

Article

Impact of Monensin Sodium and Essential Limonene Oil on the Fermentation and Chemical Composition of Total Mixed Ration Silages with Moisture Variations

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Abstract: Monensin and essential oils have antimicrobial properties that may impact silage fermentation. The present study was divided into two trials to evaluate the effects of monensin (MON) and essential limonene oils (ELO) as additives in the ensiling of total mixed ration (TMR). In the first assay, TMR was tested with sheep in growth (65% dry matter—DM) using the following treatments: control (no additive), MON35 (35 mg of monensina per kg of DM), MON45 (45 mg of monensina per kg of DM), ELO300 (300 mg of essential limonene oil per kg of DM), and ELO600 (600 mg of essential limonene oil per kg of DM). In the second assay, the same treatments were used in TMR for lactating cows under two moisture conditions (30% and 40% DM). The parameters assessed included fermentative losses, short-chain fatty acid profiles, aerobic stability (hours needed for silage to reach 2 °C above ambient), chemical composition, and in vitro DM digestibility of the silages. Treatment averages were compared using the Scott–Knott test at 5% significance. In the first assay, the treatments with ELO had the lowest ($p < 0.05$) pH values and the highest ($p < 0.05$) lactic acid concentrations, with treatment ELO600 leading to the highest ($p < 0.05$) aerobic stability (297.88 h). Only the starch contents of the ELO treatments were lower ($p < 0.05$) than the others. In the second assay, the silages with the highest moisture contents and ELO600 exhibited the lowest ($p < 0.05$) values of DM recovery, lactic acid, and pH. The highest ($p < 0.05$) lactic acid:acetic acid ratios were observed in the silages with the most moisture added with MON35 and MON45. The use of MON and ELO increased aerobic stability, with the highest ($p < 0.05$) values observed for ELO600 and MON35. The treatments with MON and ELO resulted in silages with the lowest ($p < 0.05$) fiber contents and highest ether extract and starch contents when compared with control. Thus, MON and essential oils improve fermentative quality but ELO should be used in lower doses in humid silages to avoid negative fermentation impacts.

Keywords: dairy cows; ionophore; limonene; moisture; total diets



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1. Introduction

The use of total mixed rations (TMR) in animal feed is widely adopted, which allows a nutritionally balanced diet to be provided and reduces feed selection by the animals [1]. However, the need to mix the ingredients daily can add cost to the productive process [2]. Moreover, the use of humid agro-industry coproducts is limited due to their rapid spoilage and unviable extended storage.

TMR silages offer various economic benefits, including the use of labor and machinery only during production, eliminating the need for daily mixing of ingredients [2]. They also have the potential to incorporate less palatable and more stable wet by-products from food industries [3]. TMR silages maintain high aerobic stability after silo opening and present opportunities for commercialization, particularly when stored in smaller structures such as bales, sacks, and bags [2]. Several studies have explored the effects of different ingredients and microbial additives on the fermentative and nutritional quality of TMR silage [2,4,5]. Nevertheless, few have assessed the influence of ruminal fermentation modulators such as ionophores on the fermentation process of ensiled TMR.

Monensin is one of the most studied and utilized ionophores in ruminant nutrition, acting by inhibiting Gram-positive bacteria in the rumen, which leads to lower methane production and increased animal efficiency [6]. However, its use has been banned in some European countries due to potential risks to public health and the environment [7]. Therefore, there is a search for new ruminal fermentation modulators that exert selective action on rumen microorganisms without leaving the residual effects of ionophores. Among these new modulators, essential oils (EOs) from plants have shown positive results [8].

Essential oils are concentrated plant extracts obtained from various parts of plants through methods like steam distillation or cold pressing [9]. Essential oils can help control microorganisms such as bacteria, fungi, and viruses due to their complex chemical composition, which includes compounds like terpenes, phenolics, and aldehydes [10].

Satisfactory fermentation patterns have been observed in TMR silages containing 33 mg/kg DM monensin (MON) [11]. However, it should be pointed out that all treatments tested by [11] had the same MON dose, and the lack of a control treatment prevented concluding whether MON impacts the fermentation process. Another noteworthy aspect of the research by [11] was the high dry matter (DM) contents in the TMRs at an average of 60%, since it is known that water activity is directly linked to the intensity of fermentation [12]. Since MON [13] and essential oils [10] often act by unbalancing ion concentrations in microbial cells, it is supposed that the higher the water activity in the silage (more humid silages), the greater will be the action of those additives on the microorganisms of the medium.

Moreover, under higher moisture conditions, essential oils can penetrate the forage more uniformly, which leads to a more consistent antimicrobial effect [9]. Appropriate moisture levels also ensure that the essential oils are adequately mixed throughout the silage, thus preventing areas where spoilage microorganisms can thrive [14].

In this context, the present research was based on the following hypotheses: (1) The use of MON and essential limonene oil (ELO) in TMR formulation inhibits microbial growth, thus reducing lactic acid production and improving aerobic stability without significantly altering the nutritional value of the silage. (2) The effects of MON and ELO on fermentation will be more pronounced in silages with higher moisture content. This study aimed to assess the influence of adding MON and ELO on the quality of the TMR ensilage process, and to identify the main changes in TMR fermentation at different moisture contents.

2. Materials and Methods

2.1. Experimental Assays

The research was split into two assays, with the first focusing only on identifying the possible changes in the fermentation parameters of TMR silages with the addition of MON and ELO, and the second testing the effect of adding MON and ELO under two moisture conditions.

The first assay was carried out in April 2022 at Embrapa (*Agropecuária Oeste*) (22°16'44" S, 54°49'10" W), located in the municipality of Dourados, MS, Brazil. Since that research institution focuses on lamb rearing, the TMR was formulated to meet the nutritional requirements of lambs (20 kg mean body weight) in the growth phase (mean weight gain of 300 g/day) with the intake of 0.7 kg DM/day according to the recommendations by Ref. [15] (Table 1).

Table 1. Proportions of ingredients and chemical compositions of the TMRs formulated in each of the experimental assays.

Ingredients	First Assay	Second Assay
	% of DM	% of DM
Sorghum	35.00	46.56
Ground corn kernel	41.74	28.67
Soybean meal	21.76	22.46
Calcitic lime	0.93	1.18
Dicalcium phosphate	0.56	1.11
Salt	0.01	0.02
Total	100.00	100.00
DM, %	62.45	41.00
CP, % DM	18.10	16.42
NDF, % DM	30.54	39.53
ADF, % DM	21.21	25.14
Starch, % DM	27.05	16.71
EE, % DM	2.21	2.38
Crude Ash, % DM	8.18	6.33
Lignin, % DM	2.80	4.04
NFC, % DM	42.28	36.08
BC, meqg NaOH/100 g DM	24.23	29.32

DM = dry matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether extract; NFC = non-fiber carbohydrate; BC = buffer capacity.

The first assay was conducted using a completely randomized design with four repetitions per treatment for a total of 20 experimental units. The treatments tested in that first assay were control (no additive), MON35 (35 mg of monensina per kg of DM), MON45 (45 mg of monensina per kg of DM), ELO300 (300 mg of essential limonene oil per kg of DM), and ELO600 (600 mg of essential limonene oil per kg of DM). The dose of 35 mg of MON per kg of DM was based on the manufacturer's recommendation. The dose of 45 mg of MON was selected to assess the impact of an overdose on the fermentative quality of TMR silage. The doses of 300 and 600 mg of ELO per kg of DM were determined based on previous experiments [16–18].

The second assay was developed in March 2023 at the Experimental Farm of the Federal University of Grande Dourados (UFGD) (22°13' 52.44'95" S, 54°59' 10.53'72" W) in the municipality of Dourados, MS, Brazil. In the second assay, the TMR was formulated to meet the nutritional requirements of dairy cows (500 kg body weight) in lactation (15 kg milk/day) and mean intake of 14 kg DM/day according to the recommendations by [19] (Table 1).

The second assay followed a completely randomized design in a 5 × 2 factorial arrangement with four repetitions per treatment combination for a total of 40 experimental units. The main factors corresponded to the same treatments of the first assay at two TMR moisture levels (30% and 40% DM). In order to provide a higher moisture level and maintain the proportion of ingredients in TMR formulation, distilled water had to be added when mixing the ingredients in part of the treatments of the second assay.

Both assays used sorghum (*Sorghum bicolor* L. Moench) as a source of roughage and concentrates based on ground corn, soybean meal, dicalcium phosphate, calcitic lime, and table salt. Sorghum was used in the TMR formulation because it is a crop widely used for silage production in the central region of Brazil. The proportion of ingredients used and the chemical composition of the diet in each of the experimental assays are presented in Table 1.

The additives and their respective doses were previously added to the concentrate to facilitate mixing and ensure homogenous distribution in the silage mass. In the first assay, the mix of roughage with concentrate resulted in TMR with mean contents of 62.45% DM due to the higher proportion of concentrate needed to meet the requirements of lambs. In the second assay, roughage had a higher participation and resulted in TMR with mean

contents of 41% DM, very close to the desired DM content of 40%; thus, this TMR was readily used in the experimental silos. To obtain TMR with a content of 30% DM, 171 mL of distilled water was added to the material used in each experimental silo (average of 4.25 kg material per silo). To prevent effluent formation, the material was homogenized while adding the distilled water.

Both assays employed experimental silos built using PVC pipes (10 cm diameter and 50 cm height), with a useful volume of 3.8 L. The material was manually compacted using wood rods. At the bottom of each silo, a layer of approximately 4.5 cm of sand (300 g) was placed for effluent drainage. A fine cotton fabric mesh was used to keep the forage from touching the sand. After filling, the experimental silos were sealed with double-faced (black and white) plastic film and adhesive tape and stored in the laboratory at room temperature (average of 25.15 °C) for 90 days.

During silo filling, TMR samples were collected from each treatment. The first sample, of approximately 300 g, was used to determine the chemical composition, and the second, of approximately 70 g, was frozen for later processing and to determine pH values and buffer capacity.

2.2. Methodologies Employed in the Assays

To calculate fermentation losses, all components in the silos (silo, sand, and fabric) and the TMR mass ensiled were weighed both before and after ensiling. Dry matter recovery, gas losses, and effluent production were calculated according to the equations in [20]. Dry matter recovery was calculated using the following formula:

$$DMR = 100 - \left(\frac{DMI - DMF}{DMI} \times 100 \right) \quad (1)$$

where *DMR* = dry matter recovery (% of the initial dry mass), *DMI* = initial dry mass (kg DM placed into the silos), and *DMF* = final dry mass (kg DM removed from the silos).

Gas losses were calculated using the following formula:

$$GL = \frac{WSI - WSC}{DMI} \times 100 \quad (2)$$

where *GL* = gas losses during storage (% of initial dry mass), *WSI* = initial weight of the closed silo (kg), *WSC* = weight of the closed silo when opened (kg), and *DMI* = initial dry mass (kg DM placed into the silos).

Effluent production was calculated using the following formula:

$$EP = \frac{Wf - Wi}{DMI} \times 1000 \quad (3)$$

where *EP* = effluent production (kg/t dry matter), *Wf* = final combined weight (silo + sand + fabric) in kg, *Wi* = initial combined weight (silo + sand + fabric) in kg, and *DMI* = initial dry mass (kg DM placed into the silos).

Then, the silos were opened and the material inside them was removed and homogenized for sample collection. Chemical analyses were performed using an NIRsTM DS2500 F (Foss NIR Systems, Laurel, MD, USA) spectrophotometer, using a large ring cup and the factory calibrations provided with the instrument (FOSS global calibration). Before spectral measurements, a standard protocol to ensure accuracy was performed; the spectral device was preheated for 30 min, followed by calibration with a white spectral on the calibration panel, followed by measurement of known standards to validate the model by a separate set of samples. For the measurements, labeled samples were filled into the cup and flattened to avoid translucent cavities. All near infrared spectra were collected at wavelengths between 850 and 2500 nm, registering absorbance values $\log(1/R)$ (where *r* = reflectance) at 2 nm intervals for each sample. Concentrations of dry matter (DM), crude ash, crude protein (CP), soluble protein (SP), neutral detergent insoluble protein (NDIP),

acid detergent insoluble protein (ADIP), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin, ether extract (EE), non-fiber carbohydrates (NFC), and starch were obtained via FossManager™ (Hilleroed, Denmark).

A 25 g sample of TMR was diluted in 225 mL of distilled water and manually homogenized for approximately 20 min for the production of an aqueous extract. The extract was used to measure pH both before and after silage, buffer capacity before silage, and the profile of short-chain organic acids. pH was determined using a digital potentiometer (mPA210 MS Tecno, Piracicaba, SP, Brazil), and buffer capacity was determined according to [21].

A portion of that extract was filtered using a paper filter, centrifuged for 15 min at 10,000 rpm, and the supernatant was frozen at -20°C for later analysis of volatile organic acids. The organic acids were determined using gas chromatography with a mass detector (GCMS QP 2010 Plus, Shimadzu, Kyoto, Japan) using a capillary column (Stabilwax, Restek, Bellefonte, PA, USA, 60 m, 0.25 mm \varnothing , 0.25 μm Crossbond Carbowax polyethylene glycol). The lactic acid concentration was determined using the colorimetric method proposed by [22]. The concentration of ammoniacal nitrogen ($\text{NH}_3\text{-N}$) was determined by the Kjeldahl method [23]. In vitro DM digestibility (IVDMD) was determined according to [24], with samples incubated for 48 h.

Aerobic stability (AS) was determined in all silos after they were opened. Samples (2 ± 0.005 kg) of each replicate of each treatment were freely placed in the clean experimental silos. Temperature sensors were placed in the geometric centers of the silages, and a double layer of gauze was placed on top of each experimental silo to prevent drying and contamination while allowing for air penetration. Ambient temperature, as well as the temperature of each silage, was recorded every minute, and the average was calculated every 20 min using a datalogger (RC-4, Elitech®, Canoas, RS, Brazil), which was calibrated before starting the measurements. Aerobic stability was defined as the number of hours required for the silage temperature to reach 2°C above ambient temperature [25].

2.3. Statistical Data Analysis

The data of the first assay were analyzed using the statistical program Sisvar 5.8 (build 92, 2018). Before the actual analysis, data were explored to seek disparate information (“outliers”) and for normality of residuals using the Shapiro–Wilk test. An individual observation was considered an outlier if it exceeded 3 standard deviations from the average. Data that did not attend to the normality premise were subjected to logarithmic [$\text{Log}(X + 1)$] or square root [$\text{RQ}(X + 1/2)$] transformation.

The data of the first assay were analyzed according to the following model:

$$Y_{ik} = \mu + A_i + \varepsilon_{ik} \quad (4)$$

where Y_{ik} = dependent variable, μ = overall average, A_i = effect of different additives (fixed effect; i = Control, MON35, MON45, ELO300, and ELO600), and ε_{ik} = random error associated with each observation ($k = 4$). The statistical significance was set at a p -value lower than 0.05. Post hoc means separation was conducted if the main effect of treatment was significant ($p \leq 0.05$), using the clustering algorithm of the Scott–Knott test [26], and indicated by letters in the table.

The data of the second assay were analyzed using the statistical program Sisvar 5.8 (Build 92, 2018). Assay data were analyzed according to the following model:

$$Y_{ijk} = \mu + A_i + M_j + A \times M_{ij} + \varepsilon_{ijk} \quad (5)$$

where Y_{ijk} = dependent variable, μ = overall average, A_i = effect of different additives (fixed effect; i = Control, MON35, MON45, ELO300, and ELO600), M_j = moisture effect (fixed effect; j = higher and lower), $A \times M_{ij}$ = effect of the interaction between additives and moisture, and ε_{ijk} = random error associated with each observation ($k = 4$). The statistical significance was set at a p -value lower than 0.05.

Post hoc means separation was conducted if the main effect of treatment was significant ($p \leq 0.05$), using the Scott–Knott test, and indicated by letters in the table. When the interaction of factors was significant ($\alpha \leq 0.05$), the factor combinations were read as individual treatment, clustered by Scott–Knott test, and indicated by letters in the figures.

3. Results

3.1. First Assay

No difference ($p > 0.05$) was observed between the treatments tested for gas losses, effluent production, and DM recovery (Table 2). In the first assay, the gas losses varied from 2.03 to 2.88% ensiled DM while mean effluent productions were 5.1 kg/ton DM and DM recovery losses were above 97%.

Table 2. Fermentation losses, final pH, DM recovery, aerobic stability, and silage fermentation profiles of TMRs submitted to different doses of MON and ELO (first assay).

Parameters	Treatment					SEM	<i>p</i>
	Control	MON35	MON45	ELO300	ELO600		
GL, % DM	2.88	2.03	2.27	2.18	2.28	0.35	0.50
EL, kg/t DM	6.27	4.14	5.78	5.89	3.38	1.40	0.53
DMR, %	97.38	98.18	97.92	97.95	98.16	0.26	0.23
Final pH	4.75 a	4.75 a	4.76 a	4.74b	4.73 b	0.01	0.01
Lactic ac., % DM	3.61 b	3.79 b	4.19 b	5.06 a	4.84 a	0.26	<0.01
Ethanol, % DM	0.16 b	0.14 b	0.16 b	0.21 a	0.13 b	0.01	0.03
Acetic ac., % DM	0.76 b	0.97 a	1.05 a	1.12 a	1.08 a	0.05	<0.01
Propionic ac., mg/kg DM	26.33 e	45.09 d	61.52 c	74.84 b	88.78 a	3.19	<0.01
Butyric ac., mg/kg DM	7.45	9.12	7.90	8.01	8.83	1.14	0.83
Isobutyric ac., mg/kg DM	3.10	2.05	1.90	2.93	2.07	0.52	0.36
Isovaleric ac., mg/kg DM	3.37 c	4.72 b	4.56 b	5.22 a	6.20 a	0.39	<0.01
Valeric ac., mg/kg DM	1.06	1.35	1.37	1.22	1.17	0.14	0.55
AS, hours	213.65 c	259.28 b	242.92 b	245.63 b	297.88 a	18.2	<0.01
NH ₃ -N, % TN	10.48 a	8.85 b	8.12 b	8.29 b	7.75 c	0.19	<0.01

TMR with no additive (control); TMR with 35 mg MON/kg DM (MON35); TMR with 45 mg MON/kg DM (MON45); TMR with 300 mg ELO/kg DM (ELO300); TMR with 600 mg ELO/kg DM (ELO600); GL = gas losses; EL = effluent losses; DMR = dry matter recovery; AS = aerobic stability; NH₃-N = ammoniacal nitrogen; TN = total nitrogen; ac. = acid. Means followed by different letters differ according to Scott–Knott test at 5% probability. SEM = standard error of the mean; *p* = *p*-value.

The lowest ($p < 0.05$) final pH values were observed for treatments ELO600 and ELO300, with no differences ($p > 0.05$) in pH values between the control and the treatments added with monensin (Table 2). The silages that received additives ELO300 and ELO600 had the highest concentrations ($p < 0.05$) of lactic acid (average of 4.95% of DM). No significant differences were observed for lactic acid content in the silages from treatments MON35, MON45, or control (Table 2). Acetic acid concentrations were higher ($p < 0.05$) for the silages with additives when compared with the control silages (Table 2). On average, the silages added with MON or ELO had 38.8% higher acetic acid contents when compared with the control treatment. Propionic acid concentrations increased with the use of additives, with the lowest concentration observed for the control treatment followed by MON35, MON45, ELO300, and ELO600, respectively (Table 2).

During the first assay, no difference ($p > 0.05$) was found between the treatments regarding contents of butyric acid, isobutyric acid, and valeric acid, which were, on average, 8.2 mg/kg DM, 2.41 mg/kg DM, and 1.23 mg/kg DM, respectively. Isovaleric acid concentrations were higher ($p < 0.05$) for silages ELO300 and ELO600 at 5.71 mg/kg DM on average.

Aerobic stability was impacted by the additives tested (Table 2). The silage with treatment ELO600 had the highest ($p < 0.05$) AS value (average of 297.88 h), followed by treatments ELO300, MON35, and MON45. The control treatment had the lowest AS at 231.65 h.

The $\text{NH}_3\text{-N}$ contents were the lowest for the treatments with some type of additive (Table 2). The control treatment had the highest $\text{NH}_3\text{-N}$ value with an average of 10.48% of TN. Treatment ELO600 had the lowest $\text{NH}_3\text{-N}$ value. The highest ethanol production was observed for treatment ELO300 (0.21% DM), whereas the other treatments had no significant differences between each other (Table 2).

During the first assay, differences ($p < 0.05$) were observed only for contents of DM, starch, and NFC between the treatments tested (Table 3).

Table 3. Chemical compositions of TMR silages submitted to different doses of MON and ELO (first assay).

Parameters	Treatment					SEM	<i>p</i>
	Control	MON35	MON45	ELO300	ELO600		
DM, %	63.78 a	61.94 b	62.51 b	62.14 b	61.90 b	0.22	<0.01
CP, % DM	16.58	18.00	17.60	19.16	19.18	0.74	0.11
SP, % CP	25.00	33.60	28.00	40.40	38.60	4.15	0.07
DP, % CP	57.20	61.00	56.60	64.40	65.00	2.65	0.09
NDIP, % DM	4.60	4.62	4.58	4.66	4.46	0.19	0.96
ADIP, % DM	1.04	1.02	1.12	0.98	1.10	0.09	0.84
NDF, % DM	28.20	30.86	29.56	31.66	32.42	1.21	0.14
ADF, % DM	20.09	21.08	19.85	21.25	22.25	1.14	0.21
Starch, % DM	30.12 a	27.18 a	28.26 a	25.01 b	24.76 b	1.31	0.04
EE, % DM	2.32	2.10	2.18	2.34	2.14	0.14	0.68
Crude Ash, % DM	8.00	8.24	8.04	8.30	8.36	0.14	0.31
Lignin, % DM	2.38	2.70	2.72	3.00	3.22	0.22	0.13
NFC, % DM	46.32 a	42.31 b	43.14 b	40.11 b	39.54 b	1.56	0.03
IVDMD % DM	85.47	84.26	83.23	83.96	85.05	3.21	0.98

TMR with no additive (control); TMR with 35 mg MON/kg DM (MON35); TMR with 45 mg MON/kg DM (MON45); TMR with 300 mg ELO/kg DM (ELO300); TMR with 600 mg ELO/kg DM (ELO600); DM = dry matter; CP = crude protein; SP = soluble protein; DP = degradable protein; NDIP = neutral detergent insoluble protein; ADIP = acid detergent insoluble protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether extract; NFC = non-fiber carbohydrates; IVDMD = in vitro dry matter digestibility. Means followed by different letters differ according to Scott–Knott test at 5% probability. SEM = standard error of the mean; *p* = *p*-value.

The use of additives (MON and ELO) led to silages with lower DM contents when compared with the control treatment. Starch contents were lower ($p < 0.05$) for treatments ELO300 and ELO600 (average of 24.88% of DM) when compared with MON35, MON45, and control (average of 28.52% of DM), which did not differ ($p > 0.05$) (Table 2). Contents of NFC were lower ($p < 0.01$) in the silages with some type of additive (MON or ELO), regardless of the dose employed.

3.2. Second Assay

In the second assay, differences ($p < 0.05$) were observed between the additives for gas losses (Table 4). The highest values were found for treatments MON35 and ELO600, which were 77.28% higher than in the other treatments tested. The more humid silages had gas loss values of 134.57% higher when compared with those with lower moisture. No differences ($p > 0.05$) were observed between the additives for effluent production, which were the highest in the silages with high moisture contents (average of 59.2 kg/t DM) (Table 4).

Table 4. Fermentation losses, pH, aerobic stability, and silage fermentation profiles of TMRs with different moisture contents and different doses of MON and ELO (second assay).

Parameters	Additives (A)					Moisture (M)		SEM	P		
	Control	MON35	MON45	ELO300	ELO600	High	Low		A	M	A * M
GL, % DM	1.37 b	2.13 a	1.32 b	1.39 b	2.72 a	2.51	1.07	0.22	0.03	<0.01	0.06
EL, kg/t DM	28.88	36.24	35.83	37.90	32.85	59.20	4.68	2.45	0.25	<0.01	0.41
DMR, % DM	94.58	95.29	95.95	94.06	92.66	91.45	97.56	0.38	<0.01	<0.01	<0.01
Final pH	4.01	4.07	4.05	3.96	4.17	4.06	4.04	0.03	<0.01	0.58	<0.01
Lactic ac., % DM	5.50	6.41	6.58	7.19	5.79	6.51	6.08	0.18	<0.01	0.01	<0.01
Acetic ac., % DM	0.88	0.77	0.76	0.97	1.54	1.05	0.91	0.04	<0.01	<0.01	<0.01
Lat/Ac ratio	6.50	8.4	8.94	7.49	4.77	7.64	6.8	0.4	<0.01	0.02	<0.01
Ethanol, % DM	0.23	0.28	0.31	0.26	0.25	0.33	0.20	0.02	0.37	0.01	0.42
Propionic ac., mg/kg DM	243.81 a	118.59 b	119.77 b	67.28 b	111.51 b	85.39	179.00	28.62	<0.01	<0.01	0.08
Butyric ac., mg/kg DM	76.00 a	122.89 a	44.64 b	17.42 b	18.33 b	75.66	36.07	19.27	<0.01	0.02	0.35
Isobutyric ac., mg/kg DM	38.89 c	59.11 b	75.54 a	49.90 c	38.43 c	44.27	60.49	3.04	<0.01	<0.01	0.06
Valeric ac., mg/kg DM	51.44 a	19.49 b	10.59 b	8.32 b	6.26 b	17.56	20.89	4.19	<0.01	0.37	0.58
Valeric ac., mg/kg DM	55.71 a	20.99 b	8.72 c	5.74 c	3.66 c	18.72	19.22	4.72	<0.01	0.90	0.57
AS, hours	83.94	91.56	89.13	88.94	92.35	88.55	89.81	0.607	<0.01	0.02	<0.01
NH ₃ -N, % of TN	8.46 b	8.56 b	8.58 b	8.58 b	9.34 a	9.09	8.48	0.21	<0.01	<0.01	0.06

TMR with no additive (control); TMR with 35 mg MON/kg DM (MON35); TMR with 45 mg MON/kg DM (MON45); TMR with 300 mg ELO/kg DM (ELO300); TMR with 600 mg ELO/kg DM (ELO600); GL = gas losses; EL = effluent losses; DMR = dry matter recovery; ac. = acid; Lat/Ac ratio = acetic/lactic acid ratio; AS = aerobic stability; NH₃-N = ammoniacal nitrogen; TN = total nitrogen; high moisture = 30% of DM; low moisture = 40% of DM. Means followed by different letters differ according to Scott–Knott test at 5% probability. SEM = standard error of the mean.

Significant interaction was found between TMR silage humidity and the additives tested for DMR (Figure 1). Overall, the silages with high moisture content had the lowest ($p < 0.05$) DM recovery values, especially ELO600 at only 88% DM recovery. Among the silages produced with high moisture content, the treatments using MON had the highest DM recovery values. No differences ($p > 0.05$) were observed in DM recovery for the silages with low moisture content, which were all above 97%.

An interaction ($p < 0.05$) was found between the additives tested and the moisture contents of the TMR silages, where ELO600 in the TMR with the highest moisture resulted in the highest pH values (average of 4.31) (Figure 1). The other treatments tested, irrespective of moisture (including ELO600 in the silages with lower moisture) did not differ ($p > 0.05$), with a mean pH of 4.02.

The silage with the highest ($p < 0.05$) lactic acid content (average of 8.1% of DM) was ELO300, with higher moisture (Figure 1). Only treatment ELO600 had higher ($p < 0.05$) lactic acid production in the drier silage when compared with the more humid one. For the other additives, the silages with higher moisture content always had higher lactic acid production.

Lactic acid concentration was higher ($p < 0.05$) for silage ELO600 with higher moisture (2.11% of DM) (Figure 1). The opposite behavior was observed for the control, MON35, and MON45 treatments, which had the lowest acetic acid values for the silages with higher moisture content. On average, treatment ELO600 in silages with higher moisture produced 2.45 times more acetic acid compared with the average of the other treatments.

The silages with the highest ($p < 0.05$) lactic acid/acetic acid ratios were the ones added with MON with higher moisture (Figure 1). Only ELO600 had a higher ($p < 0.05$) lactic acid:acetic acid ratio in the silage with lower moisture when compared with the silage with higher moisture. For the other additives tested, the lactic acid:acetic acid ratios were always higher or similar in the silages with higher moisture when compared with those with lower moisture. The silages with higher moisture added with ELO600 had the lowest lactic acid:acetic acid ratios (average of 2.3).

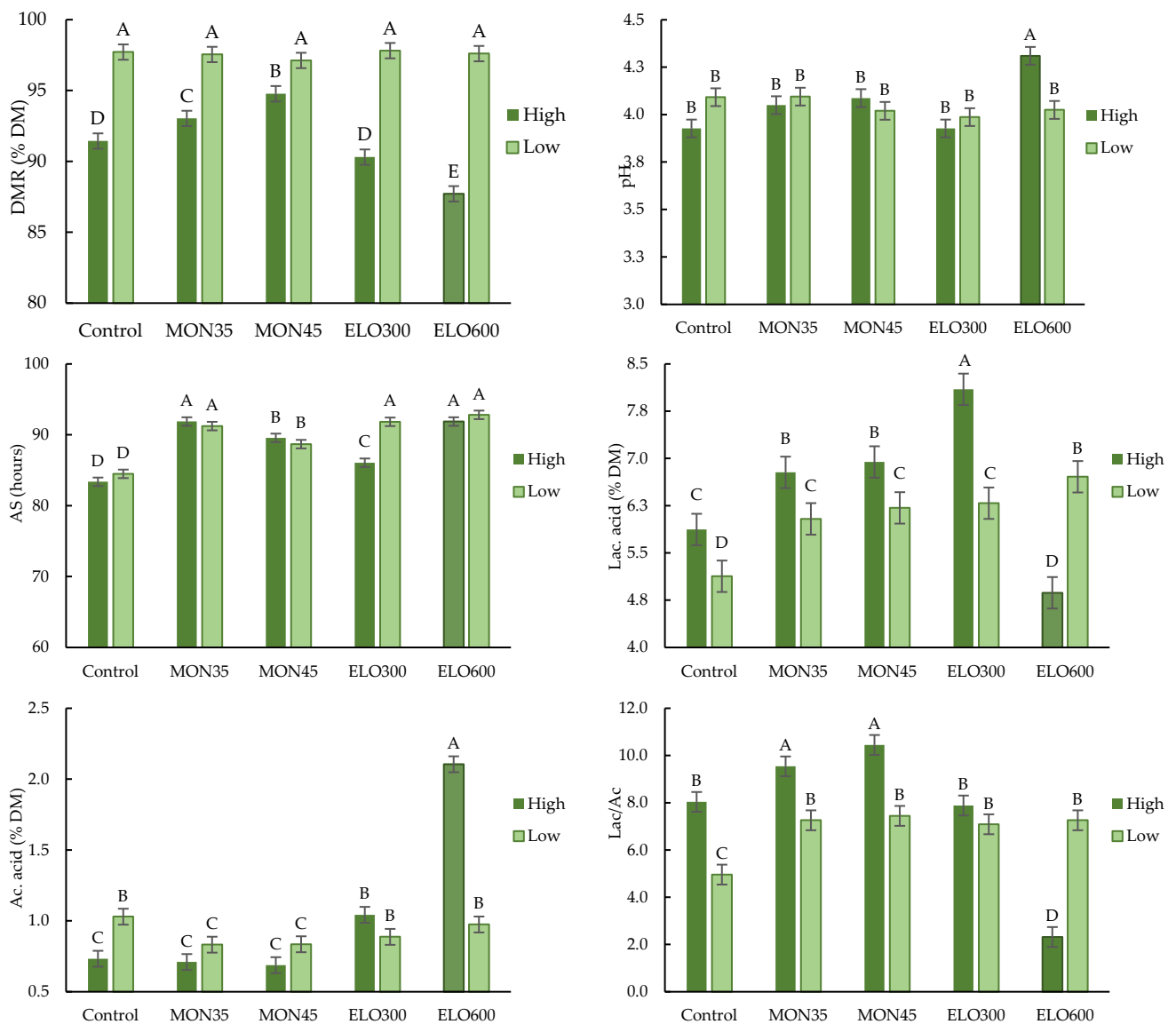


Figure 1. DM recovery (DMR), potential of hydrogen (pH), aerobic stability (AS), acetic acid content (Ac. acid), lactic acid content (Lac. acid), and acetic acid:lactic acid ratio (Lac/Ac) of the TMR silages with higher and lower moisture and MON and ELO doses (second assay). High moisture = 30% of DM; low moisture = 40% of DM. Means followed by different letters differ according to Scott–Knott test at 5% probability. I = standard error of the mean.

The additives tested did not impact ($p > 0.05$) ethanol production, while higher ethanol values were found for silages with higher moisture compared with those with lower moisture (Table 4). A difference ($p < 0.05$) was found between the silages with higher and lower moisture for propionic and butyric acid contents (Table 4). The highest butyric acid concentrations (75.66 mg/kg DM) were observed for the silages with higher moisture, whereas the opposite comparison was observed for the concentrations of propionic and isobutyric acids. Overall, the control treatment had the highest concentrations of propionic, butyric, isovaleric, and valeric acids, with a significant reduction in these acids observed when MON and ELO were added.

The $\text{NH}_3\text{-N}$ contents in the silages tested were impacted ($p < 0.05$) by the additives and moisture contents of the TMRs. Overall, the silages with higher moisture had the highest $\text{NH}_3\text{-N}$ values (9.09% of TN) compared with those with lower moisture (8.48% of TN). The

control, MON35, MON45, and ELO300 treatments had the lowest $\text{NH}_3\text{-N}$ contents, while ELO600 was the treatment with the highest $\text{NH}_3\text{-N}$ value.

Aerobic stability was affected by the interaction ($p < 0.05$) between the additives and moisture contents of the TMRs tested (Figure 1). Regardless of the moisture tested, the lowest AS values were observed in the control treatment, with an average of 83.5 h for AS failure. Both ELO600 and MON35 had the best AS values (>92 h), irrespective of silage moisture. The silages with higher moisture added with ELO300 had lower AS (average of 85.33 h) compared with those with higher moisture (average of 91.84 h).

The silages with lower moisture had the highest ($p < 0.05$) DM, NDF, and ADF contents, whereas those with higher moisture had the highest ($p < 0.05$) EE values (Table 5). All additives tested resulted in lower fiber contents and higher starch contents. Ether extract contents were higher in the treatments added with MON (Table 5). The higher IVDMD coefficient (77.56% of DM) was observed in the silages added with MON35, followed by MON45, ELO300, and ELO600 with intermediate values and the control treatment with the lowest IVDMD value (71.09% of DM) (Table 5).

Table 5. Chemical compositions of TMR silages with different doses of MON and ELO submitted to higher and lower moisture (second assay).

Parameter	Additives (A)					Moisture (M)		SEM	<i>p</i> -Value		
	Control	MON35	MON45	ELO300	ELO600	High	Low		A	M	A * M
DM, %	36.28	37.05	36.35	35.51	35.55	30.49	41.81	0.54	0.27	<0.01	0.39
Crude Ash, % DM	6.43	6.69	6.95	6.73	6.88	7.13	6.34	0.21	0.46	0.06	0.12
CP, % DM	15.62	16.27	16.86	16.97	16.43	16.76	16.52	0.21	0.24	0.43	0.38
SP, % CP	47.38	47.13	49.25	47.25	48.25	50.85	44.85	1.26	0.73	0.62	0.87
NDIP, % DM	19.04	18.53	18.58	18.34	18.73	17.26	20.03	0.81	0.98	0.14	0.64
ADIP, % DM	5.41	6.24	6.05	5.81	5.96	5.12	6.67	0.43	0.73	0.24	0.70
NDF, % DM	41.21 a	38.25 b	38.01 b	37.67 b	37.07 b	37.75	39.54	0.91	0.02	0.03	0.90
ADF, % DM	26.78 a	24.40 b	24.80 b	24.75 b	23.99 b	24.62	25.67	0.52	0.01	0.03	0.89
Lignin, % DM	4.16	3.71	3.88	3.84	3.95	3.77	4.05	0.22	0.69	0.17	0.27
Starch, % DM	16.36 b	18.55 a	18.72 a	18.45 a	19.72 a	20.01	16.72	0.68	0.02	<0.01	0.15
NFC, % DM	34.50	36.06	36.16	36.71	37.23	36.18	36.09	0.72	0.11	0.88	0.61
EE, % DM	2.77 b	3.11 a	3.21 a	2.98 b	2.87 b	3.59	2.39	0.07	<0.01	<0.01	0.42
IVDMD, % DM	71.09 c	77.56 a	73.09 b	72.73 b	72.38 b	74.26	72.79	0.86	<0.01	0.06	0.07

TMR with no additive (control); TMR with 35 mg MON/kg DM (MON35); TMR with 45 mg MON/kg DM (MON45); TMR with 300 mg ELO/kg DM (ELO300); TMR with 600 mg ELO/kg DM (ELO600); DM = dry matter; CP = crude protein; SP = soluble protein; NDIP = neutral detergent insoluble protein; ADIP = acid detergent insoluble protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; NFC = non-fiber carbohydrates; EE = ether extract; IVDMD = in vitro dry matter digestibility; high moisture = 30% of DM; low moisture = 40% of DM. Means followed by different letters differ according to Scott–Knott test at 5% probability. SEM = standard error of the mean.

4. Discussion

4.1. First Assay

The gas loss values observed for all treatments tested were less than 3% of the ensiled DM, which is characteristic of silages with good fermentative quality (prevalence of lactic fermentation), according to [12]. Effluent production in this assay was minimal, which is common in silages with high DM content [11,27]. According to [2], high DM contents (above 40%) contribute to the conservation of the ensiled material and increase DM recovery values. Similar data were reported by [11], who found DM recovery values above 96% when ensiling TMR with DM contents between 60 and 62%.

pH is an important parameter to assess the silage fermentation quality, as it is directly related with the total organic acids produced [28,29]. Silages with higher DM values, i.e., low water activity, result in higher final pH values (lower fermentation intensity) [30]. This explains the pH values observed in this assay (average of 4.74). According to [4,5,31], pH values between 4.0 and 5.0 are common in silages with DM contents above 40%, with such a pH range being sufficient to control the growth of undesirable microorganisms (particularly mold, yeast, and *Clostridium* spp.).

When assessing several studies in the literature, [18] concluded in their meta-analysis that essential oils act by inhibiting the growth of undesirable microorganisms in the silages (particularly molds and yeasts), thus improving AS. This matches the results obtained in this research, especially with the highest ELO dose (600 mg/kg DM). To a lesser extent, MON also improved silage AS compared with the control treatment. The authors of [11] observed that TMR silages with 33 mg MON/kg DM had AS values above 240 h, which corroborated the data obtained in this assay.

Another aspect that helps explain the higher AS of the treatments with MON and ELO was acetic acid production. According to [25], a major characteristic of acetic acid is to inhibit the growth of molds and yeasts and, at moderate amounts, it can contribute to improving silage stability. The mean normal concentration of acetic acid in the silage is 3 to 4% of DM, a value that is lower (0.5 to 2%) in silages with higher DM contents (45 to 55% DM) [25].

The treatments added with ELO, at either dose, had the highest lactic acid concentrations; this contradicts most data found in the literature on the use of essential oils in silages [18]. However, Ref. [32] observed that adding 300 mg/kg cumin EO had a selective antimicrobial effect, decreasing the number of harmful microorganisms (molds and yeasts) and increasing the number of beneficial microorganisms (lactic acid bacteria). Such a situation led to higher lactic acid productions and lower pH values, which corroborated the data obtained in the present research.

The control treatment had the highest butyric acid and $\text{NH}_3\text{-N}$ contents, suggesting the additives tested helped inhibit undesirable fermentations. In the research by [32] with cumin EO in oat silage, those authors also observed a reduction in $\text{NH}_3\text{-N}$ contents when adding essential oil. Essential oils help reduce proteolysis in silages primarily due to their antimicrobial and antioxidant properties. Compounds such as terpenes, phenols, and aldehydes in essential oils inhibit the growth of proteolytic microorganisms and the activity of proteolytic enzymes responsible for protein degradation during fermentation [10]. Additionally, by improving the aerobic stability of the silage, essential oils reduce the proliferation of microorganisms that cause deterioration when the silage is exposed to air, thus preserving the original quality of the proteins [18].

The antioxidant action of essential oils also plays a crucial role in protecting proteins from oxidative damage. Antioxidants present in essential oils neutralize free radicals and inhibit oxidation reactions that could degrade proteins and other nutrients in the silage [33]. Consequently, essential oils significantly contribute to preserving the nutritional quality of the silage, resulting in a final product with higher nutritional value for animals.

Similar to essential oils, monensin inhibits protein degradation by targeting Gram-positive proteolytic bacteria [34]. This antimicrobial effect fosters the growth of Gram-negative bacteria, which produce propionate rather than ammonia, thereby preserving more dietary protein for absorption [6]. While the $\text{NH}_3\text{-N}$ level is an indicator of silage quality, it is noteworthy that the values obtained in this study for all treatments were close to the ideal range (10% of total nitrogen), as recommended in the literature [12,25,29].

According to [29], some heterofermentative bacteria can produce ethanol from the fermentation products or substrates present in the medium, which explains the higher ethanol contents in the treatments added with MON and ELO. Nonetheless, the ethanol values obtained in both assays can be considered low and did not negatively impact the quality of the fermentation process [35].

Using the ELO and MON additives led to a slight reduction ($p < 0.05$) in the DM contents of the silages. It is known that determining DM in a forced-air oven causes partial loss of the volatile fatty acids produced during ensiling; hence, lower DM values are commonly observed in silages with higher organic acid contents [36]. This may help explain why the silages added with ELO and MON (higher organic acid productions) had the lowest DM values.

In the literature, some studies also observed reductions in starch and NFC contents during TMR ensiling, similarly to the findings in the present study. Researchers in [3] found

reductions by 5.5% in starch contents in TMR based on corn kernels. According to those authors, some types of microorganisms present in the silages are able to use the starch as substrate for the production of lactic acid, acetic acid, and ethanol, and the concentrations of those compounds commonly increase in the final product. The silages added with ELO300 and ELO600 had the highest lactic acid concentrations (37.11% higher than in the control treatment) and the highest starch reductions, which corroborates the statements by [3].

The small variations in nutrient levels observed in the first trial can be explained by the lower fermentation intensity in silages with low moisture content. Moisture limitation restricts the activity of microorganisms responsible for undesirable fermentations, leading to better preservation of nutrients in the silage. Additionally, in low moisture conditions, effluent production is reduced, which decreases the loss of soluble nutrients through leaching.

4.2. Second Assay

4.2.1. Effect on Fermentation Parameters

Although higher, the GL in treatments MON35 and ELO600 were below 3% of the ensiled DM, which is considered adequate according to [12]. Ref. [4] observed that TMR with higher moisture (23% of DM) associated with heterofermentative microbial additives resulted in slightly higher GL (3.7% of DM), while drier TMR silages with no inoculants (32% of DM) had lower GL values (3.1% of DM), which corroborated the data obtained in this research.

Since silage moisture is directly correlated with EL, higher EL values were expected for the more humid TMRs. According to [12], excess effluent is avoided in most cases when the DM content of the material is above 35%. The higher effluent production in the more humid silages likely contributed to their lower DM recovery values.

According to [12], lactic fermentation results in silages with minimum DM and energy losses, whereas acetic, alcoholic, and butyric fermentations result in silages with higher DM and energy losses, i.e., lower DM recovery values. Treatment ELO600, with higher moisture, experienced more intense heterofermentation and used the sugars present in the medium for higher acetic acid production, which explains the lower DM recovery values.

The more humid silages had higher lactic acid concentrations in the control, MON35, MON45, and ELO300 treatments. However, treatment ELO600, with higher moisture, resulted in a significant reduction in lactic acid production and increased pH, which was not found when the same dose was employed in drier TMR silages. The higher EO dose (600 mg/kg DM), in association with the higher medium moisture, likely potentialized antimicrobial action and favored the growth of heterofermentative bacteria (more resistant to ELO), in detriment of homofermentative bacteria (less resistant to ELO).

Limonene, an important compound of several citric oils, acts as a bactericidal and fungicidal agent by disrupting the cell membrane, increasing its permeability, and causing the loss of essential ions, leading to cell death [16]. Additionally, it inhibits ergosterol synthesis in fungal cells and can induce oxidative stress, generating reactive oxygen species that damage DNA, proteins, and lipids. These mechanisms make limonene an effective antimicrobial agent with applications in food preservation, agriculture, and medicine. Studies have shown that limonene has significant antibacterial activity against several bacterial species, including food-related microorganisms such as *Lactobacillus plantarum* and *Lactobacillus brevis*, common in silage [16].

The meta-analysis by [18] concluded that not only do essential oils inhibit the growth of bacteria harmful to the silage (especially cumin and oregano), but they also inhibit the growth of bacteria that produce the main organic acids. However, most studies used by [18] were conducted on silages with moisture contents between 22 and 35%, i.e., silages with higher moisture that likely had more effective antimicrobial action by the EOs, particularly at higher doses, which corroborates the data observed in the present research.

The higher lactic acid:acetic acid ratios observed when adding MON allow concluding that the additive modulated fermentation in the TMRs and prioritized homofermentation.

That is a positive result, since it allows using MON in the formulation of TMR to be ensiled aiming at improving the fermentation process. Moreover, [11] found that MON does not undergo microbial breakdown during the ensiling process and remains active in the diets for later action in the rumen.

A synergistic effect likely took place between the low moisture of the silages and the antimicrobial action of limonene, thus reducing the activity of undesirable bacteria, which explains the lower butyric acid values in the silages with ELO300 and ELO600. Although the control treatment had the highest butyric acid and $\text{NH}_3\text{-N}$ concentrations, they were within the acceptable range (1 g butyric acid/kg DM and 10% TN) for silages with good nutritional and sanitary quality [12,28,29]. The divergent results regarding $\text{NH}_3\text{-N}$ content can be explained by the difference in fermentation intensity of the two trials. The DM content of the first trial contributes to a lower population of *Clostridium* spp., an $\text{NH}_3\text{-N}$ -forming bacteria [37]. In the second trial, it is possible that the additive interacted with the moisture content of the material, which could be favorable for the population of saccharolytic *Clostridium* (ferment sugars into butyric acid) in the cases of the control and MON treatments, or proteolytic *Clostridium* (NH_3 -forming) in the case of ELO use [25]. This makes it clear that the choice of additive should be linked to the dry matter content of the material to be ensiled.

4.2.2. Aerobic Stability

As in the first assay, adding ELO and MON likely helped reduce the growth of undesirable microorganisms after the silages were opened (especially molds and yeasts), thus improving their AS values, especially in treatments ELO600 and MON35. It is noteworthy that, in the second assay, the silages had higher moisture and, therefore, the AS values were much lower than those obtained in the first assay. Therefore, it can be said that using MON and ELO is a more interesting strategy to preserve the quality of TMR silage, with higher moisture after opening.

Greater aerobic stability in silage offers several advantages, including preserving nutritional value by preventing the growth of undesirable microorganisms, extending the silage life after opening, reducing waste, improving animal health due to lower presence of mycotoxins, saving on feed costs, and ensuring consistency in animal diets, leading to better performance in milk and meat production [38]. These benefits make aerobic stability a crucial factor for producing high-quality silages efficiently.

4.2.3. Impact on Nutrient Composition

The reductions in NDF and ADF contents when adding MON and ELO were likely due to higher activity of fibrolytic enzymes during the fermentation process. In an experiment with alfalfa and cumin EO at 300 and 500 mg/kg DM, Ref. [32] observed reductions in NDF and ADF contents in relation to the control treatment. According to those authors, the doses employed stimulated enzyme activities that promoted cell-wall breakage and released saccharose into the medium. Such saccharose release was likely used as substrate by the LAB to produce lactic acid [29]. The same behavior was observed by [39] when adding cumin EO at 200, 300, and 500 mg/kg to oat silages. Those authors observed that cumin EO significantly reduced the cellulolytic fraction of the silages.

Ref. [2] reported that the partial break of the cell wall is favored in silages with higher moisture and that the cell wall components can be used as substrate for silage fermentation. This helps explain why, in the present experiment, NDF and ADF contents were lower in the silages with higher moisture when compared with those with lower moisture.

The increase in starch content in the TMR silages added with MON and ELO is likely related to the reduction in prolamins, the layer that protects the starch in the seed. When reviewing TMR ensiling for ruminants, Ref. [2] argued that TMR silage fermentation promotes a positive effect on protease activity, favoring prolamins reduction and, consequently, increasing starch availability in the silage. The lower prolamins content may increase the efficiency of starch detection, since the standard technique depends on the hydrolysis of

starch granules [40]. Therefore, if prolamin impacts starch detection, that error will be intrinsic in the NIR calibration equations as they are derived from the extension of the conditions observed in the standard technique [41]. Another factor that may help explain the higher starch contents is the dilution effect, since the decrease in fiber contents increases the proportion of other non-fiber nutrients in the silage, such as starch.

The higher EE contents observed in the treatments with ELO were also reported in other research in the literature [42–44]. When studying the origin of lipolysis in alfalfa silages, Ref. [45] found that the decrease in total fatty acid contents during ensiling took place mainly due to the breakdown of fatty acids C18:2n-6 and C18:3n-3. According to those authors, plant enzymes play a major role in lipolysis during alfalfa ensiling; however, several epiphyte microorganisms in alfalfa contributed much more to lipolysis. In that same research, the authors showed that homofermentative LAB purely inoculated in alfalfa silages did not impact the lipolysis of the material, but rather only the epiphytic flora contributed to it. This finding by [45] may help explain why treatments MON35 and MON45 preserved EE contents more, since, during TMR ensiling with MON, homolactic fermentation (higher lactic acid:acetic acid ratios) prevailed, thus decreasing the lipolytic action of other bacterial groups.

The increase in IVDMD in the TMR silage added with MON35 may be related to the fiber contents in that silage. Since the activity of fibrolytic enzymes was higher in that treatment, the production of other more digestible compounds, among which is lactic acid, may also have been higher. Following that assumption, the silages added with MON45, ELO300, and ELO600 may also have had the same stimulus for greater breakdown of the fibrous fraction, albeit at lower intensity. Improving feed efficiency in ruminants leads to greater weight gain or milk production, thus reducing production costs. These benefits result in more efficient, economical, and sustainable animal production.

The data obtained in this research are important, as they show growth-promoting additives can be added to TMR formulations with no harm to the fermentation process or nutritional quality of the silage. However, the effect of ionophores and essential oils on the dynamics of microbial populations over the ensiling process must still be better studied. Moreover, further studies are needed with other types of ionophores (lasalocid) and essential oils (eugenol, carvacrol, thymol, and cumin) commonly used in animal diets.

5. Conclusions

Strategic use of MON and ELO may effectively improve TMR silage quality by increasing acids production and aerobic stability, and have potential benefits for ruminant nutrition and production profitability. We demonstrated that ELO interacts with the silage moisture content on its effect on fermentation quality. Therefore, when silages present DM content above 40%, it is recommended to add the higher dose (ELO600), and for TMR silages with DM content below 30%, it is recommended to add the lower dose (ELO300).

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Abbreviations

TMR with no additive (control); TMR with 35 mg MON/kg DM (MON35); TMR with 45 mg MON/kg DM (MON45); TMR with 300 mg ELO/kg DM (ELO300); TMR with 600 mg ELO/kg DM (ELO600); GL = gas losses; EL = effluent losses; DMR = dry matter recovery; Ac. = acid; Lat/Ac ratio = acetic/lactic acid ratio; AS = aerobic stability; NH₃-N = ammoniacal nitrogen; TN = total nitrogen; DM = dry matter; CP = crude protein; SP = soluble protein; NDIP = neutral detergent insoluble protein; ADIP = acid detergent insoluble protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether extract; NFC = non-fiber carbohydrates; IVDMD = in vitro dry matter digestibility.

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