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Fermentation characteristics of elephant grass silages with macaúba cake

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ABSTRACT. The elephant grass presents problems during the fermentation of the ensiled material, being necessary use of additives. This study aimed to evaluate the population of yeast and filamentous fungi, enterobacterias, contents of acetic and butyric acids and ethanol production, in elephant grass silages added with different levels of macaúba cake at opening times. This was a 3 x 6 factorial completely randomized experimental design, with three inclusion levels of the macaúba cake (0, 10 and 20%) and six opening times (1, 5, 10, 20, 40 and 60 days after ensiling), with four replications. For all the studied variables, there was a difference in the interaction among levels and times, and an increasing linear behavior only for the contents of acetic acid, for the other variables, the behavior was quadratic. The elephant grass silages produced acetic acid, which in turn inhibited the production of yeast and filamentous fungi. There was a small development of enterobacterias only in the first opening times, and low production of butyric acid and ethanol, which indicated a material with good fermentative characteristics. The macaúba cake contributed to improve the anaerobic fermentation process, but it was not as expressive for the parameters evaluated in this work.

Keywords: enterobacterias; filamentous fungi; opening times; organic acids; yeast fungi.

Características fermentativas de silagens de capim-elefante com torta de macaúba

RESUMO. O capim-elefante apresenta problemas durante a fermentação do material ensilado, sendo necessário uso de aditivos. Objetivou-se avaliar a população de fungos leveduriformes e filamentosos, enterobactérias, teores de ácido acético e butírico e produção de etanol, nas silagens de capim-elefante adicionadas com diferentes níveis de torta de macaúba, em tempos de abertura. O delineamento experimental foi o inteiramente casualizado, em esquema fatorial 3 x 6, sendo três níveis de inclusão da torta de macaúba (0, 10 e 20%) e seis tempos de abertura (1, 5, 10, 20, 40 e 60 dias após a ensilagem), com quatro repetições. Em todas as variáveis estudadas, houve diferença na interação níveis x tempos, encontrando-se comportamento linear crescente somente para os teores de ácido acético. Nas demais variáveis, observou-se comportamento quadrático. As silagens de capim-elefante produziram ácido acético, que, por sua vez, inibiu a produção de fungos leveduriformes e filamentosos. Houve um pequeno desenvolvimento de enterobactérias, que ocorreu somente nos primeiros tempos de abertura, e baixa produção de ácido butírico e etanol, o que indicou um material com boas características fermentativas. A torta de macaúba contribuiu para a melhoria do processo fermentativo anaeróbico; porém não foi tão expressiva para os parâmetros avaliados neste trabalho.

Palavras-chave: ácidos orgânicos; enterobactérias; fungos filamentosos; fungos leveduriformes; tempos de abertura.

Introduction

Tropical forages show pronounced seasonality, which determines an uneven distribution in production during the year, with surplus of forage in the rainy season and lack in the dry season, impairing animal performance (Ferreira et al., 2014; Reis, Ruggieri, Casagrande, & Páscoa, 2009). The adoption of forage conservation techniques that preserve their nutritional characteristics and allow their use during critical periods of feeding becomes extremely necessary, standing out the ensiling process.

Elephant grass has been widely used in ensiling processes because it can have great yield potential and reasonable amounts of soluble carbohydrates (Rêgo, Cândido, Pereira, Feitosa, & Rêgo, 2010). However, this forage has a high moisture content at the time of cutting to obtain silage, which can lead to undesirable fermentation, compromising the quality of the ensiled material (Cardoso et al., 2016). Therefore, it is necessary to use a technique to raise the dry matter content, an alternative is the technique of wilting with less expense; however, the use of additives with the intention of improving their fermentation attributes and reducing the moisture, can raise nutrient contents, making it possible to guarantee a production of quality silage (Oliveira et al., 2012; Rezende et al., 2008). Biodiesel co-products are promising for use in animal feed and can be added in the silage process, since they can add nutritional value to the final product and reduce the costs of producing both biodiesel and feed to be supplied to the animal (Abdalla, Silva Filho, Godoi, Carmo, & Eduardo, 2008).

Microorganisms naturally found during the ensiling process are called epiphytic microflora, which is essential for the fermentation processes and consequently the preservation of the ensiled material. The number of microorganisms varies according to several factors, such as plant maturity, particle size, compaction, silo sealing, material conditioning, soluble carbohydrate and dry matter contents, among others (Ferreira et al., 2013).

Yeast and filamentous fungi are considered undesirable microorganisms. After opening the silos, when silage is exposed to the air, there is the development of the main silage-spoilage microorganisms, especially yeast fungi; at this moment the residual soluble carbohydrates and organic acids of the silage are metabolized with energy losses to CO_2 and water, with consequent production of heat. In this way, Borreani, Bernardes, and Tabacco (2008) correlated the aerobic degradation of silage to the elevation of its temperature, due to the release of heat by the microbial respiratory activity as well as the fermentation (metabolism) of the microbial mass. Under aerobic conditions, filamentous fungi metabolize the residual sugars or formed organic acids, raising the pH of the ensiled material. Moreover, enterobacteria are also undesirable because they compete with lactic acid bacteria for sugars at the beginning of the fermentation process, in which they produce acetic acid as the final product. These microorganisms are capable of degrading proteins, which cause a decrease in the nutritional value of the silage, provide the production of amines and branched fatty acids, representing a negative effect on animal acceptability (McDonald, Henderson, & Heron, 1991).

Considering the importance of knowing the potential of macaúba cake added when ensiling elephant grass, and the lack of studies on this biodiesel coproduct in animal feed, the objective of this study was to evaluate the effects of different levels of inclusion of macaúba cake, in different opening times, in days, in elephant grass ensiling on the population of yeast and filamentous fungi, enterobacteria, contents of acetic and butyric acids and ethanol production.

Material and methods

The experiment was conducted at the Federal University of the Jequitinhonha and Mucuri Valleys (UFVJM), Campus JK, located in the municipality of Diamantina, State of Minas Gerais, Brazil. Laboratory analyses were carried out in the Microbiology Laboratory of the Graduate Program in Biofuels and the Animal Nutrition Laboratory of the Department of Animal Science, both at UFVJM.

The co-product from the biodiesel production chain was the macaúba cake [*Acrocomia aculeata* (Jacq.) Lood. ex Mart.] (pulp and peel) obtained from Indústria DBIO, located in the municipality of Dores do Indaiá, State of Minas Gerais. Elephant grass (*Pennisetum purpurem* Schum.) was purchased from a rural property in the municipality of Lavras, State of Minas Gerais. We studied the cultivar Cameroon, subjected to a standardization cut previously made and the maintenance fertilization consisted of the formulated fertilizer NPK 8-28-16 at 400 kg ha^{-1}. At the time of cutting, forage was around 1.60 m high and 70 days of growth, 22% dry matter content (DM), with an average yield of 70 t ha^{-1} green matter, the harvest was made at 10 cm from the ground level.

The experiment was a 3 x 6 factorial completely randomized design, with three levels of inclusion of macaúba cake (0, 10 and 20% based on green matter of elephant grass) and six times (in days) of silo opening times (1, 5, 10, 20, 40 and 60 days after ensiling), with four replications, totaling 18 treatments and 72 experimental units. The statistical model was:

$$\mathrm{YijK} = \mu + \mathrm{Ni} + \mathrm{Tj} + \mathrm{NTij} + \mathrm{eijk},$$

where:
YijK = observation concerning level i of macaúba cake, at opening time j, in repetition k;
μ = overall mean;

Ni = effect of level i of macaúba cake (i = 1, 2 and 3);
Tj = effect of the opening time j (j = 1, 2, 3, 4, 5 and 6);
NTij = effect of the interaction between the level i of macaúba cake and opening time j;
eijk = experimental error associated with each observation that received the level i of macaúba cake, in the opening time j in the repetition k.

Before the ensiling process, elephant grass was chopped in a stationary forage harvester to obtain particles of approximately 2 cm. Subsequently, macaúba cake was added to the elephant grass, with the respective inclusion levels mentioned above. This material was homogenized, placed in the experimental silos made of polyvinyl chloride (PVC) tubes of 100 mm diameter and 450 mm length and compacted with the aid of wood sockets, obtaining a specific average mass of 600 kg m^{-3} of green matter of forage. Silos were sealed using the PVC cap fitted with a Bunsen-type valve to allow the escape of gases generated during the fermentation process inside the silos, the entire set was weighed upon ensiling, the cap was sealed with adhesive tape. These were placed under roof at room temperature.

In sequence, the opening of the silos occurred at the respective times (in days), according to previously set treatments, discarding the material from the top and bottom. The median portion was homogenized and separated into two sub-samples, one part for analyses of the yeast, filamentous and enterobacterias; and the other sub-sample destined to the pressing for extraction of the liquid of the silage for identification and quantification of acetic and butyric acids and ethanol production.

The quantification of the microbial population in the fresh materials, in the homogenized mixtures before ensiling the elephant grass and in the ensiled material, was performed immediately after their separation, where an aliquot of 25 g was taken and added in an Erlenmeyer containing 225 mL phosphate buffer solution, and homogenized in a Shaker (sample homogenizer) at 28°C for 20 minutes, representing the dilution of 10^{-1}. Subsequently, successive dilutions were performed until obtaining 10^{-6} dilutions.

The culture media used were Violet Red Bile Agar (VRBA), Yeast Extract Peptone Glucose (YEPG) and Dichloran Rose Bengal Chloramphenicol Base (DRBC), selective for the growth of enterobacterias, yeast and filamentous fungi, respectively. The microbial population was counted on dishes containing values up to 300 colony forming units (CFU) and transformed into log 10 base (log CFU g^{-1}) (Table 1).

For the evaluation of acetic and butyric acids and ethanol production, the liquid extract obtained from the silage was analyzed in a high-performance liquid chromatograph (HPLC), through a Biorad C18 (Reverse Phase) column, the eluent used was H_2SO_4 (0.0025 mol L^{-1}).

In the statistical analysis, data were subjected to analysis of variance, using the statistical software Genes (Cruz, 2013) and whenever significant effects were detected in relation to inclusion levels and opening times, the regression analysis was applied. The regression models tested were the first and second degree polynomial models, being kept the most parsimonious based on the significance at 5% probability and higher coefficient of determination.

Table 1. Microbial population of elephant grass, macaúba cake and their mixtures before ensiling (log CFU g^{-1}).

Material	Entero	YF	FF
Elephant grass	8.16	3.20	-
Macaúba	-	-	-
10% Macaúba	7.99	3.47	4.67
20% Macaúba	7.63	2.93	4.65

-: Variable not evaluated. Entero: enterobacterias; YF: yeast fungi; FF: filamentous fungi.

Results and discussion

As for the microbial population, there was an effect on the enterobacterias (Entero) and filamentous fungi (FF), which were affected by the opening times and by the interaction levels x times. On yeast fungi (YF), these were influenced by the inclusion levels of macaúba cake (MC), opening times and interaction levels x times.

A quadratic behavior was observed for the Entero population in the elephant grass silages in the interaction levels x times (Table 2). In the initial phase of the fermentation process of the ensiled material, Entero was found, and subsequently these were eliminated inside the silos, at the three levels of macaúba cake and throughout the opening times. The presence of this microbial population in the first days of ensiling was expected, since the growth of this microbial population is directly related to the presence of oxygen, that is, it development occurs in the first days of ensiling, in which they consume the oxygen present, and decreases rapidly with acidification of the medium, being inhibited by lactic acid bacteria, with a rapid drop in pH.

In silages with only grass (0% MC), at times 1 and 5 (days after ensiling), the population of these microorganisms were larger, compared to the silages added of 10 and 20% MC (Table 2), which may be justified by the greater presence of Entero in the fresh material of elephant grass before ensiling (Table 1). However, the counting lasted until the tenth day only in silage which was added with 20%

MC. These results indicate the competition of Entero with the beneficial microorganisms of the silage, which may have occurred only in the initial phase of the fermentation process, being a positive factor.

In the interaction levels x times, quadratic behavior was found for yeast fungi (YF) at all MC inclusion levels along silo opening times, in days after ensiling (Table 3). The highest quantification (maximum point) of the yeast fungi population of elephant grass silages was 3.88, 4.07 and 3.72 log CFU g^{-1} obtained at 18, 1 and 20 days after ensiling with addition of 0, 10 and 20% MC, respectively. After these periods, it was observed that there were decreases in the amount of yeast fungi at all levels of inclusion and throughout the opening times of the silos, and that in turn, no presence of these microorganisms was detected in the last opening time, at 60 days, after ensiling.

Table 2. Population of enterobacteria (log CFU g^{-1}) of elephant grass silages according to the inclusion levels (IL) of the macaúba cake and silo opening times, in days after ensiling, with the respective regression equations, coefficients of determination (R^2) and coefficient of variation.

IL	Opening times						Regression equations	R^2
(%)	1	5	10	20	40	60		
0	6.07	5.79	ND	ND	ND	*ND*	Y = 5.9211 − 0.3722X + 0.0047X2	0.86
10	4.15	5.70	ND	ND	ND	ND	Y = 4.7588 − 0.2890X + 0.0036X2	0.81
20	4.73	3.03	4.79	ND	ND	ND	Y = 5.0397 − 0.2414X − 0.0026X2	0.88
CV							39.8	

ND: undetected, CV: coefficient of variation.

The highest number of YF colonies with a 10% MC addition in the early opening times of the silos may be related to the epiphytic population of YF of the fresh material (Table 1).

Table 3. Population of yeast fungi (log CFU g^{-1}) of elephant grass silages according to the inclusion levels (IL) of the macaúba cake and silo opening times, in days after ensiling, with the respective regression equations, coefficients of determination (R^2), and coefficient of variation.

IL	Opening times						Regression equations	R^2
(%)	1	5	10	20	40	60		
0	4.01	3.13	3.13	3.74	3.48	ND	Y = 3.1763 + 0.0770X − 0.0021X2	0.93
10	5.40	3.10	3.19	3.50	3.24	ND	Y = 4.0682 − 0.0009X − 0.0010X2	0.85
20	2.75	3.62	3.70	2.82	3.48	ND	Y = 2.8259 + 0.0888X − 0.0022X2	0.92
CV							12.3	

ND: undetected, CV: coefficient of variation.

From 15 days of storage, the yeast fungus population was higher in the silage without addition of MC, compared to the other treatments, which shows that this biodiesel co-product was efficient in reducing the development of this microbial group in the silages of elephant grass along the opening times of the silos, favoring the fermentation process.

The count of this microbial population in the silages was considered low, being smaller than those found by Gonçalves et al. (2014) and Cardoso et al. (2016) in silages of elephant grass alone. Another factor that serves as a basis for considering that the population of yeast fungi was low is the small production of ethanol (Table 7).

The presence of yeast fungi in the medium is undesirable because they consume the soluble carbohydrates, reducing the amount of these compounds in the ensiled material, consequently affecting the development of lactic acid bacteria, promoting increase of other fractions of the forage, mainly fiber (Evangelista, Siqueira, Lima, Lopes, & Rezende, 2009).

There was a quadratic behavior in the interaction levels x times for the filamentous fungi population of elephant grass silages (Table 4). Considering the inclusion level of 0%, the highest count (maximum point) of FF (5.28 log CFU g^{-1}) was found at approximately 27 days; at the 10 and 20% MC levels, the largest populations obtained (maximum points) were 5.30 and 5.24 log CFU g^{-1} at 23 and 22 days, respectively (Table 4).

Table 4. Population of filamentous fungi (log CFU g^{-1}) of elephant grass silages according to the inclusion levels (IL) of the macaúba cake and silo opening times, in days after ensiling, with the respective regression equations, coefficients of determination (R^2) and coefficient of variation.

IL	Opening times						Regression equations	R^2
(%)	1	5	10	20	40	60		
0	5.05	5.29	5.31	5.18	5.20	4.94	Y = 5.146 + 0.0106X − 0.0002X2	0.81
10	5.11	5.21	5.31	5.12	5.28	4.65	Y = 5.101 + 0.0180X − 0.0004X2	0.90
20	5.16	5.21	5.22	5.20	5.23	4.96	Y = 5.152 + 0.0087X − 0.0002X2	0.96
CV							4.0	

CV: coefficient of variation.

After this period, the microbial population reduced in the inclusion levels throughout the opening times. In the initial phase of the fermentation process, that is, in the first opening time, it was verified that the FF population did not vary much in the different levels of inclusion, whose coefficient of variation was 4.0, which can be based on the initial amount of FF colonies of the fresh material in the silage (Table 1). In the last opening time, at 60 days, the observed values were low at all levels of inclusion, especially in the final stage of the fermentation process, without impairing the quality of the silages.

Silage spoilage is especially related to the population of fungi, as they degrade a wide variety of nutrients, including sugars and lactic acid through respiration, as well as structural carbohydrates. Some species of the genus *Aspergillus, Fusarium* and *Penicillium* grow in silages where there is air penetration, which contribute to losses on the surface of the silo during discharging and in cases of improper compaction and sealing. Besides, they produce toxins that are harmful to animals and man. As in yeast fungi, some species of filamentous fungi can grow at low oxygen levels (McDonald et al., 1991); however, in this present work, the quantities found are considered low. Thus, the low amounts of yeast and filamentous fungi in elephant grass silages are due to the inhibitory effect caused by acetic acid (Table 5), which showed opposite behavior, that is, as the production of acetic acid increased there was a reduction in the population of yeast and filamentous fungi.

There was an effect on acetic acid contents of elephant grass silages according to levels x times interaction, with an increasing linear behavior. The levels of acetic acid found in elephant grass silages increased linearly with MC levels along the opening times of the silos. As the storage time of ensiled material was extended, the contents of this acid increased at all levels of inclusion MC (Table 5).

Table 5. Acetic acid contents (%) of elephant grass silages according to the inclusion levels (IL) of macaúba cake and the silo opening times, in days, after ensiling, with the respective regression equations, coefficients of determination (R^2) and coefficient of variation.

IL	Opening times						Regression equations	R^2
(%)	1	5	10	20	40	60		
0	0.03	0.06	0.07	0.10	0.07	0.17	$Y = 0.0431 + 0.0018X$	0.85
10	0.04	0.09	0.12	0.13	0.13	0.19	$Y = 0.0736 + 0.00190X$	0.88
20	0.02	0.10	0.12	0.14	0.14	0.25	$Y = 0.0624 + 0.0028X$	0.88
CV						13.3		

CV: coefficient of variation.

The acetic acid estimated contents of elephant grass silages ranged from 0.04 to 0.15% at the 0% MC level, from 0.07 to 0.19% with the addition of 10% MC and from 0.06 to 0.23% with the inclusion of 20% MC, over the opening times of the silos (Table 5). Thus, the MC levels and opening times of the silos contributed to the increase in the acetic acid, with higher contents of this acid when 20% MC was added at 60 days and that the lowest values were found when the silage was exclusively made of elephant grass (0% MC), followed by 10% MC (Table 5). The contents found at all inclusion levels of MC and silo opening times were lower than 0.8%, considered a positive factor, since according to Rodrigues et al. (2007), values above this critical limit are related to undesirable changes during the ensiling process. Acetic acid has the potential to be a great inhibitor of filamentous and yeast fungi, microorganisms that are precursors of aerobic degradation of silages and responsible for the consumption of carbohydrates in the process with the release of CO_2 resulting from their metabolism (Danner, Holzer, Mayrhuber, & Braun, 2003). This inhibitory activity of acetic acid on filamentous and yeast fungi was observed since they presented a reduction in their populations (Table 3 and 4) as the acetic acid increased; these fungi detrimental to the fermentation process. Thus, it is possible to confirm that acetic acid contributed to the improvement of the preservation of the ensiled material by preventing the further development of these undesirable microorganisms.

There was an effect on butyric acid contents of elephant grass silages as a function of levels x times interaction, with a quadratic behavior (Table 6). From the fifth day after ensiling, we observed the absence of butyric acid in the levels of 10 and 20% of macaúba cake. However, in the silage of elephant grass alone (0% MC), production was still recorded in this period, only after 10 days of ensiling at all levels of inclusion macaúba cake, no butyric acid contents were found in the silage.

Table 6. Butyric acid contents (%) of elephant grass silages according to the inclusion levels (IL) of macaúba cake and the silo opening times, in days, after ensiling, with the respective regression equations, coefficients of determination (R^2) and coefficient of variation.

IL	Opening times						Regression equations	R^2
(%)	1	5	10	20	40	60		
0	0.01	0.05	0.0	0.0	0.0	0.0	$Y = 0.03 - 0.001X + 0.00002X2$	0.61
10	0.06	0.0	0.0	0.0	0.0	0.0	$Y = 0.03 - 0.002X + 0.00003X2$	0.67
20	0.08	0.0	0.0	0.0	0.0	0.0	$Y = 0.04 - 0.003X + 0.00004X2$	0.67
CV							36.9	

CV: coefficient of variation.

The low content of that organic acid indicates no clostridial fermentation, since this microbial population is responsible for the production of butyric acid, indicating good quality of elephant grass silages. Bacteria of the genus *Clostridium* promote undesirable secondary fermentations, protein and lactic acid degradation, characterizing low quality silages (McDonald et al., 1991). In addition, they reduce the nutritional value of silages and increases the pH values with the reduction of the lactic acid contents of the medium.

For ethanol contents, there was an effect of levels x times interaction, with a quadratic behavior (Table 7). The contents of ethanol found in elephant grass silage in the macaúba cake levels and silo opening times made it possible to point out that its productions were low. Thus, the data verified in the literature on ethanol from silages of elephant grass alone are higher than those found in the present study (Rodrigues et al., 2007), which is a positive factor, since the ethanol production triggers losses of DM in the ensiled material.

Table 7. Ethanol contents (%) of elephant grass silages according to the inclusion levels (IL) of macaúba cake and the silo opening times, in days, after ensiling, with the respective regression equations, coefficients of determination (R^2) and coefficient of variation.

IL	Opening times						Regression equations	R^2
(%)	1	5	10	20	40	60		
0	0.05	0.11	0.11	0.15	0.08	0.14	Y = 0.08 + 0.0019X – 0.00002X2	0.46
10	0.06	0.08	0.09	0.08	0.07	0.11	Y = 0.08 – 0.0003X + 0.00001X2	0.68
20	0.04	0.10	0.10	0.11	0.07	0.13	Y = 0.07 + 0.0008X – 0.00002X2	0.51
CV							14.7	

CV: coefficient of variation.

Although ethanol production was low, it can be observed that in the exclusive silage of elephant grass, there was higher production of ethanol, compared to that added with macaúba cake, especially in the last opening times.

The elephant grass silages produced acetic acid, which in turn inhibited the production of yeast and filamentous fungi. There was a small development of enterobacterias only in the first opening times, and low production of butyric acid and ethanol, which indicated a material with good fermentative characteristics.

Conclusion

The macaúba cake contributed to improve the anaerobic fermentation process of elephant grass silages, but it was not as expressive for the parameters evaluated in this work. As for the storage time of the elephant grass silages added with macaúba cake, it is recommended the storage for at least 60 days, so that the fermentation processes, mainly related to the microorganisms and the production of organic acids, occur, characterizing a food of quality for the animals.

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