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'Alecrim Pimenta' nanoformulated essential oil (*Lippia sidoides*) as additive in consortium silages

Óleo essencial nanoformulado de alecrim pimenta (*Lippia sidoides*) como aditivo em silagens consorciadas

Bráulio Maia de Lana Sousa[1*], Sebastião de Jesus Santos[1], Alfredo Acosta Backes[1], Camilla Mendonça Silva[1], Jailson Lara Fagundes[1], Arie Fitzgerald Blank[1], José Ricardo dos Santos Filho[1]

[1]Universidade Federal de Sergipe (UFS), São Cristóvão, Sergipe, Brazil.
[*]Corresponding author: brauliosousa@academico.ufs.br

Abstract
The presence of spoilage microorganisms in forage interferes with the fermentation process of silage, due to competition with lactic acid bacteria for substrate, generating losses and influencing the nutritional value of the ensiled material. Thus, the objective is to evaluate the effect of "Alecrim Pimenta" essential oil (*Lippia sidoides*) and nanoformulated thymol on microbiological, fermentative and aerobic stability profile of sorghum (*Sorghum bicolor* cv. BRS Ponta Negra) silage intercropped with Paiaguás grass (*Urochloa brizantha* cv. BRS Paiaguás). A 4 x 3 factorial design was adopted, with four additives applied to the silages (control treatment; nanoformulated "Alecrim Pimenta" essential oil (OEN); 62% nanoformulated thymol; and 100% pure nanoformulated thymol), associated with three silo period times (15, 30 and 45 days), with five replications per treatment, totaling 60 mini silos. The *Clostridium* population was higher in the control treatment and in the OEN. The *Lactobacillus* population decreased with the increase in silo opening time. Higher aerobic stabilities were recorded in silages with 100% nanoformulated thymol with opening at 15 days; and silages with 62% nanoformulated Thymol (opening period at 30 and 45 days). Silages with 100% thymol provided higher losses of dry matter, gases and effluents, while the use of OEN provided lower losses of dry matter and gases. Silos opened at 45 days showed higher losses of dry matter, gases and effluents. Sorghum and Paiaguás grass silages that received nanoformulated thymol were more efficient in controlling *Clostridium* and *Lactobacillus* populations, and this additive improved the aerobic stability of the silage.
Keywords: aerobic stability; silage microbiology; sustainability; thymol

Resumo
A presença de microrganismos deterioradores na forragem interfere no processo fermentativo da silagem, devido a competição com as bactérias ácido láticas por substrato, gerando perdas e influenciando o valor nutritivo do material ensilado. Assim, objetiva-se avaliar o efeito do óleo essencial de Alecrim pimenta (*Lippia sidoides*) e do timol nanoformulado sobre perfil microbiológico, fermentativo e estabilidade aeróbia de silagem do consórcio de Sorgo (*Sorghum bicolor* var. Ponta Negra) com capim Paiaguás (*Urochloa brizantha* cv. Paiaguás). Foi adotado esquema fatorial 4 x 3, quatro aditivos aplicados nas silagens (tratamento controle; óleo essencial de Alecrim pimenta nanoformulado (OEN); timol nanoformulado 62%; e timol nanoformulado 100% de pureza), associados a três tempos de abertura do silo (15, 30 e 45 dias), com cinco repetições por tratamento, totalizando 60 mini silos. A população de *Clostridium* foi maior no tratamento controle e no OEN. A população de *Lactobacillus* reduziu com o aumento no tempo de abertura do silo. Maiores estabilidades aeróbica foram registradas em silagens com timol nanoformulado 100% com abertura aos 15 dias; e silagens com Timol nanoformulado 62% (tempos de abertura aos 30 e 45 dias). Silagens com timol 100% proporcionaram maiores perdas de matéria seca, gases e efluentes, enquanto que o uso de OEN proporcionou menores perdas de matéria seca e gases. Silos com abertura aos 45 dias apresentaram maiores perdas de matéria seca, gases e efluentes. Silagens de Sorgo e capim Paiaguás que recebram timol nanoformulado foram mais eficientes em controlar as populações de *Clostridium* e *Lactobacillus*, bem como este aditivo melhorou a estabilidade aeróbica da silagem.
Palavras-chaves: estabilidade aeróbica; microbiologia da silagem; sustentabilidade; timol

1. Introduction

The essential oil extracted from "Alecrim pimenta" (*Lippia sidoides* Cham.) has fungicidal, bactericidal, molluscicidal, and larvicidal properties [1,2], mainly due to the high content of thymol (approximately 62%), its major constituent [3]. Notably, these specificities allow essential oils to be applied in preserved forages as a chemical additive [4], with possible antifungal and bactericidal character [5], improving silage fermentation quality, especially because of the low environmental persistence and low toxicity of their chemical constituents [6].

Despite the pharmacological potential of essential oils, relatively few studies have reported their use as additives for forage preservation. Soycan-Önenç et al. [7] showed that mold formation was inhibited in the aerobic period by the addition of cinnamon essential oil at 400 mg/kg fresh forage in field pea silage. However, when this

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substance was combined with thymol, an increase in crude protein and dry matter, a decrease in CO_2 content, and inhibition of mold formation after aerobic exposure were observed. Besharati et al. [8] investigated the effects of essential oils applied as an additive in lucerne silage on aerobic stability and fermentation patterns, and revealed a significant reduction in silage pH.

In this context, to enhance the antimicrobial and antifungal bioactivity of essential oils, nanoformulation is a promising approach for designing new materials at the nanometer scale [9]. Indeed, it is possible to improve the characteristics of active ingredients, water solubility, storage form and thermal stability, allowing the controlled release of valuable compounds, such as thymol in reduced concentrations [10, 11].

In view of the importance of essential oils, this study supports the hypothesis that the nanoformulated "Alecrim pimenta" essential oil can improve the fermentation process when applied to sorghum silages with Paiaguás grass; and that its bioactivity can be ascribed only to the nanoformulated thymol. Thus, the present study aimed to determine the effects of the nanoformulated "Alecrim pimenta" (*Lippia sidoides*) essential oil, 62% nanoformulated thymol and 100% pure nanoformulated thymol on the microbiological and fermentative profile of sorghum silage intercropped with Paiaguás grass.

2. Material and methods

This study was carried out in the municipality of São Cristóvão, Sergipe, Brazil (10°55′27′′ S, 37°11′57′′ W and altitude 5 m), from May 2018 to May 2019. According to the Köppen classification, the climate of the region is Aw tropical. The average annual rainfall in São Cristóvão is 1,200 mm, with an average temperature of 25.5 ºC and average relative humidity of 75%.

The experiment was conducted in a completely randomized block design, in a 4 x 3 factorial arrangement, with four types of antimicrobial agents added to the ensiled material of sorghum intercropped with Paiaguás grass (control group with water; nanoformulated "Alecrim pimenta" (*Lippia sidoides*) essential oil; 62% nanoformulated thymol; and 100% pure nanoformulated thymol); associated with three silo opening times (15, 30, and 45 days). Each treatment consisted of five replications, totaling 60 experimental units (PVC mini-silos).

Lippia sidoides essential oil was purchased from Produtos Naturais Ltda (Pronat Ltda, Ceará, BR). The phytochemical composition was determined using a GC-MS/FID (QP2010 Ultra, Shimadzu Corporation, Kyoto, Japan) equipped with an AOC-20i autosampler (Shimadzu).

The phytochemical analysis showed the presence of thymol (62.05); p-cymene (13.65); (E)-caryophyllene (6.74); myrcene (3.51); γ-terpinene (2.15); α-terpinene (1.25); thymol methyl ether (1.14); terpinen-4-ol (0.71); α-thujene (0.50); α-terpineol (0.32); α-pinene (0.64); caryophyllene oxide (0.70); carvacrol (0.63); linalool (0.30); 3-thujen-2-one (0.21); 6,7-epoxymyrcene (0.12); (E)-β-ocimene (0.14); (Z)-β-ocimene (0.12); isoterpinolene (0.15); BHA (0.22); viridiflorene (0.44); β-bisabolene (0.14); α-cadinene (0.19); oct-1-en-3-ol (0.14); α-copaene (0.31); aromadendrene (0.55); α-humulene (0.34); limonene (0.78); 1,8-cineole (0.61); α-3-carene (0.21); γ-elemene (0.14); p-cymenene (0.11); and <0.1% of [isopentyl acetate, 2-methylbutyl acetate, camphene, α-pinene, α-phelandrene, cis-sabinene hydrate, 3-thujen-2-one, butyl acetate and miltail-4(12)-ene].

The nanoformulation of *Lippia sidoides* essential oil and thymol was carried out at the Pharmacotechnical Development Laboratory (Department of Pharmacy, Federal University of Sergipe). A surfactant and co-surfactant mixture was prepared at a 1:1 ratio of Tween 80 and Propylene glycol under magnetic stirring for 10 minutes. Then, the active ingredient (*Lippia sidoides* essential oil - oil phase) of density 0.983 g/mL was slowly added. The mixture of surfactant-cosurfactant, water, and oil (ratio of 9.0:0.5:0.5, respectively) was homogenized by magnetic stirring at room temperature [12]. The system was kept under agitation until complete homogenization. The solutions were stored in amber glass bottles at room temperature (26°C ± 3°C), humidity of 50% ± 10%, in the dark.

The forage plants sorghum (*Sorghum bicolor* var. Ponta Negra) and Paiaguás grass (*Urochloa brizantha* cv. Paiaguás) were used for silage production, cultivated in intercropping in an integrated crop-livestock system.

Regarding the establishment characteristics, sorghum was planted at a row space of 0.55 m, with 11 plants/m linear. The sowing of Paiaguás grass was carried out in the sorghum planting line, in association with establishment fertilization and a density of 7 kg/ha of viable pure seeds.

At 100 days after sowing, the forages were harvested manually using a backpack brush cutter. Then, the material was mechanically chopped into 3-cm particles, using a stationary cutter. The chopped material was homogenized and subdivided into four 90-kg portions per type of treatment plus control, for application of the corresponding additive.

The proportion of each forage for the composition of silages was 92.0% sorghum and 8.0% Paiaguás grass [13]. Sorghum presented, on average, 2.66% of green leaf blades, 69.71% of stems, 6.04% of dead leaf blades, and 21.59% of tassels. Paiaguás grass had, on average, 28.66% of leaf blades, 62.78% of stems and 8.56% of dead forage.

The concentration of each additive in the ensiled mass was 400 mg/kg. The application of the additives was carried out by spraying, diluted in 300 mL of distilled water. The control treatment had only 300 mL of distilled water.

Posteriorly, the green forage was stored in PVC mini-silos of 6 kg capacity, with 150 mm diameter and 500 mm length. The ensiled material was compacted with an iron pendulum and the silos were closed with PVC lids equipped with "Bunsen" valves, sealed with adhesive tape [14]. The mini-silos were stored until the opening period (15, 30, and 45 days after closing the silo).

Phytochemical characterization of the ensiled biomass was determined according to the methodology of AOAC [15]. The intercropped silage exhibited 29.1% dry matter, 9.7% crude protein, 65.4% neutral detergent fiber, 38.5% acid detergent fiber, 6.7% lignin, 3.8% ether extract, and 7.9% ash.

After opening the silos in their respective periods (15, 30 and 45 days after the ensiling process), a 2-kg sample was removed and homogenized; 500 g of biomass were removed and frozen for further analysis of pH and microbiology. A second portion was stored in styrofoam boxes without lids, kept at a controlled room temperature (25°C) in a closed room for aerobic stability assessment. Specifically, the temperature was retained at 25°C, using an air conditioning system, and monitored with a thermometer attached to the inside wall of the room.

To observe the biomass temperature, a digital thermometer with stainless-steel probe (model AK05, AKSO Produtos Eletrônicos, Rio Grande do Sul, Brazil) was attached to the center of mass in each styrofoam box.

Aerobic stability test was performed based on the difference between silage temperature and ambient temperature, defined as the number of hours that silage temperature remained up to 2ºC above ambient temperature, according to the methodology developed by Cantoia Junior et al. [6]. After 24 h of aerobic exposure, pH values were determined, later at 12-hour intervals until 240 h [14]. The pH of the biomass was measured according to AOAC [15].

To determine dry matter loss by gases and effluents, and dry matter recovery, the methodology proposed by Pinedo et al. [16] was applied. To measure microbial populations (*Clostridium* ssp., *Lactobacillus* ssp., *Enterobacteria* ssp., fungi, and yeasts), 10 g of fresh silage were diluted in 90 mL of distilled water for 30 seconds in an industrial blender [17]. Afterwards, the solution was filtered, and 1.0 mL was mixed with 9.0 mL of phosphate buffer solution (pH 7.2), obtaining dilutions from 10^{-2} to 10^{-6}. Then, a duplicate plating of each dilution was performed.

Microbiological analysis was conducted using specific culture media, according to the following protocols: *Lactobacillus* MRS agar and reinforced *Clostridium* agar to determine lactic acid bacteria and *Clostridium* sp., respectively (48 h incubation at 37 °C); "Potato Glucose" agar for the enumeration of fungi and yeasts, after seven days at ambient temperature; and Violet Red Bile agar for the isolation of *Enterobacter* sp. (48 h incubation at 32°C). The culture media were prepared according to the manufacturer's recommendations, and sterilized by autoclaving at 121ºC for 15 min.

Statistical analysis was performed using SAS software [18], and data were submitted to analysis of variance. When a significant effect of the interaction between the additive and silo opening time was verified, one of the factors was fixed. Mean values were compared using the Tukey test with 5% significance.

3. Results and discussion

Interaction (P<0.05) was observed between the type of additive and the silo opening time on the maximum and accumulated temperature and aerobic stability period (Table 1). The silages treated with the nanoformulated "Alecrim pimenta" essential oil with a fermentation time of 30 and 45 days reached the maximum temperatures. Additionally, there was an effect of the 62% nanoformulated thymol, suggesting a low efficiency in the control of microorganisms after opening. However, in general, lower maximum and accumulated temperatures were recorded with 100% nanoformulated thymol.

Table 1. Maximum and accumulated temperatures and aerobic stability of sorghum silage intercropped with Paiaguás grass with different additives and silo opening times

Silo opening time (days)	Additive				CV (%)[1]
	Control	NEO	62% thymol	100% thymol	
Maximum temperature (ºC)					
15	30.7 ab	31.2 Bb	32.1 Aa	29.8 Db	0.8
30	30.9 Ba	31.5 Ab	30.5 Cb	30.5 Ca	0.9
45	30.6 Cb	32.6 Aa	31.8 Ba	30.8 Ca	1.6
CV (%)[1]	0.5	0.7	1.5	1.3	
Accumulated temperature (ºC)					
15	615.1 Cab	623.6 Bb	641.8Aa	595.6 Db	1.9
30	618.9 Ba	629.7 Ab	609.7 Cb	607.1 Ca	2.2
45	612.5 Cb	651.2 Aa	637.1 Ba	615.3 Ca	3.0
CV (%)[1]	0.4	0.7	1.5	1.2	
Aerobic stability (h)					
15	30.2 Bb	36.8 Bb	38.4 Bb	52.6 Aa	9.4
30	31.0 Bb	42.2 Ba	50.2 Aa	38.2 Bb	7.9
45	40.6 Ba	40.4 Ba	52.6 Aa	40.6 Bb	8.8
CV (%)[1]	3.78	2.50	3.89	4.67	

For each characteristic, means followed by the same capital letter (in rows) are not statistically different (P>0.05, Tukey test). [1]CV: Coefficient of variation.

The aerobic stability test revealed that silages treated with 100% pure nanoformulated thymol fermented for 15 days reached the aerobic stability breakdown after 52.6 h; a similar effect was verified for 62% nanoformulated thymol (opening time: 30 and 45 days). Regarding the control group and silages treated with NEO, the increase in opening time provided a longer period to initiate aerobic stability breakdown (Table 1).

The findings demonstrated that 100% pure nanoformulated thymol was efficient in reducing the increase in mass temperatures, and suggested that the addition of essential oil failed to control the proliferation of spoilage microorganisms, which increase the temperature. According to Hodjatpanah-Montazeri et al. [19], the action against EO pathogens is favored under anaerobic conditions, while essential oils have a pH-dependent effect, with higher efficiency at lower pH [5]. Moreover, possibly the concentration used in this study was low. Cantoia Junior et al. [6] reported that sugarcane silage treated with high levels of lemongrass essential oil (120g/kg) remained stable for two weeks.

The pH values of sorghum silages intercropped with Paiaguás grass varied on days 0, 2, and 3 during aerobic stability analysis (P<0.05) as a function of the type of additive and the silo opening time. Higher pH values were recorded for silage treated with 100% nanoformulated thymol (Figure 1A). Three days after opening the silo, higher pH values were found for the control group, and reduced pH for silages with 62% nanoformulated thymol. With respect to the silo opening times, fermentation after 15 days of exposure provided the highest pH values (Figure 1B).

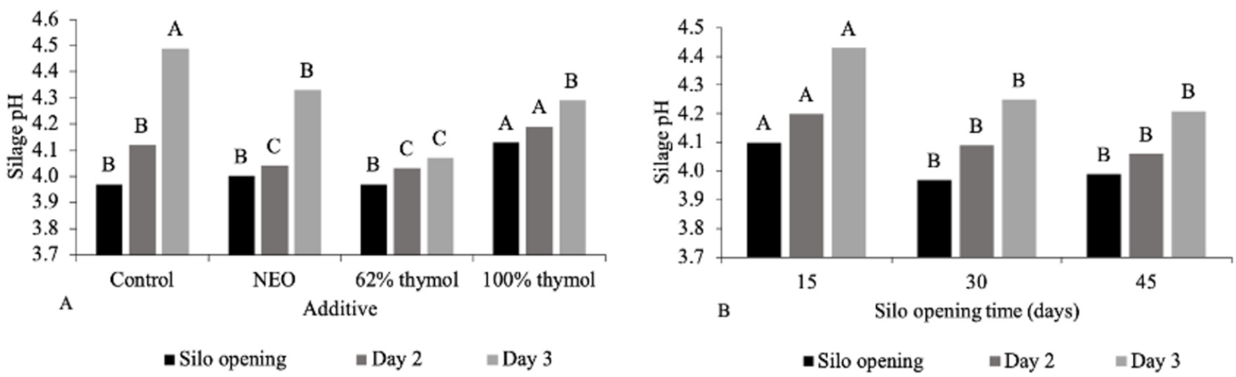

Figure 1. pH values during aerobic stability of sorghum silage intercropped with Paiaguás grass with different additives (1A) and silo opening times (1B). *For each characteristic, means followed by the same capital letter (in rows) are not statistically different (P>0.05, Tukey test).

There was interaction (P<0.05) between treatment and silo opening time on pH values during stability on days 1, 4, and 5. On day 1 of aerobic stability, silage pH did not vary between the additives used when the silo opening time was 15 days (Table 2). However, at 30 and 45 days, an increase in pH was observed in silages with 100% nanoformulated thymol. On days 4 and 5, the control treatment showed high pH values at all opening times. The silage with NEO also showed high pH values on day 5. In general, increasing silo opening time from 15 to 30 and 45 days reduced silage pH, except on day 1 for the groups treated with NEO, 62% nanoformulated thymol and 100% nanoformulated thymol (Table 2).

Despite the pH variation of silages treated with the additives compared to the control group, low silage pH levels (<4.5) were recorded in the first three days of aerobic stability, thereby indicating good forage conservation [20]. From the fifth day, all silages showed pH values above 4.5. Nevertheless, the lower pH values obtained in silages with 62% and 100% nanoformulated thymol demonstrated that this substance was more efficient in controlling the development of aerobic microorganisms, such as yeasts and fungi. Chaves et al. [5] reported that the inclusion of essential oils in barley silage decreased yeast growth after seven days of aerobic exposure.

Regarding silage fermentation losses, the use of 100% nanoformulated thymol resulted in greater loss of dry matter, gases, and effluents, while the treatment with NEO provided a lower loss of dry matter and gases in sorghum silage intercropped with Paiaguás grass (Figure 2A). Higher losses of dry matter, gases, and effluents were recorded at 45 days of silo opening time (Figure 2B).

Table 2. pH values during aerobic stability of sorghum silage intercropped with Paiaguás grass with different additives and silo opening times

Silo opening time (days)	Additive				CV (%)[1]
	Control	NEO	62% thymol	100% thymol	
The first day after silo opening					
15	4.07 Aa	4.00 Aa	3.99 Aa	4.10 Aa	2.09
30	3.99 Bb	3.96 Ba	3.94 Ba	4.13 Aa	3.99
45	3.99 Bb	3.96 Ba	3.99 Ba	4.17 Aa	1.56
CV (%)[1]	0.79	1.64	1.53	1.23	
The fourth day after silo opening					
15	5.18 Aa	4.59 Ba	4.69 Ba	4.43 Ba	1.89
30	5.05 Ab	4.58 Ba	4.33 Bb	4.34 Bb	3.57
45	5.03 Bb	4.45 Ab	4.22 Db	4.33 Ab	1.65
CV (%)[1]	1.48	2.74	3.78	1.37	
The fifth day after silo opening					
15	5.90 Aa	5.59 Aa	5.15 Ba	4.84 Ca	3.76
30	5.77 Ab	5.52 Aa	4.85 Ba	4.63 Bb	5.19
45	5.70 Ab	5.50 Ba	4.45 Bb	4.59 Bb	3.46
CV (%)[1]	1.06	3.32	5.91	4.95	

For each characteristic, means followed by the same capital letter (in rows) are not statistically different (P>0.05, Tukey test). [1]CV: Coefficient of variation.

The gas and dry matter losses may be related to the silage fermentation profile, due to the presence of lower lactic acid-producing bacteria. Effluent loss is also undesirable, as it indicates nutrient losses. According to Driehuis et al. [20], this material contains high content of organic compounds, including sugars, organic acids, proteins, minerals (calcium, potassium, and magnesium), and other constituents of the ensiled forage.

In this study, the better efficiency of nanoformulated thymol compared to nanoformulated "Alecrim pimenta" essential oil may be related to the biotechnological process applied to these compounds. Nanoformulation is a potential alternative due to the protection of the bioactive molecules from degradation and evaporation losses and controlled release of the active constituents, increasing their power of action [21]. When applied to essential oils, nanoformulation promotes higher surface area, greater solubility, smaller particle size, and higher mobility, thus contributing to a better performance of essential oils when compared to conventional additives [22, 23], such as bacterial cultures, carbohydrate sources, organic acids and preservatives.

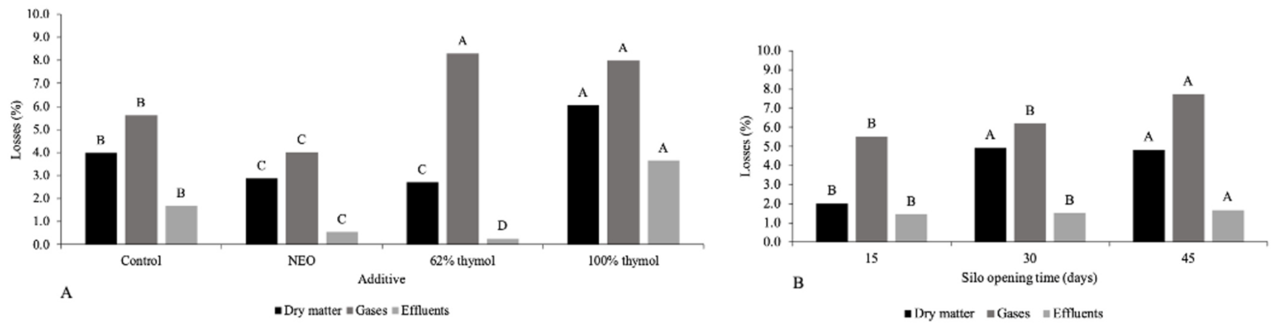

Figure 2. Fermentation losses in the sorghum silage intercropped with Paiaguás grass with different additives (2A) and silo opening times (2B). *For each characteristic, means followed by the same capital letter are not statistically different (P>0.05, Tukey test).

Clostridium and *Lactobacillus* populations in sorghum silage intercropped with Paiaguás grass were affected (P<0.05) by the type of additive. *Clostridium* was higher in the control group (4.76 log CFU/g) and in the treatment with nanoformulated essential oil (4.58 log CFU/g), compared to the 62% and 100% nanoformulated thymol (3.86 and 3.69 log CFU/g, respectively) (Figure 3).

In turn, greater growth of *Lactobacillus* was observed in the control treatment (4.58 logs cfu/g). Lower values were recorded for the samples treated with 62% and 100% thymol (3.70 and 3.62 log CFU/g, respectively). A reduction in *Lactobacillus* was observed with increasing silo opening time (P<0.05), totaling 4.13 log CFU/g (15 days), 3.99 log CFU/g (30 days), and 3.78 log CFU/g (45 days) (Figure 4).

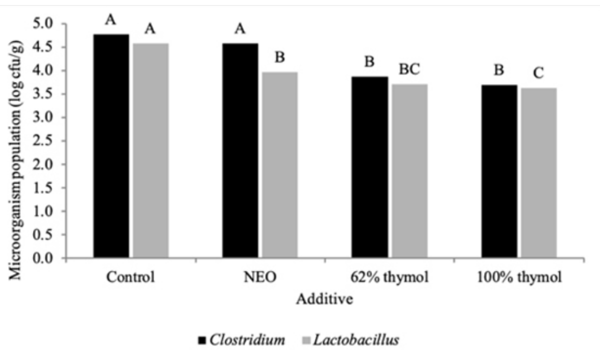

Figure 3. *Clostridium* and *Lactobacillus* populations in sorghum silage intercropped with Paiaguás grass using different additives. *For each bacterial strain, means followed by the same letter are not significantly different (P>0.05, Tukey test).

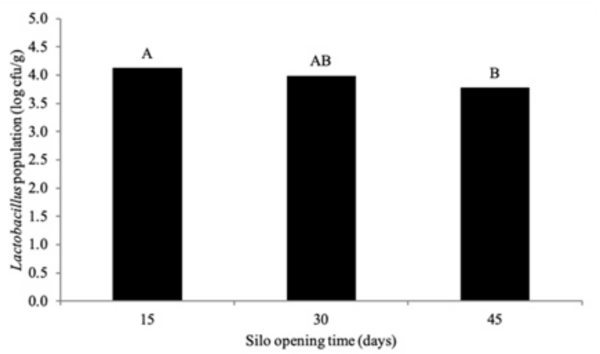

Figure 4. *Lactobacillus* spp. in sorghum silage intercropped with Paiaguás grass with different silo opening times. *Means followed by the same capital letter (in rows) are not significantly different (P>0.05, Tukey test).

In general, the increase in silo opening time resulted in a lower *Lactobacillus* count, but without compromising silage pH at the moment of opening the silo (<4.2). This demonstrates flexibility for the producer as to how long the silo remains closed. However, the shorter silo opening time (15 days) can compromise silage quality if it is not used rapidly, since aerobic stability was lower and pH values during this stability were higher in comparison to 30 and 45 days.

The results demonstrated that the use of 62% and 100% thymol reduced the *Clostridium* and *Lactobacillus* populations. Inhibition of *Clostridium* growth in silage can be associated with good fermentation since this endospore-forming bacteria is responsible for butyric acid fermentation [24]. Butyric acid production indicates silage deterioration owing to the presence of undesirable microorganisms, therefore, high levels can compromise silage quality [25].

On the other hand, the reduction in *Lactobacillus* count is undesirable in the process since these lactic-acid bacteria are essential for achieving high-quality silage fermentation. By inhibiting bacteria growth, there was possibly a reduction in the production of organic acids, such as lactic acid in silages treated with additives, thus increasing pH. However, this finding did not negatively affect the fermentation process, because the pH values were 3.97 (control silage), 4.00 (silage with nanoformulated EO), 3.97 (silage with 62% nanoformulated thymol), and 4.13 (silage with 100% nanoformulated thymol). These values are within the optimal pH range (3.8 to 4.2) for good forage conservation [20].

Lactic acid is the primary end product of traditional crop silages, including corn, grass, and alfalfa [26]. Importantly, lactic acid bacteria mainly metabolize water-soluble carbohydrates into organic acids [6].

In the present study, the absence of effects of nanoformulated "Alecrim pimenta" (*Lippia sidoides* Cham.) essential oil on silage fermentative characteristics may be associated with the expressive variation in the concentration of compounds with microbial activity, resulting in reduced bactericidal activity [27, 28], since, in general, the use of thymol alone provided silages with good fermentation profile. Furthermore, some secondary compounds present in small amounts in essential oils, such as γ-terpinene and p-cymene, interfere with the antimicrobial property, facilitating the permeability of carvacrol and thymol in the bacterial cell [29].

In general, knowledge of the action of essential oils as silage additives is still scarce. Therefore, further in-depth studies are needed to elucidate the mechanisms of action of these compounds as an additive to control losses in the fermentation of the ensiled material.

4. Conclusion

The nanoformulated thymol is more efficient in controlling fermentation processes and *Clostridium* population, in addition to improving aerobic stability of sorghum silage with Paiaguás grass.

Declaration of conflict of interest

The authors declare that there is no conflict of interest.

Author Contributions

Conceptualization: B.M.L. Sousa, A.C. Backes and A.F. Blank. *Data curation*: B.M.L. Sousa, J.L. Fagundes. *Investigation*: S.J. Santos and J.R. Santos Filho. *Methodology*: A.C. Backes and A.F. Blank. *Project Management and Validation*: B.M.L. Sousa, A.C. Backes and A.F. Blank. *Writing (original draft)*: S.J. Santos. *Writing (proofreading and editing)*: C.M. Silva.

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