http://dx.doi.org/10.1590/1678-4162-12984

Arq. Bras. Med. Vet. Zootec., v.75, n.5, p.981-992, 2023

Elephant grass silage inoculated with cellulolytic fungi isolated from rumen

[*Silagem de capim-elefante inoculada com fungos celulolíticos isolados do rúmen*]

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ABSTRACT

The objective was to evaluate the inoculation with *Aspergillus terreus* and/or *Trichoderma longibrachiatum* on fermentation, chemical and microbiological composition of elephant grass 'Cameroon' silage (*Cenchrus purpureus*). Treatments were *A. terreus* at 10^5 colony forming units (CFU)/g (AT15), *T. longibrachiatum* at 10^5 CFU/g (TL20), a mixture of both at 10^5 CFU/g (MIX), and a control group without inoculation (CONTR). The design was completely randomized with seven replicates. The MIX silage was most stable, while CONTR, AT15, and TL20, had lower dry matter losses. There was no effect of inoculation in the chemical composition of silages. Only MIX silage (4.40) had pH above the minimum of 4.2 for humid grass silage and above the control (4.05). Bacteria from *Diplococcus* genus was identified at the opening of TL20 and CONTR silages. After air exposure, the population of rods, *Lactobacillus*, and total lactic acid bacteria was higher in theTL20 and MIX. The inclusion of a *T. longibrachiatum* and *A. terreus* mixture increases dry mater loss and silage pH. *T. longibrachiatum* was more efficient in maintaining populations of total lactic acid bacteria after opening; therefore, this strain has potential as an additive for elephant grass 'Cameroon' silage.
keywords: *Aspergillus terreus*, *Cenchrus purpureus* 'Cameroon', dry matter loss, silage quality, *Trichoderma longibrachiatum*.

RESUMO

O objetivo foi testar a inoculação com Aspergillus terreus *e* Trichoderma longibrachiatum *sobre a fermentação, a composição bromatológica e microbiológica de silagem de capim-elefante cultivar 'Cameroon' (*Cenchrus purpureus*). Os tratamentos foram* A. terreus *a 10^5 unidades formadores de colônias (UFC)/g (AT15),* T. longibrachiatum *a 10^5 UFC/g (TL20), a mistura de ambos a 10^5 UFC/g (MIX), cada, e um controle não inoculado (CONTR). O delineamento foi inteiramente ao acaso, com sete repetições. A silagem MIX foi mais estável após abertura, enquanto CONTR, AT15 e TL20 apresentaram menor perda de massa seca. Não houve efeito de inoculação sobre a composição bromatológica das silagens. Apenas a silagem MIX (4,40) apresentou pH acima do mínimo de 4,2 para silagem de capim úmido e superior ao controle (4,05). Bactérias do gênero* Diplococcus *foram identificadas na abertura das silagens TL20 e CONTR. Após exposição ao ar, a população de bastonetes,* Lactobacillus *e bactérias láticas totais foram maiores em TL20 e MIX. A mistura de* T. longibrachiatum *e* A. terreus *aumenta a perda de matéria seca e o pH da silagem.* T. longibrachiatum *é mais eficiente em manter as populações de bactérias láticas totais após a abertura. Portanto, essa cepa tem potencial como aditivo para silagem de capim-elefante 'Cameroon'.*

Palavras-chave: Aspergillus terreus, Cenchrus purpureus *'Cameroon', perdas de matéria seca, qualidade da silagem,* Trichoderma longibrachiatum

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Submitted: January 30, 2023. Accepted: May 10, 2023.

INTRODUCTION

Grass silage is the main forage conservation option in tropical environments, and elephant grass 'Cameroon', from the specie *Cenchrus purpureus* (Schumach.) Morrone (Syn. *Pennisetum purpureum* Schumach.) is highlighted for its high forage production, although it has high fiber and moisture contents. These characteristics can compromise the fermentation process and reduce the consumption and utilization of forage by ruminants.

Recent research has shown the beneficial effects of some species of ruminal cellulolytic fungi in the ensiling process (Lee *et al.*, 2015; Wang *et al.*, 2019a). These fungi produce enzymes that directly contribute to breaking down the lignocellulosic biomass (Duarte *et al.*, 2021; Alves *et al.*, 2021). Degradation of the complex carbohydrate structure can improve fiber digestibility and release soluble carbohydrates that can be used in the fermentation process. Lee *et al.* (2015) found a significant reduction in the fiber content of rice straw silage inoculated with three species of anaerobic fungi from goat rumen, and the use of the ruminal fungus *Piromyces* sp. improved forage conservation and the fiber degradation rate in maize silage (Wang *et al.*, 2019a).

Our studies have indicated the presence of facultative anaerobic cellulolytic fungi in the rumen of cattle fed with lignified tropical forages (Abrão *et al.*, 2014, 2017, 2018). In these studies, isolates of *Trichoderma longibrachiatum* and *Aspergillus terreus* were selected considering the potential for fibrolytic enzyme production (cellulase, avicelase, xylanase, and phenol oxidase), the absence of mycotoxins, and the easy cultivation under laboratory conditions. These anaerobic facultative fungi produced high level of enzymes capable to broke complex lignocellulosic compounds in submerged fermentation of sugarcane bagasse or *Urochloa decumbens* in pH ≤4.5 (Alves *et al.*, 2021; Duarte *et al.*, 2021). Glucose liberation could favor the growth of acid lactic bacteria and these characteristics can indicate the biotechnological potential of these species as silage inoculants.

Isolates of T. *longibrachiatum* and *A. terreus* have being able to ferment plant biomass as substrate in solid state fermentation (Jahromi *et al.*, 2012; Li *et al.*, 2022). Therefore, we believe that these isolates from bovine digestive tract could also grow, considering the changes in pH, humidity, and substrate presents in the ensiling process. Its growth may improve aspects like pH, dry mass losses, and chemical and microbiological composition of silage.

The objective in this study was to evaluate the effect of inoculation with isolates of *A. terreus* and *T. longibrachiatum* on aerobic stability, loss, chemical composition, and the microbial population of elephant grass 'Cameroon' silage.

MATERIALS AND METHODS

Elephant grass 'Cameroon' (*Cenchrus purpureus* (Schumach.) Morrone) was grown in Montes Claros, North Minas Gerais state, Brazil (16º41′10.05″ S; 43º50′33.56″ W). The climate is tropical subhumid (Aw) according to the Köppen classification and is marked by a dry season from May to October and a rainy period from November to April.

The experimental design was completely randomized with four experimental groups and seven replicates as follows: control without additive (CONTR), *A. terreus* isolate (AT15), *T. longibrachiatum* isolate (TL20), and a mixture (MIX) of *A. terreus* and *T. longibrachiatum*. These fungi were inoculated at a concentration of 1.0×10^5 CFU/g of fresh forage weight.

Elephant grass 'Cameroon' (2.0m tall and 90 days of regrowth) was manually harvested in October 2019 at 10cm above ground level. Subsequently, it was diced in a forage chopper regulated for particle size from 0.5 to 2.5cm and stored in plastic bags.

The dry matter of fresh grass was determined using a moisture analyzer (Shimadzu model MOC63, Japan), and forage pH was measured 30 min after immersion of 9.0g of fresh chopped forage in 60mL of distilled water with a pH meter (PG1800 GEHAKA®, São Paulo, Brazil), with a result of 6.28.

The fungal strains were collected from the rumen of Nellore steers that were raised in an extensive system on *Urochloa decumbens* cv. Basilisk, with mineral supplementation containing urea

(Abrão *et al.*, 2014). These fungi were identified by analyzing the macroscopic and micromorphological characteristics, as well as the ribosomal DNA sequences obtained from the amplification of the ITS region of the rDNA (ITS1 – TCCGTAGGTGAACCTGCGG and ITS4 –TCCTCCGCTTATTGATATGC) (White *et al.*, 1990). The products were analyzed in DYEnamicTM (Amersham Biosciences, USA) using the MegaBACETM 1000 automated sequencing system at the Genome Analysis and Gene Expression Center. The sequences obtained were analyzed using BLASTn v. 2215 of BLAST 2.0 (Altschul, 1997). This species was considered an isolate with a similarity of 99% or more. The strains were deposited in GenBank and were identified as *Aspergillus terreus* [KF781532] and *Trichoderma longibrachiatum* [KF781535].

These strains were selected because they had greater proportions in the bovine digestive tract, they did not produce mycotoxins, and they had higher production of fibrolytic enzymes (Abrão *et al.*, 2014, 2017).

The forage was ensiled in silo tubes with a packing density of 700 kg/m³ (or 700g/dm³) of fresh matter. The experimental silos were made with PVC tubes with a diameter of 10cm and a height of 40cm (3.141dm³). Each silo had a fixed lid at the base and a movable lid at the top with a Bunsen-type valve for gases to escape. A bag of non-woven fabric with 200g of dry sand was placed into the silo and used as effluent drain. Over the bag, a protective mesh was placed to separate the forage. The sand was pre-dried in a forced circulation oven for 72 h at 65ºC. Each complete structure (silo + sand drain + protective mesh) was identified and weighed using a semi-analytical scale before the ensiling process.

The amount of 2.2kg of fresh forage was inoculated according to the experimental group: AT15 – 45mL inoculum and 55mL sterile culture medium, TL20 – 55mL inoculum and 45mL culture medium, and MIX – 45mL AT15 and 55mL TL20 solutions. The control group consisted of 100mL of sterile culture medium to standardize the amount of nutrients added to the culture medium. Subsequently, the forage containing the inoculants and the control were stored in the experimental PVC tube silos. After packing the forage, the silos were closed, sealed, re-weighed, and stored for 31 days.

After 31 days of fermentation, the silos were opened to determine the microbiological communities, chemical composition, loss of DM and effluents, and aerobic stability. Fermented forage was transferred to sterile plastic bags and homogenized. A 5g aliquot was removed and mixed in 30mL of sterile 0.9% NaCl solution and 15mL of sterile glycerin and kept in an ultra-freezer for subsequent microbiological analysis.

After the storage period, the full and empty silos were weighed to determine the total and effluent losses. Total dry matter loss was estimated as the difference between the initial and final dry mass weight of the experimental silos in relation to the amount of dry mass ensiled, discounting the weight of the tube, cover, sandbag at the beginning of ensiling process, according to Schmidt *et al.* (2011). The measurement of effluent loss was performed according to Jobim *et al.* (2007). After the estimation of the losses, the same procedure used to determine the pH of fresh forage was used on the silage. Each experimental unit had the pH determined individually.

To determine the microbiological analysis, samples of 5g of fresh silage were diluted in 45mL of sterile 0.9% NaCl solution, and vortexed for 5min. Subsequently, 10µl aliquots of the 10^{-1} to 10^{-10} dilutions were inoculated into sterile *Petri* dishes containing MRS agar medium (Merck KGaA®, Darmstadt, Germany) for lactic acid bacteria growth; potato dextrose agar (KASVI®, Terámo, Italy) containing 1.5% chloramphenicol solution (10%/v) for fungal growth, and MacConkey agar (KASVI®, Terámo, Italy) for Enterobacteriaceae growth. The MRS plates were incubated at 37°C for 48h in anaerobic jars with carbon dioxide reactors (Permution ®, Curitiba, PR, Brazil), while the MacConkey and potato dextrose agar plates were incubated at 37°C in a BOD oven and monitored for two and seven days, respectively.

For analysis of *Clostridium spp*., 50µL of 10^{0} to 10^{-3} dilutions were inoculated by *Pour-plate* onto *Petri* dishes with TST agar (Isofar, Rio de Janeiro, Brazil), containing the antibiotics polymyxin sulfate B (12 000IU/mL) and nystatin (100 000 IU/mL). After solidification of the

inoculated medium, the plates were incubated in anaerobic jars with carbon dioxide reactors (Permution ®, Curitiba, PR, Brazil) and incubated at 37°C for 72h.

The colony-forming units (CFU/mL) verified in each culture medium were differentiated according to the colony's morphological structures (colony color, size, and shape) and quantified with a colony counter (Murray *et al.*, 2007). The fungi were isolated and evaluated on slides using the microculture method and subsequently identified using an optical microscope with 40 and 100 × objective lenses according to St-Germain and Summerbell (2011).

All procedures for microbiological analysis were conducted in triplicate for the fresh forage and the experimental silage units, both at the time of opening and after the loss of stability (10 days of air exposure).

To determine the chemical composition of the silage, a sample of approximately 300g from each experimental unit was placed in an oven with forced air circulation at 55°C for 72h to determine the pre-dry matter. The samples were ground in a Willey grinder with 1 mm diameter sieves and analyzed according to the AOAC (2005) for dry matter (DM, method 934.01), ash (method 942.05), crude protein (CP, method 954.01), and ether extract (EE, method 920.39). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) analyses were performed according to Van Soest et al. (1991) using an ANKOM200 fiber analyzer unit (ANKOM Technology Corporation, Fairport, New York, USA). Non-fiber carbohydrates were calculated as follows: NFC = 100 – (% NDF + % EE + % CP + % Ash).

The remainder of the silage from each repetition was homogenized and placed in an open pot and exposed to air in a room with controlled temperature (25.0℃ ± 1.3℃). The pH of the silage was evaluated every 24 h over 10 days. Jobim *et al.* (2007) stated that aerobic stability represents the time required for silage to reach two pH units above the initial value. At the end of the stability assessment period, a 5g aliquot of the forage was preserved in an ultrafreezer with 30mL of sterile 0.9% NaCl solution and 5mL of sterile glycerin for microbiological analysis after air exposure and stability breakage.

The Shapiro-Wilk test was used to verify if the values of each variable satisfied a normal distribution, while the Cochran and Bartlett test was used to assess the results for homogeneity of variances. Microbial populations were estimated as colony-forming units (CFU) per gram of DM forage or silage and were transformed into $\log_{10}x$. The silage parameters, aerobic stability, and microbial data were evaluated in a completely randomized design, considering the four experimental groups (AT15, TL20, MIX, and CONTR), with seven replications.

Analysis of variance was performed, and the averages were compared using Duncan's test at the level of 5% probability. The microbial data were analyzed as a split-plot design 4 × 2 considering four inoculum types and two periods (after opening and after break stability). These data were tested considering 10% of probability. We used the package Easyanova, version 4.0, in the statistical software R (R Core Team, 2021).

RESULTS AND DISCUSSION

There was a high count of yeast fungus (2.0×10^6 CFU/mL), mycelial fungi (3.36×10^5 CFU/mL) and Enterobacteriaceae (1.0×10^6 CFU/mL) in the grass before ensilage. The yeasts present at the beginning of the fermentation process can impair fermentation by competing with lactic acid bacteria (LAB). Wambacq *et al.* (2016) reported the presence of fungi of the *Aspergillus*, *Fusarium*, *Penicillium*, and *Trichoderma* genera in maize and grass forage before ensiling. Fresh elephant grass showed low LAB counts ($<10^5$ CFU/mL) that were identified as Diplococci. This result corroborates the study by Ferreira *et al.* (2013), who reported that tropical grasses have low colonization with these bacteria.

Before ensilage, elephant grass forage showed the following composition: crude protein – 41.6 g/kg, ethereal extract – 18.0 g/kg, NDF – 702.8 g/kg, mineral matter – 125.7 g/kg, DM – 219.4 g/kg, NFC –111.9 g/kg, and pH – 6.28. The grass forage must have dry matter content between 28 and 40% and at least 6-8% of soluble

carbohydrate to favor the fermentation process and improve the lactic acid production.

The different types of inoculums influenced the pH at the silo opening (pH), loss of dry matter, time to break the stability (TBE), and effluent losses ($P<0.05$). The pH value at the opening was significantly influenced by the type of inoculant (Table 1). The CONTR, AT15, and TL20 groups had a pH below 4.2 and did not differ from each other (Tab. 1); however, the silage inoculated with MIX showed a pH above the recommended value for humid grass silage (4.2). Therefore, the silages of the CONTR, AT15, and TL20 groups were well preserved during the storage period, since at the opening of the silos, they had pH values within the appropriate threshold of 3.8 to 4.2 proposed by McDonald *et al.* (1991). The pH value is considered one of the main factors that influence the quality of the silage and represents the intensity of fermentation (Liu *et al.*, 2020). Wang *et al.* (2019a) evaluated the effect of inoculation with the ruminal fungus *Piromyces sp*. in the silage of whole corn plants and reported a pH below 4.0, after opening at 30 and 60 days of fermentation.

The silages inoculated with AT15, TL20, and CONTR showed lower DM losses and did not differ from each other, while the MIX showed a loss of MS significantly higher than the CONTR. Inoculation with two fungi in the same population (10^5 each) may have contributed to increase soluble carbohydrate consumption and DM loss when compared to the CONTR. Despite this result, inoculation with the cellulolytic fungus *Trichoderma reesei* reduces the DM loss in *Pennisetum sinese* silage after 60 days of storage (Li *et al.*, 2018). The reduction in dry matter loss is associated with a rapid reduction in pH and with factors that favor lactic fermentation.

Table 1. Characteristics of pH, time to break stability (days), loss of dry mass (%), and effluent (g/kg of fresh mass) of elephant grass silages with fungi from the rumen of cattle

Types of silage	pH at the opening	Loss of dry matter (%)	Time to break stability (days)	Effluent losses (g/kg)
AT15	4.10b	3.89ab	6.1ab	22.9a
TL20	4.17b	3.86ab	6.4ab	23.5a
MIX	4.40a	4.56a	7.4a	23.2a
CONTR	4.05b	3.13b	4.4b	19.4b
CV (%)	5.71	17.83	28.36	12.15
p-value	0.0441	0.0104	0.0272	0.0329

AT15: *Aspergillus terreus* – 1.0×10^5 colony forming units per gram of fresh fodder weight; TL20: *Trichoderma longibrachiatum* – 1.0×10^5 CFU/g of fresh mass weight; MIX: *A. terreus* the *T. longibrachiatum*, each with 1.0×10^5 CFU/g of fresh mass weight; CONTR: not inoculated; CV: coefficient of variation (%) Means followed by the same letter in the column do not differ by Duncan's test at 5 % probability.

The silages inoculated with AT15, TL20, and MIX produced more effluent than those uninoculated CONTR (Table 1). However, all the loss values were considered acceptable based on the criteria of Silva *et al.* (2019). In this study, inoculation with cellulolytic fungi was an important factor in the production of effluents since the breaking of the cell wall structure could be the result of the action of fungi and their enzymes. By producing highly active cellulases, hemicellulases, stearases, and cellulosomes, fungi degrade plant cell walls, which exposes the cell content and favors the action of other microorganisms (Wang *et al.*, 2011, 2019a) that results in more effluent. Effluent loss is directly related to the moisture content of the ensiled mass. These losses can reduce the nutritional value since the effluent contains soluble compounds from the cellular content (Silva *et al.*, 2019). The application of increasing levels of cellulase and xylanase in elephant grass silages significantly increased the production of effluents (Lemos *et al.*, 2020).

The aerobic stability of silage is the resistance of the ensiled mass to spoilage after exposure to air. Spoilage is represented by the oxidation of organic acids, ethanol, and water-soluble carbohydrates, resulting in an increase in pH and a reduction in digestibility and energy content (Jobim *et al.*, 2007). The silages inoculated with MIX were more stable and presented a significantly higher time to break (TBE) than CONTR. After the fourth day of aerobic

exposure, there was a break in the stability of the CONTR silage, whereas for silages treated with AT15 and TL20, this occurred on the sixth day, and with the MIX treatment, the TBE occurred after the seventh day (Tab. 1; Fig. 1).

Exposure to oxygen promotes the consumption of lactic acid by yeasts, which quickly increases the pH of the silage (Duniere *et al.*, 2017). However, the low production of lactic acid observed in silages with pH values greater than 4.2 can result in a longer time to break stability, as observed in the MIX. In addition, the greater number of fungi inoculated in the MIX treatment may have resulted in increased competition with yeasts in the post-opening period and, consequently, greater stability.

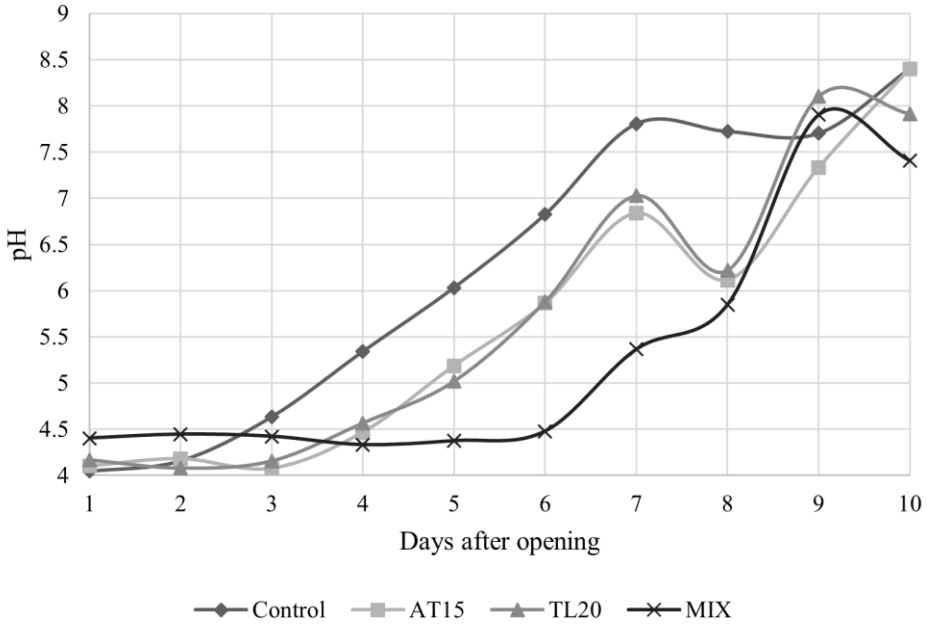

Figure 1. Average pH values during 10 days of aerobic exposure of elephant grass silage inoculated with *Aspergillus terreus* at 1.0 × 10^5 CFU/g of fresh mass weight (AT15), *Trichoderma longibrachiatum* at 1.0 × 10^5 CFU/g of fresh mass weight (TL20), MIX of the two fungi at 1.0 × 10^5 CFU/g of fresh mass weight each (MIX), and uninoculated silage (Control).

The different types of inoculums did not influence the content of mineral matter (MM), crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), acid detergent fiber (ADF), and non-fibrous carbohydrates (NFC) (Table 2) (P>0.05). The MM values were considered high for the evaluated forage. According to Silva *et al.* (2019), elephant grass 'BRS Capiaçu' harvested after 70 days had an MM content of 98.0 g/kg. The crude protein content was considered low in fresh forage, which could be a limitation for the fiber usage by ruminal microorganisms (Silva *et al.*, 2019). The grass silage has low EE content, and the lack of inoculum effect was expected. The value of NFC was low in all treatments (Table 2). Value of NFC from 199.7 to 235.2 g/kg were observed in elephant grass forage harvested from 70 to 98 days (Araújo *et al.*, 2020) and 89.4 g/kg was observed in elephant grass 'BRS Capiaçu' (Monção *et al.*, 2020).

Table 2. Content of mineral matter (MM), neutral detergent fiber (NDF), acid detergent fiber (ADF), crude protein (CP), ether extract (EE), and non-fibrous carbohydrates (NFC) from elephant grass 'Cameroon' silages with fungi from bovine rumen

Types of silage	Content (g/kg)					
	MM	NDF	ADF	CP	EE	NFC
AT15	112.2	743.0	419.5	37.9	24.1	82.7
TL20	117.3	722.6	424.6	40.0	23.3	96.7
MIX	115.7	748.1	439.8	35.5	25.0	75.6
CONTR	114.8	737.8	418.8	36.4	24.6	86.3
CV (%)	4.60	2.68	4.67	8.26	21.95	18.02
p-value	0.4099	0.2077	0.2904	0.1226	0.9604	0.1834

AT15: *Aspergillus terreus* –1.0 × 10^5 colony forming units per gram of fresh fodder weight; TL20: *Trichoderma longibrachiatum* –1.0 × 10^5 CFU/g of fresh mass weight; MIX: *A. terreus* the *T. longibrachiatum*, each with 1.0 × 10^5 CFU/g of fresh mass weight; CONTR: not inoculated; CV: coefficient of variation (%).

The values of NDF and ADF were high and like those observed in elephant grass 'BRS Capiaçu' with 150 days of regrowth (711.1 and 502.6 g/kg, respectively). Despite the lack of inoculum effect on NDF and ADF, the fungus *T. longibrachiatum* and *A. terreus* produced high levels of cellulase, carboxymethylcellulase and avicelase in medium with the tropical forage *Urochloa decumbens* and sugar cane bagasse (Alves *et al.*, 2021; Duarte *et al.*, 2021) indicating its potential to break complex carbohydrates and provide soluble substrates for silage fermentation. In another study, Wang *et al.* (2019a) reported that inoculation of maize silage with the ruminal fungus *Piromyces sp*. CN6 CGMCC 14449 increased NDF degradation by 8.28% and acid detergent fiber (ADF) by 10.5 % in an *in vitro* fermentation test.

The microorganisms in silage play a fundamental role in the fermentation process (Wang *et al.*, 2019b). In this study, a significant interaction was detected between the periods (after opening and after break stability) and the type of inoculation on the level of lactic acid bacteria of the *Diplococcus* genus (P <0.005; Table 3). These bacteria were significantly more populous in CONTR and TL20 than in the other inoculants at the opening; however, subsequently the mean counts of these bacteria were similar between the types of inoculation. For CONTR and TL20, the population was higher at the opening than at 10 days after opening, unlike the MIX, which had a significantly greater count of *Diplococcus* individuals after 10 days of opening (P <0.01; Fig. 2).

For the *Lactobacillus* population, an effect of the type of inoculation (P <0.10) and time of evaluation (P <0.0001) was observed. At the opening, the average count of 1.28 × 10^7 CFU/mL (5.8295) was statistically higher than that 10 days after opening, with 5.51 × 10^6 CFU/mL (3.0710). This result indicates that the total absence of oxygen was important for the maintenance of the *Lactobacillus* population, and that the opening resulted in a decrease in the count.

For the population of *Lactobacillus* spp. classified as gram-positive *Streptobacillus*, the opening count was 2.42 × 10^6 CFU/mL (3.9416), which was statistically higher than the value observed 10 days after opening of 2.08 × 10^3 CFU/mL (1.1541). A significant effect was observed on the gram-positive rods from the type of inoculation (P < 0.05) and the evaluation period (P < 0.001), with a higher count in silages inoculated with TL20 and MIX 10 days after opening (Tab. 3).

The inoculation of *T. longibrachiatum* was important for maintaining larger populations of *Lactobacillus* spp. after breaking stability. This species of fungus produces cellulases, xylanases, and phenoloxidases that solubilize lignin and allow cellulose to break down into glucose, which is used exclusively by LAB to produce lactic acid. Lactic acid, in turn, reduces the pH, justifying the greater quantity of rods and longer time to break the stability of the silage treated with TL20 and MIX (Abrão *et al.*, 2014, 2017, 2018; Ávila *et al.*, 2014; Muck *et al.*, 2018).

Table 3. Log of average colony forming units (CFU log) per gram of silage and standard error of the mean (SE) for microorganisms present in elephant grass 'Cameroon' silages inoculated with *Aspergillus terreus* –1.0 × 10^5 CFU/g of fresh mass weight (AT15), *Trichoderma longibrachiatum* –1.0 × 10^5 CFU/g of fresh mass weight (TL20), with the mix of these two fungi each with 1.0 × 105 CFU/g of fresh mass weight (MIX), or uninoculated (CONTR) at the time of opening and 10 days after opening

Variables	Average CFU/g (Log CFU) at the opening			
	CONTR	AT15	TL20	MIX
Diplococcus	3.01a	0.00b	1.89ab	0.00b
Lactobacillus spp.	5.36	5.97	5.53	6.13
Bacillus	3.96	4.95	5.42	5.96
Streptobacillus	5.06	2.63	3.37	3.02
Total lactic acid bacteria	6.54	5.97	6.63	6.17
Clostridium spp.	0.00	1.10	0.48	2.25
Enterobacteriacea	0.00	0.00	0.42	0.56
Yeasts	0.89	1.50	0.00	0.67
Mycelial fungi	0.78	2.56	2.18	3.25
	Average CFU/g (Log CFU) 10 days after opening			
	CONTR	AT15	TL20	MIX
Diplococcus	0.00	0.00	0.00	2.34
Lactobacillus spp.	0.91b	0.83b	5.10a	3.26ab
Bacillus	0.91b	0.83b	5.10a	2.48ab
Streptobacillus	0.00	0.00	0.00	0.78
Total lactic acid bacteria	1.89b	1.58b	5.10a	5.38a
Clostridium spp.	0.83	0.00	1.33	0.89
Enterobacteriacea	0.00	0.52	0.00	0.00
Yeasts	9.52	9.35	9.13	8.74
Mycelial fungi	6.66	7.24	7.69	7.61

The same letters on the line for the point of opening the silages or for 10 days after opening do not differ from each other by Duncan's test at 10 % probability.

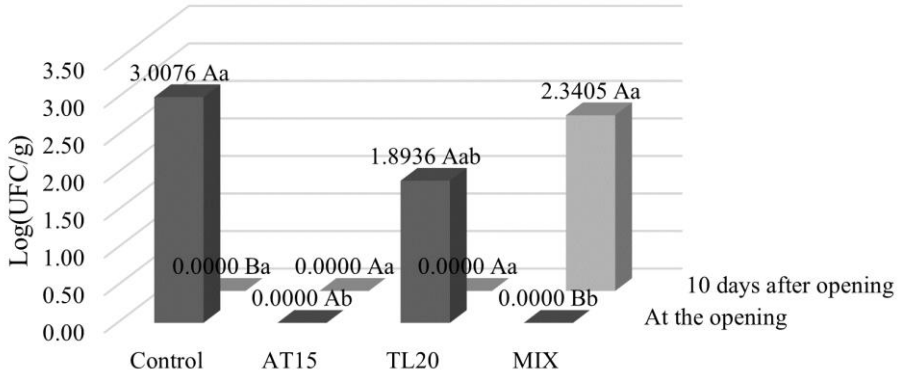

Figure 2. Averages of colony forming units (CFU log) per gram of elephant grass 'Cameroon' silage for *Diplococcus* grown in MRS medium from samples inoculated with *Aspergillus terreus* (AT15), *Trichoderma longibrachiatum* (TL20), with the mix of these two fungi (MIX), or not inoculated (Control) at the time of opening and 10 days after opening. Means followed by the same lowercase letter within each evaluation period and uppercase within each type of inoculation do not differ by Duncan's test (P <0.10).

When evaluating the effect of the type of inoculation on the *Lactobacillus* population, variation between experimental groups was found after 10 days of opening. Thus, the TL20 treatment showed a higher count and did not differ statistically from the MIX, indicating that the presence of *T. longibrachiatum* affected the *Lactobacillus* population after opening. The *Lactobacillus* counts of TL20 during the opening and after 10 days were similar; therefore, TL20 may have released enzymes that favored the growth of lactic acid bacteria.

Cai *et al.* (1998) reported that *Leuconostoc*, *Pediococcus*, and *Enterococcus* spp. begin lactic fermentation at the start of the ensiling process, and lactic acid bacteria in the form of rods dominate in the later stage. According to Wang *et al.* (2019b), the LAB prevents the aerobic deterioration of the silage, due to the production of lactic acid and reduction of the pH. The conversion of lactic acid to acetic acid inhibits the growth of yeasts and fungi (Liu *et al.*, 2020).

For total lactic acid bacteria (LAB), there was a significant effect of the type of inoculation (P <0.0086), the time of evaluation (P <0.001), and the interaction between the factors (P <0.0628). At the time of opening, the LAB counts were similar between the different types of inoculation. However, after 10 days of exposure to air, the counts of these bacteria were significantly higher for TL20 and MIX (Fig. 03). For the CONTR and AT15 silage, the total lactic bacteria counts were higher at the time of opening compared to the final period. For TL20 and MIX, this count was similar between the two periods (Fig. 3). This result also indicates that the inoculation of *T. longibrachiatum* is important for maintaining populations of total lactic acid bacteria.

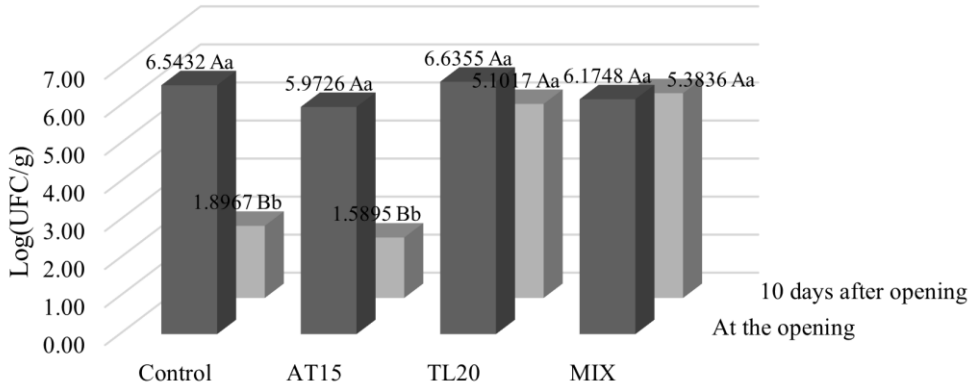

Figure 3: Colony forming units average per gram (CFU log) for total lactic acid bacteria grown in MRS medium from elephant grass 'Cameroon' silages inoculated with *Aspergillus terreus* (AT15), *Trichoderma longibrachiatum* (TL20), with the mix of these two fungi (MIX), or not inoculated (Control) at the time of opening and 10 days after opening. Means followed by the same lowercase letter within each evaluation period and uppercase within each type of inoculation do not differ by Duncan's test (P <0.10).

For bacteria of the genus *Clostridium*, the count was low and there was no significant difference in the type of inoculation or time of evaluation (Tab. 3; P> 0.10). Most *Clostridium spp.* bacteria in silage are inactive spores (Zheng *et al.*, 2017). The growth of these group occurs when LAB are inactive and the pH is very high, or when there is a high moisture content in the silage. Under anaerobic conditions, *Clostridium spp.* use soluble compounds from the cellular content of plants as a growth substrate (Zheng *et al.*, 2017)

In this study, the count of Enterobacteriaceae was low, and there was no influence of the type of inoculation (P> 0.05; Tab. 04). Wang *et al.* (2019b) and Zheng *et al.* (2017) also detected low enterobacterial counts in silage inoculated with *Lactobacillus plantarum* strain L20JPL65 and correlated the result with the rapid decline in the pH

of the silage. During ensiling, the presence of Enterobacteriaceae is undesirable, as it can compete for substrates with LAB (Wang *et al.*, 2019b; Ávila and Carvalho, 2020). Also, some species in this family can degrade proteins and produce ammonia and biogenic amines, which are undesirable in silage (McDonald *et al*., 1991).

In this study, there was no influence of the type of inoculation on the populations of yeast and mycelial fungi, also known as mold. However, it was found that the mean concentrations of yeasts and mycelial fungi were statistically higher after 10 days of opening (1.45×10^{10}, and 1.43×10^{9}, respectively) than at the time of opening the silage (4.16×10^{7}, and 2.42×10^{7}, respectively) (Tab. 3, P <0.001). The smaller population of fungi at the opening of the silos can be attributed to the lower availability of oxygen during the fermentation process. After exposure to oxygen, the population of these fungi increased and showed a greater diversity of genera. Additionally, fungi of the genus *Trichoderma* and *Aspergillus*, showing morphological characteristics of the inoculants, were observed (table 4) promoting higher time of break of stability (Fig 1)

Yeast causes dry matter losses from silage, as they metabolize soluble carbohydrates and produce alcohol (Ávila *et al.*, 2014). Yeast deteriorates silage, has a tolerance to acidic environments, and remains viable in the absence of oxygen. Besides, when silage is exposed to air, yeasts show rapid growth, which causes more dry matter losses (Ávila and Carvalho, 2020). Romero *et al.* (2017) reported that inoculation with strains of *Lactobacillus buchneri* and fibrolytic enzymes of *Trichoderma reesei* in oat silage did not alter the fungal community.

In this study, 10 fungal isolates were identified in the silage at the time of opening and 44 were observed 10 days after opening. At the opening of the silos, 100% of the isolates corresponded to the genus *Aspergillus* spp., and after exposure to air, the genera *Aspergillus* spp. (45.45%), *Emmonsia* spp. (36.36%), *Trichoderma* spp. (13.63%), and *Rhizopus* spp. (4.54%) were found (Tab. 4). The CFU/g count of fungal isolates from the main genus of *Aspergillus* increased from 10 at the time of opening to 20 after exposure to oxygen (Tab. 4). According to Ávila and Carvalho (2020), the main genera of fungi present in different silages are *Aspergillus*, *Acremonium*, and *Alternaria*. In temperate climates, the main fungal species found in silage are *Penicillium roqueforti* and *P. paneum*, but in tropical climates, *Aspergillus fumigatus* is more common (Wambacq *et al.*, 2016).

Table 4. Distribution of genera of mycelial fungi isolated from elephant grass silages (*Pennisetum purpureum* cv. Cameroon) inoculated with *Aspergillus terreus* (AT15), *Trichoderma longibrachiatum* (TL20), with the mix of these two fungi (MIX), or not inoculated (CONT) at the time of opening and 10 days after opening

Genres	Opening								
	Total	CONT	%*	AT15	%*	TL20	%*	MIX	%*
Aspergillus spp.	10	1	10.0	3	30.0	3	30.0	3	30.0
Trichoderma spp.	0	-	-	-	-	-	-	-	-
Rhizopus spp.	0	-	-	-	-	-	-	-	-
Emmonsia spp.	0	-	-	-	-	-	-	-	-
Total	10								
	10 days after opening								
	Total	CONT	%*	AT15	%*	TL20	%*	MIX	%*
Aspergillus spp.	20	5	11.4	6	13.6	7	15.9	2	4.5
Trichoderma spp.	6	2	4.5	1	2.3	1	2.2	2	4.5
Rhizopus spp.	2	-	-	1	2.23	-	-	1	2.3
Emmonsia spp.	16	3	6.8	4	9.1	5	11.4	4	9.1
Total	44								

Total = total of fungal isolates * Frequency is equal to the number of observations of each fungus divided by the number of isolates.

Ávila and Carvalho (2020) highlighted that the production of active mycotoxins can occur even at low fungal counts. Therefore, the presence of fungi in silage is associated with oxygen and an

increase in pH (Ávila and Carvalho, 2020). The acidic and anaerobic conditions of the silage are unfavorable for the growth of fungi (Romero *et al.*, 2017); however, many species of fungi can remain as spores in silage and reactivate when exposed to oxygen (McAllister *et al.*, 2018).

CONCLUSIONS

There is no influence of the fungus inoculation on the chemical composition of elephant grass 'Cameroon' silage. Inoculation with a mixture of *Aspergillus terreus* and *Trichoderma longibrachiatum* increases the dry matter losses and the pH at the silo opening, but also increases the time to break stability. Inoculation with *T. longibrachiatum* was more efficient in maintaining populations of total lactic acid bacteria after opening; therefore, this strain has the potential as an additive for elephant grass silage.

ACKNOWLEDGEMENTS

The research was supported by Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (project 310898/2018-8 and 433089/2016-4), Pró-Reitoria de Pesquisa da Universidade Federal de Minas Gerais (PRPq-UFMG, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, and Connan – Animal Nutrition.

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