table 1. The ingredient proportions and chemical composition of the TMRs formulated for both experimental trials.

Ingredients	% of DM
BRS Capiaçu grass	33.21
Ground corn	43.38
Soybean meal	19.67
Calcitic limestone	1.83
Dicalcium phosphate	1.90
Total	100.00
DM, % FM	35.04
Ash, % DM	6.30
Crude protein, % DM	18.10
Neutral detergent fiber, % DM	35.54
Acid detergent fiber, %DM	23.29
Lignin, % DM	3.40
Ether extract, % DM	2.70
Starch, % DM	22.00
Non-fiber carbohydrates, % DM	37.01
Buffering capacity, meq NaOH/100 g DM	13.63
pH	6.78

FM = fresh matter; DM = dry matter; NaOH = sodium hydroxide.

The additives tested and their respective doses were pre-mixed into the concentrate to ensure a uniform distribution throughout the ensiled mass. The forage was then mixed with the concentrate, ensuring homogeneity and complete additive distribution. The resulting mixture was used to fill the experimental silos.

PVC pipes (10 cm in diameter, 50 cm in height) with a usable volume of 3.8 L were used as experimental silos. A layer of approximately 4.5 cm of sand (300 g) was placed at the bottom of each silo for effluent drainage. A fine cotton fabric mesh was used to prevent direct contact between the forage and the sand. The material was manually compacted using wooden rods to achieve an average density of 700 kg of fresh matter/m^3. After filling, the silos were sealed with black-and-white, double-faced plastic film and adhesive tape and stored at room temperature for 110 days.

2.3. Sample Collection, Fermentative Losses, and Analytical Methodologies

During silo filling, two TMR samples were collected from each treatment in both experimental periods. The first sample (approximately 300 g) was used to determine the DM content and the chemical–bromatological composition. The second sample (approximately 70 g) was frozen for the later determination of the pH and the buffering capacity.

To calculate fermentative losses, all silo components (silo, sand, and cotton fabric mesh), as well as the TMR mass, were weighed before and after ensiling. Dry matter recovery (DMR), gas losses (GLs), and effluent losses (EL) were calculated according to ref. [9].

After the silo opening, all the contents were removed and homogenized for sampling. A sample of approximately 300 g from each experimental silo was sent to the laboratory for chemical–bromatological and fermentative profile analyses. The chemical–bromatological composition of the samples was determined using a Foss 5000 NIR spectrophotometer (Eden Prairie, MN, USA), calibrated by Dairy One Forage Laboratory (Ithaca, NY, USA) using WinISI version 4.6.11 (FOSS Analytical A/S, Hillerød, Denmark). The following components were determined as follows: dry matter (DM), ash, crude protein (CP), ammoniacal nitrogen (NH_3-N), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin, ether extract (EE), non-fiber carbohydrates (NFCs), starch, and the total digestible nutrients (TDN) [10].

The pH (before and after ensiling), buffering capacity (only before ensiling), and short-chain organic acid profiles were determined using an aqueous extract from the TMR. For extract preparation, 25 g of TMR was diluted in 225 mL of distilled water and manually homogenized for about 30 min. The extract's pH was measured using a digital potentiometer (mPA210 MS Tecnopon, Piracicaba, Brazil), and its buffering capacity was assessed according to ref. [11]. Organic acids were determined by gas chromatography with mass spectrometry (GCMS QP 2010 Plus, Shimadzu, Kyoto, Japan) using a capillary column (Stabilwax, Restek, Bellefonte, PA, USA, 60 m × 0.25 mm ID, 0.25 µm polyethylene glycol cross-bond). The lactic acid (LA) concentration was determined using the colorimetric method proposed by ref. [12].

Aerobic stability (AS) was determined after the silo opening. Samples (2 ± 0.005 kg) from each replicate were placed loosely into clean experimental silos. Temperature sensors were inserted into the geometric center of each silage mass, and a double layer of gauze was placed over each silo to avoid drying and contamination while allowing air penetration. Ambient and silage temperatures were recorded every minute, with averages calculated every 20 min using a data logger (RC-4, Elitech®, São Paulo, Brazil). AS was defined as the number of hours until the silage temperature rose to 2 °C above the ambient temperature [13].

2.4. Data Analysis

The data were analyzed using the "stats" package in R software (v. 4.2.1; R Studio v. 2023.06.1). Prior to the analysis, the Shapiro–Wilk test was used to assess residual normality. The non-normal data were transformed using the Box–Cox method [14]. Outliers were removed if values exceeded 3 standard deviations from the mean. The data were evaluated using polynomial regression with the following model:

$$Yik = \mu + poly(D)i + Pj + \epsilon ik$$

where Yik = a dependent variable for the ith dose level and the kth replicate; μ = the overall mean; Di = the linear or quadratic effect of the dose (i = 4, indicating four dose levels); Pj = the random effect of the season (j = 2, two seasons); and ϵik = the experimental error.

Treatment differences were considered significant when $p \leq 0.05$. The data are reported as the least square means (LSMeans) with the standard error of the mean (SEM). The statistically significant regression results are shown in figures, along with the corresponding regression equations.

3. Results

3.1. First Trial—TMR Silage with Different Doses of LEO

DMR was significantly affected by the LEO dose, showing a progressive increase as the additive dose increased. Both the linear and the quadratic models were significant ($p < 0.01$) for DMR (Table 2).

Table 2. Fermentation losses, pH, ammoniacal nitrogen, and fermentative parameters of TMR silages with different doses of LEO.

Parameters	LEO (mg/kg DM)				SEM	*p*-Value	
	Control	200	400	600		L	Q
DMR, % of DM	94.09	95.66	95.98	96.09	0.177	<0.001	<0.001
GLs, % DM	1.24	1.96	2.44	1.24	0.165	0.74	<0.001
ELs, kg/ton DM	63.66	55.15	51.17	52.49	2.557	0.07	0.28
pH	3.83	3.91	3.88	3.88	0.011	0.23	0.06
NH_3-N, % TN	5.14	5.25	5.36	5.27	1.000	0.18	0.25
LA, % DM	3.27	5.14	5.75	5.64	0.242	<0.001	<0.001
Ethanol, % DM	0.24	0.12	0.12	0.13	1.092	0.04	0.04
AA, % DM	1.16	1.26	1.15	1.14	0.031	0.41	0.19
PA, mg/kg DM	109.53	114.36	94.38	94.36	4.656	0.11	0.79
Iso-but, mg/kg DM	2.63	1.93	3.04	2.78	0.221	0.44	0.63
BA, mg/kg DM	23.13	19.80	29.30	31.40	2.773	0.17	0.59
Iso-val, mg/kg DM	2.79	2.49	2.49	2.67	1.074	0.45	0.02
VA, mg/kg DM	6.00	3.27	1.60	1.86	1.242	0.09	0.47
AS, hours	111.99	110.36	114.47	99.81	1.684	0.01	0.01

LEO = limonene essential oil; DMR = dry matter recovery; GLs = gas losses; DM = dry matter; ELs = effluent losses; NH_3-N = ammoniacal nitrogen; TN = total nitrogen; LA = lactic acid; AA = acetic acid; PA = propionic acid; Iso-But = isobutyric acid; BA = butyric acid; Iso-Val = isovaleric acid; VA = valeric acid; AS = aerobic stability; SEM = standard error of the mean; L = linear effect; Q = quadratic effect.

However, the quadratic model provided a better fit to the data (best model), with the DMR curve peaking at a dose of 473 mg/kg DM (Figure 1). GLs also followed a quadratic pattern, with the maximum production observed at a dose of 372 mg/kg of LEO on a DM basis, reaching 2.55%, while the control treatment showed the lowest value of 1.16% (Figure 1).

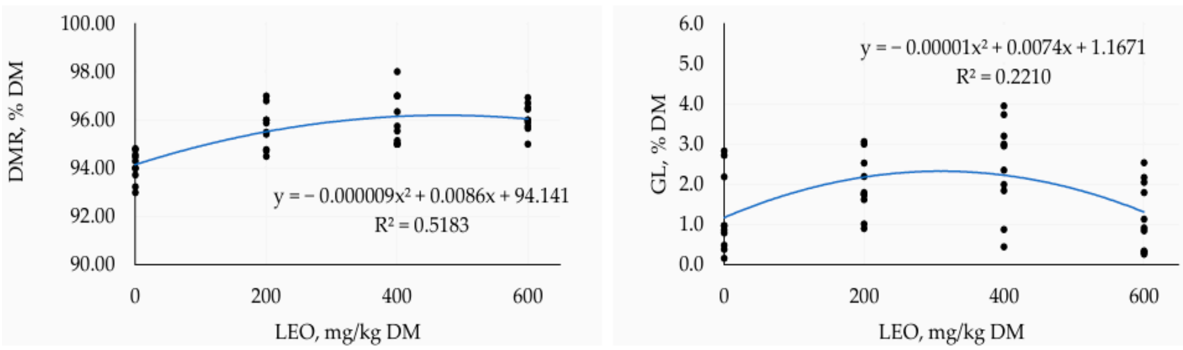

Figure 1. Dry matter recovery (DMR) and gas losses (GLs) of TMR silages with different doses of LEO. Black dots represent individual data points from the experimental silos, and the blue line represents the quadratic regression curve fitted to the data.

The concentration of LA (Figure 2) increased with the increment of the LEO dose, with the 456 mg/kg DM dose showing the highest LA production (5.87% of DM), while the control dose showed the lowest value for this organic acid (3.2% of DM). Ethanol concentrations (Figure 2) decreased in the silages as the LEO dose increased, following a quadratic trend. The ethanol value in the control was 0.252% of DM, while at the dose of 392 mg/kg, it was reduced to 0.13% of DM (p = 0.04).

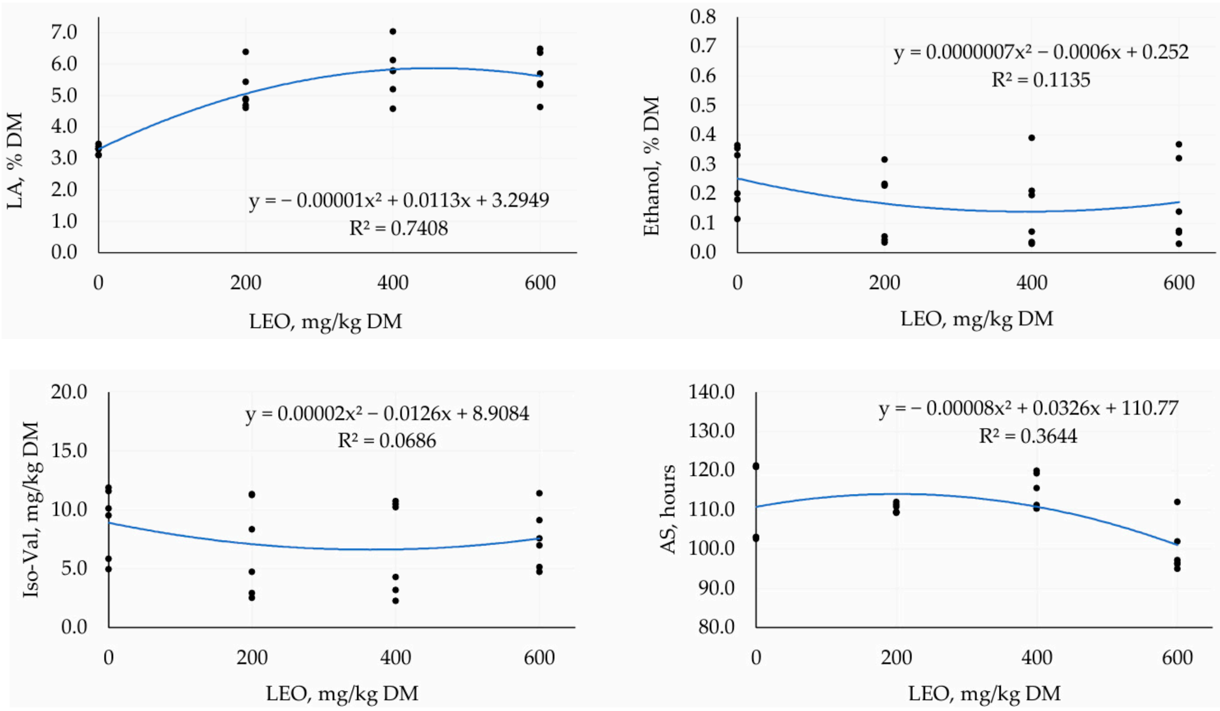

Figure 2. Lactic acid (LA), ethanol, isovaleric acid (Iso-Val) concentrations and aerobic stability (AS) of TMR silages with different doses of LEO. Black dots represent individual data points from the experimental silos, and the blue line represents the quadratic regression curve fitted to the data.

In the case of isovaleric acid, a negative quadratic effect was observed (p = 0.02), decreasing from 8.90 mg/kg DM in the control treatment to 6.61 mg/kg DM at the dose of 365 mg LEO/kg DM (estimated according to the model). According to the model, the best AS value (114 h) was achieved at the dose of 203.7 mg of LEO/kg DM, which was higher than the lowest observed value (101 h) at the 600 mg/kg DM dose.

The chemical-bromatological parameters evaluated at different LEO doses are presented in Table 3. Significant variations were observed in some components, such as dry matter (DM) and mineral ash (AS), as indicated by the p-values.

Table 3. Chemical–bromatological composition of TMR silages with different doses of LEO.

Parameters	LEO (mg/kg DM)				SEM	p-Value	
	Control	200	400	600		L	Q
DM, % FM	34.78	36.43	38.32	37.30	0.541	0.04	0.18
AS, % DM	8.13	7.42	7.32	6.93	0.170	0.01	0.59
CP, % DM	17.63	17.74	17.29	16.07	0.504	0.20	0.46
NDF, % DM	35.13	37.42	33.97	34.70	0.845	0.54	0.66
ADF, % DM	23.27	24.78	21.83	23.07	0.612	0.53	0.91
LIG, % DM	2.97	3.33	2.72	3.15	0.169	0.95	0.89
ST, % DM	22.40	22.40	24.02	24.15	0.723	0.23	0.96
EE, % DM	3.22	2.97	2.72	3.18	0.110	0.72	0.10
NFCs, % DM	37.12	34.75	38.72	39.33	0.790	0.14	0.34
TDN, % DM	69.83	69.00	70.83	70.67	0.417	0.21	0.67

LEO = limonene essential oil; DM = dry matter; FM = fresh matter; AS = ash; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; LIG = lignin; ST = starch; EE = ether extract; NFCs = non-fibrous carbohydrates; TDN = total digestible nutrients; SEM = standard error of the mean; L = linear effect; Q = quadratic effect.

In Figure 3, it can be observed that for each mg of LEO added per kg of DM of the silage, there was an increase of 0.047% in the DM content, indicating a positive linear effect. However, for ash, there was a linear decrease of 0.00185%. The addition of LEO had a significant effect on the DM and ash contents of the silage.

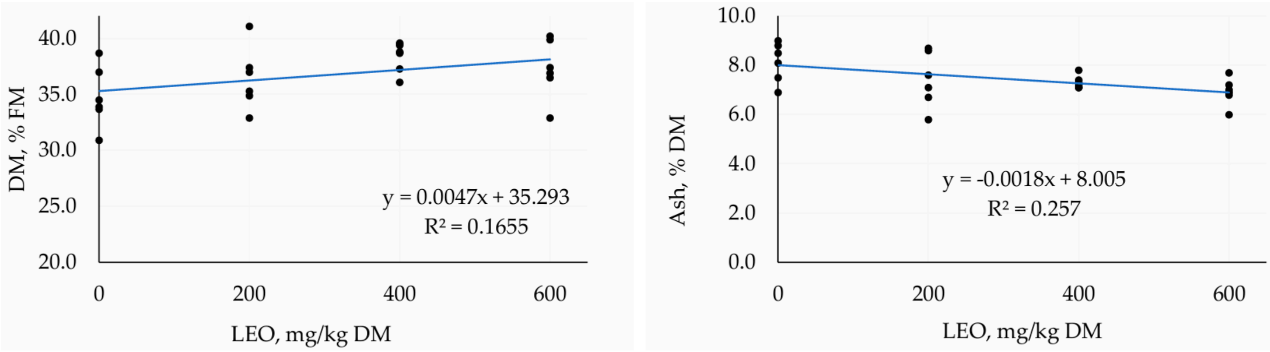

Figure 3. Dry matter (DM) and ash contents of TMR silages with different doses of LEO. Black dots represent individual data points from the experimental silos, and the blue line represents the quadratic regression curve fitted to the data.

3.2. Second Trial—TMR Silage with Different Doses of BCC

Table 4 presents the parameters related to fermentation losses, pH, ammoniacal nitrogen, and organic acids in total mixed ration (TMR) silages with different doses of BCC.

Table 4. Fermentation losses, pH, ammoniacal nitrogen, and fermentation parameters of TMR silages with different doses of BCC.

Parameters	BCC (mg/kg DM)				SEM	*p*-Value	
	Control	200	400	600		L	Q
DMR, DM%	94.09	95.73	97.08	97.06	0.230	<0.001	<0.001
GLs, % DM	1.07	0.99	1.91	0.72	0.008	0.85	0.18
ELs, kg/ton DM	63.66	51.44	46.02	44.57	3.301	0.01	0.29
pH	3.83	3.89	3.85	3.87	0.010	0.43	0.36
NH_3-N, % TN	5.14	5.26	5.26	5.40	1.000	0.04	0.95
LA, % DM	3.27	6.33	5.70	6.02	0.288	<0.001	<0.001
Ethanol, % DM	0.26	0.12	0.22	0.14	0.020	0.15	0.54
AA, % DM	1.16	1.24	1.32	1.36	0.063	0.16	0.87
PA, mg/kg DM	114.29	122.07	132.60	122.19	1.004	0.66	0.62
Iso-but, mg/kg DM	2.63	1.67	2.81	4.45	0.380	0.05	0.07
BA, mg/kg DM	24.10	36.06	29.09	36.58	2.243	0.14	0.56
Iso-val, mg/kg DM	8.98	9.84	7.63	8.27	0.653	0.47	0.94
VA, mg/kg DM	1.43	1.47	1.85	2.04	0.143	0.09	0.85
AS, hours	111.99	122.50	131.16	119.84	2.477	0.16	0.02

BCC = blend of cinnamaldehyde and carvacrol; DMR = dry matter recovery; GLs = gas losses; DM = dry matter; ELs = effluent losses; NH_3-N = ammoniacal nitrogen; TN = total nitrogen; LA = lactic acid; AA = acetic acid; PA = propionic acid; Iso-But = isobutyric acid; BA = butyric acid; Iso-val = isovaleric acid; VA = valeric acid; AS = aerobic stability; SEM = standard error of the mean; L = linear effect; Q = quadratic effect.

DMR increased as the BCC dose was elevated (Figure 4). The DMR values ranged from 95.03% in the control treatment to 98.159% at the dose of 548 mg/kg (the best dose according to the quadratic prediction model). The values for ELs exhibited a negative linear behavior in response to the BCC doses, with an approximate reduction of 20 kg of effluent per ton of DM when comparing the control treatment with the maximum BCC dose tested.

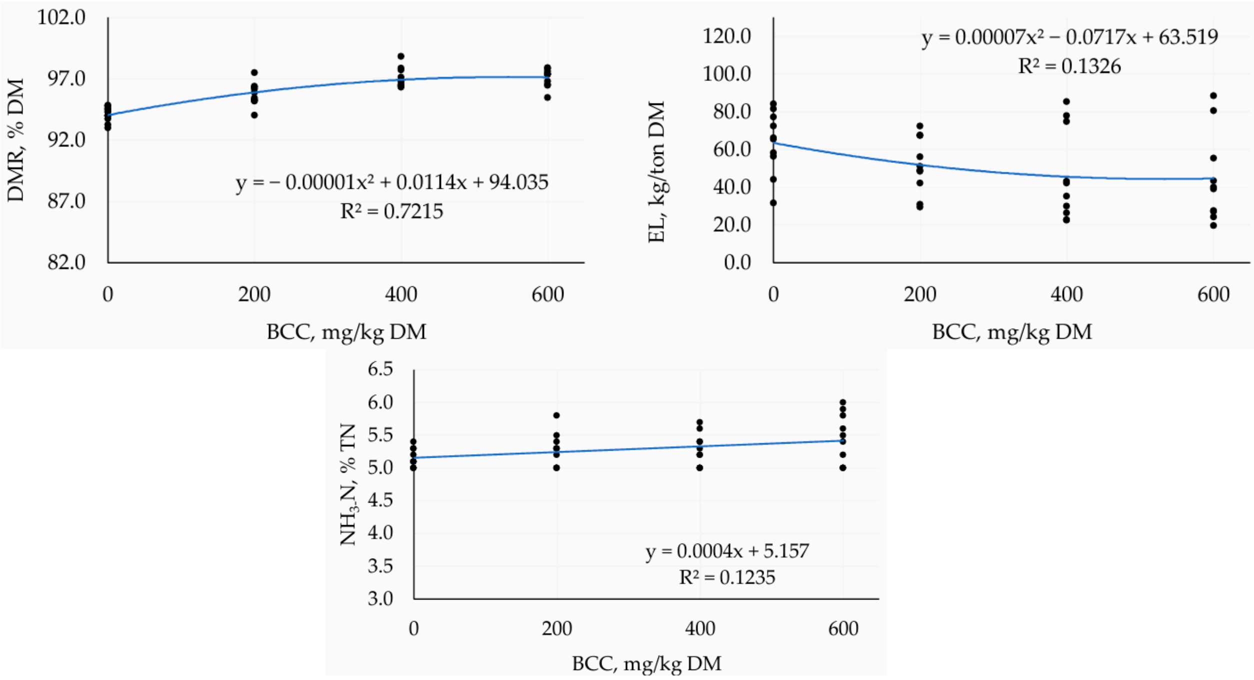

Figure 4. Dry matter recovery (DMR), effluent losses (ELs), and ammoniacal nitrogen (NH_3-N) of TMR silages treated with different doses of BCC. Black dots represent individual data points from the experimental silos, and the blue line represents the quadratic regression curve fitted to the data.

The concentration of NH_3-N showed a significant increase (p = 0.04) with the addition of BCC. According to the prediction model (Figure 4), the concentration of NH_3-N increased from 5.14% of the total nitrogen (TN) in the control treatment to 5.40% of the TN at the dose of 600 mg/kg DM, leading to an increase of 0.00043% of NH_3-N per the TN for each mg of BCC.

It can be seen in Table 5 that the use of BCC was effective in increasing LA production, reducing ethanol production, and improving the AS of the silages, especially at higher doses. However, the effect on other fermentation acids was limited.

Table 5. Chemical–bromatological composition of TMR silages with different doses of BCC.

Parameters	BCC (mg/kg DM)				SEM	*p*-Value	
	Control	200	400	600		L	Q
DM, % FM	34.78	36.77	35.58	34.38	0.562	0.65	0.17
AS, % DM	8.13	7.50	7.78	7.48	0.124	0.13	0.49
CP, % DM	18.24	17.96	18.24	17.94	0.188	0.73	0.97
NDF, % DM	35.13	38.28	39.05	41.20	0.960	0.02	0.78
ADF, % DM	23.27	25.23	26.68	27.73	0.695	0.01	0.70
LIG, % DM	2.97	3.18	3.35	3.50	0.186	0.18	0.91
ST, % DM	22.40	22.07	21.55	18.82	0.738	0.08	0.40
EE, % DM	3.22	3.25	3.20	2.75	0.130	0.12	0.24
NFCs, % DM	37.12	35.30	33.90	32.75	0.827	0.04	0.83
TDN, % DM	69.83	69.17	68.33	67.50	0.419	0.02	0.91

BCC = blend of cinnamaldehyde and carvacrol; DM = dry matter; FM = fresh matter; AS = ash; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; LIG = lignin; ST = starch; EE = ether extract; NFCs = non-fibrous carbohydrates; TDN = total digestible nutrients; SEM = standard error of the mean; L = linear effect; Q = quadratic effect.

The LA and iso-butyric acid concentrations in TMR silages treated with BCC provided a detailed view of the fermentative profile of the silages and their relationship with the observed nutritional composition. The concentration of LA (Figure 5) showed a significant increase with the increase in the BCC dose, ranging from 3.50% in the control treatment to 6.406% at the dose of 412 mg/kg (the best dose according to the prediction model). For iso-butyric acid, there was a linear increase with the BCC doses, ranging from 2.63 mg/kg DM in the control treatment to 4.45 mg/kg DM at the dose of 600 mg/kg.

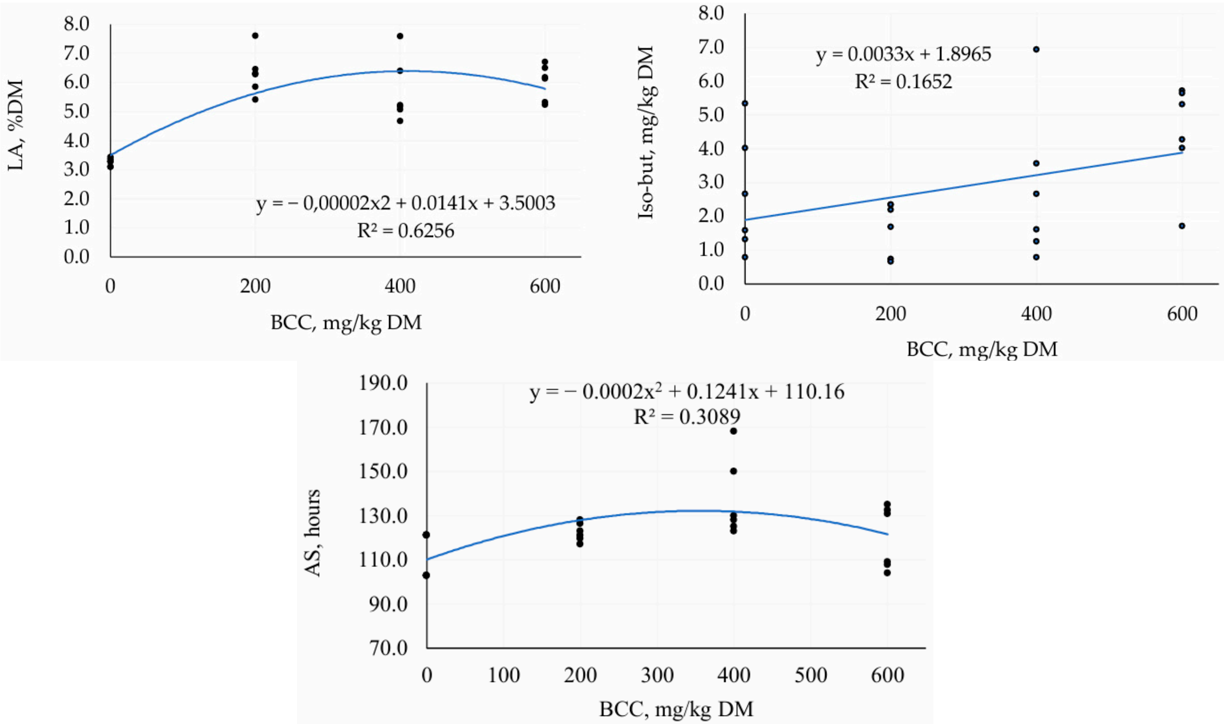

Figure 5. Lactic acid (LA) and isobutyric acid (Iso-but) concentrations and aerobic stability (AS) of TMR silages treated with different doses of BCC. Black dots represent individual data points from the experimental silos, and the blue line represents the quadratic regression curve fitted to the data.

AS was also affected by the use of BCC (Figure 5). AS exhibited a quadratic pattern, with the dose of 359 mg/kg DM being the most effective for providing the highest AS value (131 h).

The nutritional composition of the TMR silages with different doses of BCC is presented in Table 5. Significant differences ($p < 0.05$) were observed in the fiber fractions, especially NDF and ADF. Additionally, variations in NFCs and the TDN contents were also noted (Figure 5).

In the control treatment, NDF represented 23.27% of the DM, while at the highest BCC dose (600 mg/kg DM), the concentration increased to 27.73% of the DM. For NDF, a positive linear behavior was observed ($p = 0.01$), indicating that for each increment of 1 mg of BCC/kg DM, there was an increase of 0.095% in the NDF content of the silage. Similarly, ADF also increased with the increment of the BCC dose. The ADF concentration ranged from 35.13% in the control to 41.20% at the dose of 600 mg/kg, so for ADF, each 1 mg of BCC added per kg of DM resulted in an increase of 0.074% in the ADF content (Figure 6).

On the other hand, the concentrations of NFCs and the TDN decreased as the BCC dose increased, meaning that for each increment of 1 mg of BCC/kg of DM, there was a reduction of 0.0007% and 0.039% of DM for NFCs and the TDN, respectively.

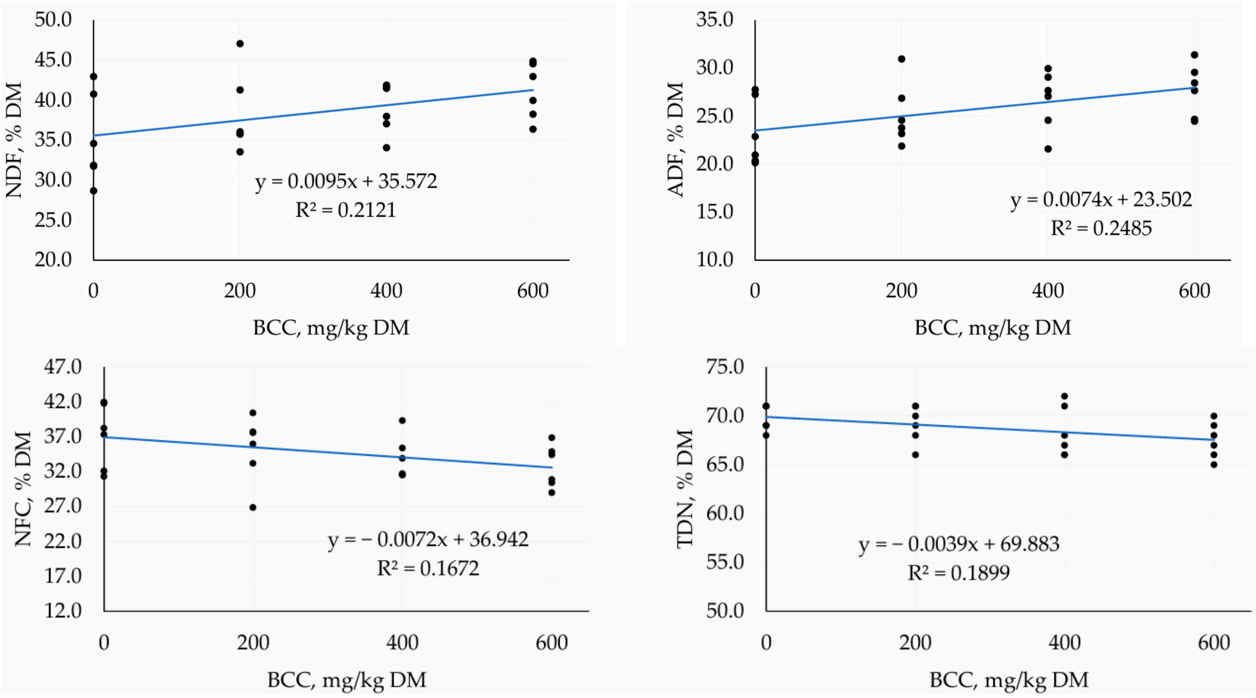

Figure 6. Neutral detergent fiber (NDF), acid detergent fiber (ADF), non-fibrous carbohydrates (NFCs) and total digestible nutrients (TDN) of TMR silages treated with different doses of BCC. Black dots represent individual data points from the experimental silos, and the blue line represents the quadratic regression curve fitted to the data.

4. Discussion

4.1. First Trial—TMR Silage with Different Doses of LEO

Silages with high DMR values tend to exhibit a greater predominance of lactic fermentation compared to others, as observed in this study. According to ref. [15], lactic fermentation is the most desirable during ensiling, as it converts feed sugars into lactic acid (LA), resulting in lower energy losses and a rapid pH drop, which inhibits the growth of undesirable microorganisms. This process helps preserve nutrients, improves the silage quality, enhances its palatability for animals, and ensures long-term preservation [2]. Therefore, promoting lactic fermentation is an effective strategy for preserving silage quality, and the use of substances that favor homofermentation is a research line that deserves further exploration.

In the present study, increasing doses of LEO resulted in higher DMR, possibly due to the greater preservation of the ensiled material (greater LA production) and the control of pathogenic microorganisms. Similar results were reported by ref. [16], who observed that sweet orange essential oil also enhanced DMR at doses of 400 and 600 mg/kg DM (94.1% and 92.5% of DM ensiled). On the other hand, ref. [5] found no significant differences in the DMR of TMR silages based on sorghum, although LA contents were higher than in the control. Both authors attributed the antimicrobial action of the essential oils used as the main factor for the effective lactic fermentation over other fermentations.

The antimicrobial mechanism of action of LEO is directly related to its ability to destabilize microbial cell membranes [17]. Being a hydrophobic compound, LEO accumulates in the lipid bilayer of the cytoplasmic membrane, altering its fluidity and increasing permeability [17]. As a result, there are leakages of ions, nucleotides, and other essential molecules, leading to the loss of cellular homeostasis and eventually cell death [18]. Studies show that LEO exhibits antibacterial activity against both Gram-positive and Gram-negative strains, and that its antimicrobial efficacy depends on several factors, including a medium pH In media with a pH near 4.0, its bactericidal activity is more intense compared to media with a pH of around 7.0 [18]. Thus, the more acidic the silage, the more effective this essential oil is in controlling undesirable strains, especially those resistant to low pH environments, such as yeasts. These explanations are supported by the values observed for pH and DMR variables (Table 2).

Although LEO and other essential oils exhibit strong activity against various groups of microorganisms, the literature shows that the required doses vary according to the microorganism type [19]. In the study by ref. [20], the use of essential oils in silage reduces fermentability parameters and the content of organic acids, such as lactic, acetic, and propionic acids, by inhibiting harmful microorganisms like enterobacteria, clostridia, yeasts, molds, and the undesirable bacteria responsible for producing acetic and butyric acids, which impair the silage quality. Additionally, these oils affect cell membrane permeability and inhibit nutrient transport, limiting the growth of microorganisms such as *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, and *Lactobacillus brevis*. It is also important to consider forage management since the sugar content can serve as a substrate for unwanted microorganisms during storage, making essential oils an effective strategy to preserve the silage quality [21,22]. Thus, ref. [19] concluded that essential oils generally exhibit selective inhibition, requiring low concentrations to inhibit pathogens and higher concentrations to combat LA bacteria, making them promising for use in silages. Similarly, this may have occurred in the present experiment with LEO, explaining the higher LA values observed in treated silages.

The use of LEO also reduced ethanol production in this experiment, likely due to yeast control. Ref. [23] observed lower yeast populations and a reduced ethanol concentration after adding cinnamon, oregano, and sweet orange essential oils (120 mg/kg DM) to barley silages (p = 0.001), suggesting essential oils have potential to control undesirable microorganisms. Ref. [6] also reported that moderate doses of lemongrass essential oil (1.53–2.22 mL/kg) effectively reduced ethanol production and fermentative losses in sugarcane silages. Additionally, they observed that lemongrass oil inclusion linearly reduced yeast and mold counts, improving the AS of silages.

In this study, AS showed a quadratic pattern and is related to AA production (due to antifungal action) and yeast control. However, higher LA contents (although desirable and beneficial for conservation) can reduce the AS of silages. According to ref. [15], while lactic fermentation is desirable for reducing the silage's pH and improving conservation, it can increase the potential for mold and yeast growth, especially when the silage is exposed to oxygen. This occurs because LA can serve as a substrate for these microorganisms, compromising the AS.

In this context, AA plays a crucial role as it not only reduces the pH but also helps inhibit fungal and yeast growth, improving AS and reducing losses after oxygen exposure. According to ref. [13], undissociated organic acids in silage easily pass through microbial cell membranes, where they dissociate and release H^+ ions, lowering the intracellular pH. This imbalance forces microbial cells to expend energy to maintain the pH, and when excessive, this can lead to cell death. Therefore, the lower the dissociation of the organic acid in the medium, the more effective its passage through microbial cell membranes, enhancing microbial growth control. AA dissociates less than LA due to its higher pKa, facilitating its membrane permeability. Silages with more than 0.8% undissociated AA (on a fresh matter basis) remain stable in air, whereas lower concentrations make silage unstable [13].

In the present study, AA contents showed no significant variation with LEO doses, remaining low compared to the values identified in the literature. This helps explain why treatments with higher LA concentrations showed lower AS. Different results were obtained by ref. [16], who investigated the effect of sweet orange essential oil in corn silage at doses of 200, 400, and 600 mg/kg DM. The authors observed a quadratic effect ($p = 0.022$) on the AA concentration, with the 600 mg/kg DM dose resulting in a 72.72% increase in AA compared to the control.

A meta-analysis by ref. [20] on the effects of various essential oils on the nutritional quality of different silages concluded that, in general, essential oil use tends to increase silage DM, CP, and EE contents. According to the authors, adding essential oils reduces harmful microorganism populations, minimizing nutrient losses in silage. Thus, higher levels of digestible nutrients are commonly observed. This is consistent with the DM contents observed in this experiment, which varied with the increasing essential oil doses.

According to ref. [1], even in well-preserved silages, the ash content tends to increase compared to the original material, due to the decomposition of some organic matter. Therefore, very high ash levels in silage may indicate the greater degradation of organic components during fermentation. In this experiment, ash decreased with the increasing LEO doses, possibly due to the lower consumption of organic nutrients as this additive was added to the mixtures.

Overall, the lack of variation in the nutrient contents in the silages produced with LEO doses is a positive outcome. An additive that improves fermentation without significantly altering nutritional value is a desirable feature.

4.2. Second Trial—TMR Silage with Different Doses of BCC

In this study, silages treated with BCC showed increased NDF and ADF levels, which may be explained by enzymatic activity stimulation, promoting cell wall breakdown and sucrose release into the medium, increasing fiber concentrations. This released sucrose was likely used by LA bacteria to produce LA, as reported by ref. [5]. The greater availability of fermentable substrate may have favored increased LA production, contributing to faster silage acidification and more-efficient fermentation. Similar results were observed by ref. [24], who found higher LA concentrations in silages treated with essential oils, indicating these compounds promote more effective fermentation, helping preserve silage nutrients.

Moreover, BCC use was effective in preserving DM during fermentation, indicating it helped increase DMR during ensiling—a positive reflection for the nutritional silage quality. Ref. [25], studying corn silages treated with essential oil blends, found similar results, with higher DMR in essential oil treatments, attributed to effective microbial control and greater fermentation efficiency. These findings are also supported by ref. [4], who reported higher DMR in silages treated with essential oils compared to the control.

Lactic fermentation promotes lower fermentative losses, such as effluent loss. When BCC was included, lower nutrient leaching was observed, which is essential to maintain the silage's nutritional quality. Ref. [26] also found that essential oil blends did not significantly alter effluent loss, but in this study, the more-effective nutrient loss control suggests that BCC had a positive effect in this regard.

The presence of isobutyric acid in silage may indicate undesirable fermentation by *Clostridium* spp., which are harmful due to their limited contribution to pH reduction and their potential to compromise silage quality by promoting the growth of spoilage microorganisms [2]. Nevertheless, it is important to highlight that the concentrations of isobutyric acid observed across all the treatments were low and did not negatively affect the nutritional quality of the silage. Additionally, BCC addition appeared to control ethanol production in the silages, reflecting greater efficiency in controlling yeast-driven fermentations.

Yeasts can compromise silage's AS, as higher yeast populations during fermentation tend to accelerate their growth after silo opening [15]. BCC addition increased resistance to aerobic spoilage, with the 400 mg/kg DM dose showing the highest stability. In addition to the antimicrobial effect of BCC described by ref. [27], an increase in the AA content (1.32% of DM) was also observed, which may have further contributed to yeast and mold control. Ref. [28] also reported greater AS in essential-oil-treated silages, due to increased AA concentrations.

On the other hand, the increase in NH_3-N concentrations in silages with higher BCC doses may reflect greater proteolytic activity, indicating increased protein degradation in silage. However, this rise in NH_3-N concentration did not compromise nutritional quality, as reinforced by ref. [4], who reported that essential oils may alter protein structures without significantly affecting the silage quality.

BCC use in silages effectively promoted more-efficient fermentation, controlled undesirable microorganisms, and improved AS. Although increases in iso-butyric acid and NH_3-N were observed, they were not sufficient to negatively affect DMR. The reduction in effluent loss indicates that essential oils may be a promising strategy for improving silage quality without significantly impairing its nutritional value.

5. Conclusions

LEO and BCC acted as effective fermentation modulators in TMR silages, contributing to the reduction of fermentative losses and the increase of lactic acid production, without significantly compromising the aerobic stability or the nutritional value of the silages. Doses ranging from 400 to 600 mg/kg of dry matter were the most effective for the majority of the evaluated parameters. However, further studies are recommended to assess the potential effects of these dosages on diet palatability and voluntary feed intake in order to ensure their practical applicability in livestock production systems.

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