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Exogenous Fibrolytic Enzymes and Length of Storage Affect the Nutritive Value and Fermentation Profile of Maize Silage

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Abstract: The addition of exogenous fibrolytic enzymes (EFEs) and length of storage can affect the quality of maize silage. Therefore, the objective of this study was to evaluate the fermentative profile and the nutritive value of maize silage treated with different doses of EFEs ensiled for 30, 60, or 90 days. The study was designed as completely randomized in a split-plot arrangement of treatments, where four doses of EFEs were assigned to the main plot and three lengths of storage to the sub-plot, with four replicates per treatment. Treatments were: Control, E100 (EFEs at 100 g/ton dry matter (DM)), E150 (EFEs at 150 g/ton DM) and E200 (EFEs at 200 g/ton DM). The EFE treatment did not increase the digestibility of nutrients but increased the acetic acid concentration (1.87 vs. 1.18% DM), while decreasing the content of ethanol (0.02 vs. 0.08% DM), ethyl lactate (7.50 vs. 15.9 mg/DM) and ethyl acetate (5.58 vs. 10.6 mg/DM). Prolonged storage increased DM losses (7.05 vs. 2.32%) and acetic acid content (2.19 vs. 1.03% DM), but decreased ethanol concentration (0.02 vs. 0.09% DM). In conclusion, the addition of EFEs in maize silages did not affect nutrient digestibility and DM losses during fermentation, but it slightly decreased the concentrations of ethanol and esters and increased the acetic acid content. Although statistically significant, such differences may not be relevant biologically, due to the relatively low concentrations of ethanol and esters in all treatments.

Keywords: fermentation; length of storage; nutrient digestibility; xylanase

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1. Introduction

Maize silage is the most used roughage in the diets of dairy cows in Brazil [1] and worldwide [2]. Improvements in the nutritive value of maize silage can result in a reduction in diet costs and in greater milk production [3].

Forage digestibility is a limiting factor to the intake of digestible energy by dairy cows, and the use of exogenous fibrolytic enzymes (EFEs) in maize silage production has been studied as an alternative to catalyze the depolymerization of fiber, which is the main barrier to nutrient availability [4,5]. Although the major fractions of maize silage consist of fibrous components (stems, leaves, husks, and cobs), high-quality maize silages have a considerable amount of grains. The grain fraction is a potential substrate for the action of EFEs, which can act to improve the silage quality through hydrolyzation of the cell wall (mainly arabinoxylan) in the kernel endosperm, which encapsulates starch [6]. The use of EFEs, with xylanase as the main activity, is known to improve in vitro digestion of silages [7,8]. Furthermore, cellulases and xylanases could promote the production of water-soluble carbohydrates (WSC) from fiber hydrolysis, which could result in an increase of lactic acid content [9].

Because the pH of the silage is lower and its temperature at fermentation onset is relatively higher than that in the rumen, the application of EFEs at ensiling, rather than providing it directly to the animal, can optimize the enzyme's activity and its effects [10]. However, studies have shown inconsistencies regarding the effects of EFEs when applied at ensiling [11], and the efficacy of EFEs in improving the silage nutritive value is dependent on their application rate [12,13].

In addition to EFE application at ensiling, the length of storage can affect the nutritive value of maize silages. Increased length of storage has previously been shown to increase the concentration of soluble protein and ammonia nitrogen, as well as the starch digestibility [14,15]. Therefore, we hypothesized that EFE treatment could increase the solubilization of fiber components, including the cell walls in the kLernel endosperm, allowing more substrate to be fermented, altering the fermentative profile and enhancing the nutrient availability and digestibility. Furthermore, we hypothesized that the effect of EFE application rate on silage quality and nutritive value is affected by storage length. The aim of this experiment was to evaluate the effects of EFE application rate, storage time and their interaction on fermentation characteristics and nutritive value of maize silage.

2. Materials and Methods

2.1. Ensiling

The maize hybrid (Pioneer P2866H) was grown at the Luiz de Queiroz College of Agriculture—University of Sao Paulo, Piracicaba, Sao Paulo, Brazil. The maize crop was harvested manually at 15 cm stubble height on 11 September 2018, at half of the milk line. It was chopped in a stationary chopper (Trapp, Jaraguá do Sul, SC, Brazil) adjusted to reach a mean particle size of 10 mm. The chemical composition (mean ± SD) of the maize plant before ensiling was 31.5 ± 0.56% of Dry Matter (DM); 52.8 ± 2.7% of neutral detergent fiber (NDF); 9.1 ± 0.18% of crude protein (CP); 2.6 ± 0.20% of ether extract (EE); 71.1 ± 1.47% of DM in situ digestibility; and 60.8 ± 2.83% of NDF in situ digestibility, with a pH value of 4.89 ± 0.22. The mix of exogenous fibrolytic enzymes (EFEs) (Rovabio Advance L2, Adisseo, Commentry, France) used in the present study was in liquid form and contained two main active enzymes, endo-1,4-β-xylanase (E.C. number 3.2.1.8) and endo-1,3(4)-ß-glucanase (E.C. number 3.2.1.6), obtained from *Talaromyces versatilis* strains, as declared by the manufacturer. The guaranteed concentration was 12,500 VU/mL (1.8%) of xylanase and 8600 VU/mL (1.2%) of ß-glucanase. The product also contained sorbitol (25.2%) and potassium sorbate (0.11%). One visco-unit (VU) of xylanase or ß-glucanase is defined as the quantity of enzyme required to hydrolyze the substrate (wheat arabinoxylan or barley ß-glucan, respectively), reducing the solution viscosity by one unit per minute, at 30 °C and pH 5.5. The endo-ß-glucanase to xylanase ratio of the product was 0.69:1. Chopped maize was mixed well before four samples of 7 kg each were randomly selected from the initial pile. From each of these four samples, subsamples were allocated to the following four treatments: control (untreated), EFEs at 100 g/ton DM (E100), EFEs at 150 g/ton DM (E150) and EFEs at 200 g/ton DM (E200). The enzymes were diluted in 100 mL of deionized water and applied by hand-spraying the solution onto the material. The control silage was treated with 100 mL of deionized water, exclusively. Samples of approximately 500 g of treated forage were placed into low-density polyethylene bags, 250 × 350 mm in size with 0.20 mm of thickness (ZPP Embalagens, Santa Rita do Passa Quatro, SP, Brazil), and vacuum sealed. The bags were stored at room temperature (24.6 °C ± 0.97) for 30, 60 and 90 days. The vacuum bags were weighed right after sealing and before each opening time to determine the DM losses, according to Jobim et al. [16]. In total, there were 48 ensiling bags, and each bag was exclusively opened once.

2.2. Chemical Analysis

After 30, 60 and 90 days of ensiling, the silos were opened and samples were collected and frozen at −40 °C. The frozen samples were thawed and dried in a forced-air oven for 72 h at 55 °C and ground to pass through a 1 mm mesh screen (Wiley mill, Arthur H.

Thomas, Philadelphia, PA, USA). Next, the subsamples were analyzed for absolute DM content and ash, according to the Association of Official Analytical Chemistry [17]. The NDF content was analyzed according to Van Soest et al. [18] with a heat-stable α-amylase and sodium sulfite, using a fiber analyzer (Marconi, Piracicaba, SP, Brazil), without ashing. The nitrogen was analyzed by the Dumas method [19], using a nitrogen analyzer (FP-2000A, Leco Corp., St. Joseph, MI, USA). Crude protein (CP) was obtained by using the factor 6.25 × N%. Samples were also analyzed for water-soluble carbohydrates (WSC) after being submitted to 4 h of extraction using an 80% ethanol solution, using the phenol-sulfuric acid assay [20]. The starch content was analyzed using the enzymatic hydrolysis method according to Hall [21], and the concentrations of WSC and starch were determined using a spectrophotometer (Janway 6305, Marconi, Piracicaba, SP, Brazil), with the absorbance set at 490 nm for WSC and 510 nm for starch. The ammonia nitrogen was analyzed according to the method of Chaney and Marbach [22].

Subsamples of 25 g of maize silage were mixed with 225 g of deionized water for 4 min at 152 rpm using a stomacher (Nova Ética, Vargem Grande Paulista, SP, Brazil). The extract was filtered through 3 layers of cheesecloth, and the pH was measured using pH meter (DM20, Digimed Analítica, SP, Brazil). Then, the extract was centrifuged at 10,000× *g* for 15 min at 4 °C. The lactic acid content of the filtrate was determined according to the method of Pryce [23] using a spectrophotometer at a wavelength of 565 nm. Concentrations of VFA, alcohols, and esters were analyzed using a gas chromatograph GCMS QP 2010 plus and mass detector (Shimadzu, Kyoto, Japan) with a capillary column (Stabilwax cross bond carbowax polyethylene glycol) 60 m in length with a diameter of 0.25 mm (Restek, Bellefonte, PA, USA). Detection limit of trace compounds (e.g., esters) was 1 mg/kg DM. The DM content was corrected for volatile compounds using the equation proposed by Weissbach [24], in which 2,3-butanediol and esters were added: DMcorr (% as fed) = DM (% as fed) + 0.08 × lactic acid (% as fed) + 0.77 × 1,2-propanediol (% as fed) + 0.87 × 2,3-butanediol (% as fed) + 0.95 × VFA (% as fed) + esters (% as fed) + alcohols (% as fed).

2.3. In Situ Nutrient Digestibility

Approximately 15 g of the samples were placed into 10 × 20 cm woven bags with a porosity of 50 ± 10 microns (R1020 Forage Bag, ANKOM Technology, Macedon, NY, USA). The samples were not dried or processed, and they were degraded in the rumen as they were fed [25]. The ratio of sample size to free bag surface area was 37.5 mg/cm^2. Each bag was tied 1 cm below the top with rubber bands, and clips were used to attach it to a chain. To allow adequate nutrient degradation, the chain with the bags was placed in the ventral sac of the rumen for 48 h. Two fistulated dry cows (Holstein) fed 55% (DM) of maize silage and 45% (DM) concentrate (maize, soybean meal, and mineral and vitamin supplement) were used to incubate the silages. Each cow received a replicate of the samples, and blank bags were added to correct for the empty bag weight of the bag tare. All the bags were removed from the rumen simultaneously. The bags were placed into an ice bucket and washed using a washing machine. The bags were dried for 48 h at 60 °C and then weighed to calculate the DM digestion. The residual samples after incubation were analyzed for NDF and starch content, as previously described, to estimate the respective digestibility. The incubation in rumen-cannulated cows was approved by the animal welfare committee of the University of São Paulo, protocol number: 2017.5442.11.4.

2.4. Statistical Analyses

The data were analyzed using the MIXED procedure of SAS software (3.8, enterprise edition, SAS Institute Inc., Cary, NC, USA), as a completely randomized design with a split-plot arrangement of treatments, where four doses of EFEs were assigned to the main plot and three lengths of storage to the sub-plot, with four replicates per treatment. The model included the fixed effects of EFEs, length of storage and interaction between EFEs and length of storage. The effect of replicate nested within EFEs was used as error term for the main plot. The best covariance structure was defined by the smallest value for corrected

Akaike's information criterion among the variance components, compound symmetry, first-order autoregressive or unstructured.

For the 48 h in situ digestibility assay, cows were included in the model as random effect. The residual option was used to establish the degrees of freedom for the tests of the fixed-effects model. When the global *p*-value was significant for the main effects of dose and storage length and their interactions, the Tukey's honest significant difference test was used to determine significant differences between least square means (LS means) in the main effects and in the interaction. Means were considered significantly different when $p \leq 0.05$ and were considered to have a tendency to significance when $0.05 < p \leq 0.10$.

3. Results

3.1. Nutritive Value

The characteristics related to the chemical composition and nutrient digestibility of the maize silages treated with different doses of EFEs and ensiled for 30, 60 or 90 days are shown in Table 1. Averaged over storage lengths, the DM content was lower ($p < 0.01$) for E150 and E200 than for the control and E100. Considering the length of storage, the DM content decreased ($p < 0.01$) as the time increased from 30 to 90 days, when averaged over EFE treatment. The DM digestibility was unaffected by the enzyme treatment, but it tended ($p = 0.10$) to be higher at 60 days than at 30 and 90 days of storage. The content and digestibility of NDF increased from 30 to 60 days of storage, with no further increase to 90 days of storage. There was a tendency for a dose × storage length interaction ($p = 0.08$) for NDF digestibility, in which E150 and E200 tended to have a greater NDF digestibility than the E100 and control after 90 days of ensiling, whereas no dose effects were found for the shorter storage periods.

Table 1. Chemical composition and 48 h in situ digestibility of maize silages treated with exogenous fibrolytic enzymes (EFEs) at 0, 100, 150 and 200 g/ton of DM ensiled for 30, 60 and 90 days.

	DM %	DMD [1] % DM	NDF % DM	NDFD [1] % NDF	Starch % DM	StarchD % Starch	CP % DM	NH_3-N %Total N
30 d								
Control	32.0	70.3	51.8	55.1	31.8	76.2 [ab]	9.4	8.0
E100	31.3	71.4	49.0	54.4	33.0	72.0 [ab]	9.6	2.6
E150	31.0	72.2	49.0	55.9	31.2	79.1 [ab]	9.7	3.2
E200	31.0	71.5	48.5	55.5	33.4	79.3 [ab]	9.5	5.9
60 d								
Control	31.7	71.6	56.8	60.9	31.2	70.6 [b]	9.8	7.7
E100	31.5	73.3	52.0	60.8	35.1	82.3 [a]	9.8	4.5
E150	30.0	73.5	54.5	62.3	31.1	80.3 [ab]	10.3	3.0
E200	30.3	72.2	50.8	56.4	32.5	77.3 [ab]	10.1	5.0
90 d								
Control	30.5	70.3	51.0	57.2	30.4	71.2 [b]	10.0	6.3
E100	30.7	69.9	51.8	55.9	31.8	76.5 [ab]	10.3	10.9
E150	29.3	72.4	51.5	60.8	32.7	78.6 [ab]	10.6	10.6
E200	29.8	73.1	52.5	60.2	32.9	79.6 [ab]	10.1	14.9
SEM [2]	0.22	1.57	0.007	1.76	1.43	3.06	0.23	0.52
EFE doses								
Control	31.4 [a]	70.8	53.2	57.7	31.2	72.6	9.8	7.0
E100	31.1 [a]	71.5	50.9	57.0	33.3	76.9	9.9	6.3
E150	30.1 [b]	72.7	51.7	59.6	31.7	79.4	10.2	5.5
E200	30.3 [b]	72.3	50.6	57.4	32.9	78.7	9.9	8.8

Table 1. *Cont.*

	DM %	DMD [1] % DM	NDF % DM	NDFD [1] % NDF	Starch % DM	StarchD % Starch	CP % DM	NH_3-N %Total N
SEM [2]	0.15	1.36	0.006	1.49	0.96	2.79	0.13	0.36
Length of storage								
30 d	31.3 [a]	71.4	49.6 [b]	55.2 [b]	32.4	76.7	9.6 [b]	5.2 [b]
60 d	30.9 [b]	72.6	53.5 [a]	60.1 [a]	32.5	77.6	10.0 [a]	5.0 [b]
90 d	30.1 [c]	71.4	51.7 [ab]	58.5 [ab]	32.0	76.5	10.3 [a]	10.3 [a]
SEM [2]	0.13	1.28	0.005	1.47	0.83	2.66	0.12	0.26
p-value								
Dose	<0.01	0.22	0.12	0.06	0.24	0.09	0.17	0.68
Storage length	<0.01	0.10	0.01	<0.01	0.86	0.64	0.01	0.01
D × L [3]	0.19	0.47	0.22	0.08	0.65	0.01	0.91	0.18

[a–c] Means in a column with different superscripts differ ($p < 0.05$).[1] DMD—DM digestibility; NDFD —NDF digestibility.[2] SEM—Standard error of means. [3] D × L—Interaction between dose and storage length.

The starch content was unaffected by the enzyme treatment or by the length of storage ($p > 0.10$; Table 1). There was an interaction ($p = 0.01$) between dose and length of ensiling for starch digestibility. The enzyme did not affect the starch digestibility at 30 and 90 days, but at 60 days of storage, the E100 had higher starch digestion than the control treatment.

The ammonia-N concentration was unaffected by the EFE treatment, but when the storage length was increased from 60 to 90 days, it was increased by 5.3% units.

3.2. Fermentation Characteristics

Table 2 shows the main characteristics related to silage fermentation. The DM content corrected for volatile compounds tended to follow the same pattern as the uncorrected DM content, in which E150 and E200 had lower DM content than the control and E100 ($p < 0.01$). Additionally, the DMcorr decreased from 60 to 90 days of storage ($p < 0.01$). The DM losses were not affected by enzyme treatment, but they were affected by the length of storage ($p < 0.01$). There was a one-fold increase in DM losses from 30 to 60 days and an approximately half-fold increase from 60 to 90 days of storage. The pH of all silages, regardless of enzyme treatment, reached its lowest value ($p = 0.05$) at 60 days of storage.

There was an interaction ($p = 0.01$) between EFE dose and length of storage for WSC content, in which E150 had a higher WSC content than the control at 60 days of storage, whereas no effect of EFEs was found at other storage lengths (Table 2). There was also an interaction between EFE dose and length of storage ($p = 0.01$) for the concentration of lactic acid. At 60 days of storage, silages treated with E150 had a higher concentration of lactic acid than the control, whereas no differences were found at 30 and 90 days of storage. The acetic acid content was higher in silages treated with E150 and E200 than in the control at all storage periods ($p = 0.01$). The difference in acetic acid concentration between EFEs and control silages was greater at 90 days than at 30 and 60 days. Generally, the acetic acid concentration increased with storage time for both treated and untreated silages. There was an interaction between dose and length of ensiling for propionic acid content ($p = 0.01$). The concentration of this acid increased over time, and at 90 days of storage, it was lower in E200 than in the other treatments. Butyric acid content was low but increased over time ($p = 0.01$).

Table 2. Fermentation characteristics of maize silages treated with exogenous fibrolytic enzymes (EFEs) at 0, 100, 150 and 200 g/ton of DM ensiled for 30, 60 and 90 days.

	DM Corr [1] %	DM Losses %	pH	WSC % DM	Lactic Acid % DM	Acetic Acid % DM	Propionic Acid mg/kg DM	Butyric Acid mg/kg DM
30 d								
Control	32.7	0.92	4.26 ^{a}	4.01 ^{abc}	2.87 ^{abcd}	0.68 ^{f}	80.8 ^{d}	15.0
E100	31.9	4.01	4.32 ^{a}	3.58 ^{bc}	2.87 ^{abcd}	1.13 ^{e}	90.5 ^{d}	15.3
E150	31.5	1.99	4.21 ^{ab}	4.16 ^{abc}	3.53 ^{abc}	1.03 ^{e}	85.8 ^{d}	11.5
E200	31.6	2.34	4.19 ^{ab}	3.94 ^{bc}	4.25 ^{a}	1.26 ^{de}	101 ^{d}	10.8
60 d								
Control	32.3	3.25	4.00 ^{c}	3.33 ^{c}	2.65 ^{bcde}	1.22 ^{de}	151 ^{cd}	20.8
E100	32.2	4.09	4.00 ^{c}	4.05 ^{abc}	3.30 ^{abc}	1.55 ^{cd}	196 ^{cd}	9.0
E150	31.0	5.38	4.00 ^{c}	5.53 ^{a}	4.29 ^{a}	1.82 ^{bc}	421 ^{c}	15.8
E200	30.9	6.02	4.09 ^{bc}	4.16 ^{abc}	4.07 ^{ab}	1.70 ^{bc}	305 ^{cd}	19.0
90 d								
Control	31.4	6.02	4.22 ^{ab}	4.25 ^{abc}	1.74 ^{de}	1.63 ^{bcd}	1107 ^{a}	23.5
E100	31.5	6.75	4.17 ^{ab}	4.76 ^{ab}	1.13 ^{e}	2.00 ^{b}	1059 ^{a}	15.8
E150	30.2	8.52	4.16 ^{abc}	4.25 ^{abc}	2.10 ^{cde}	2.46 ^{a}	1071 ^{a}	29.0
E200	30.8	6.90	4.19 ^{ab}	4.54 ^{abc}	1.56 ^{de}	2.65 ^{a}	728 ^{b}	15.0
SEM [2]	0.27	1.062	0.036	0.294	0.303	0.083	51.35	3.34
EFE doses								
Control	32.1 ^{a}	3.40	4.16	3.86	2.42	1.18	446	19.8
E100	31.9 ^{a}	4.95	4.16	4.13	2.43	1.56	448	13.3
E150	30.9 ^{b}	5.30	4.12	4.65	3.31	1.77	526	18.8
E200	31.1 ^{b}	5.09	4.16	4.21	3.29	1.87	378	14.9
SEM [2]	0.16	0.615	0.020	0.170	0.198	0.048	44.35	1.57
Length of storage								
30 d	31.9 ^{a}	2.32 ^{c}	4.25	3.92	3.38	1.03	89.6	13.1 ^{b}
60 d	31.6 ^{a}	4.69 ^{b}	4.02	4.27	3.58	1.57	268	16.1 ^{ab}
90 d	31.0 ^{b}	7.05 ^{a}	4.19	4.45	1.63	2.19	991	20.8 ^{a}
SEM [2]	0.14	0.533	0.018	0.147	0.171	0.042	38.41	1.35
p-value								
Dose	<0.01	0.21	0.42	0.02	0.01	<0.01	0.07	0.19
Storage length	<0.01	<0.01	<0.01	0.05	<0.01	<0.01	<0.01	0.01
D × L [3]	0.30	0.26	0.05	0.01	0.01	0.01	0.01	0.14

$^{a–f}$ Means in a column with different superscripts differ ($p < 0.05$).[1] DM corr—Dry matter corrected for silage volatile compounds, using Weissbach (2009) equation.[2] SEM—Standard error of means.[3] D × L—Interaction between dose and storage length.

Concentrations of alcohols and esters in the maize silages treated with increased doses of EFEs are presented in Table 3. The ethanol content was lower ($p < 0.01$) in EFE silages than in the control, and the lowest concentration of ethanol was observed when applying the highest dose of EFEs. The ethanol concentration decreased over time ($p < 0.01$). There were interactions between EFE dose and storage length for 1,2-propanediol, 2,3-butanediol and 1-propanol. At 30 days, E200 had higher concentration of 1,2-propanediol ($p < 0.01$) than the control. At 60 days, E150 had higher 2,3-butanediol content than the control ($p = 0.01$). At 90 days of storage, the control treatment had a higher concentration of 1-propanol than silages treated with EFEs ($p < 0.01$). The ethyl esters were affected by the EFE doses and the lengths of storage. Concentrations of ethyl lactate ($p < 0.01$) and ethyl acetate ($p = 0.01$) were lower in E200 than in the control. Regarding the length of storage, the ethyl lactate concentration gradually decreased from 30 to 90 days ($p < 0.01$) and the ethyl

acetate content decreased from 30 to 60 days but remained constant from 60 to 90 days of storage ($p < 0.01$).

Table 3. Concentrations of alcohols and esters (DM basis) in maize silages treated with exogenous fibrolytic enzymes (EFEs) at 0, 100, 150 and 200 g/ ton of DM ensiled for 30, 60 and 90 days.

	Ethanol % DM	1,2-Propanediol mg/kg DM	2,3-Butanediol mg/kg DM	1-Propanol mg/kg DM	Ethyl Lactate mg/kg DM	Ethyl Acetate mg/kg DM
30 d						
Control	0.140	690 ^{b}	131 ^{d}	2.75 ^{b}	18.8	17.7
E100	0.090	1152 ^{ab}	189 ^{d}	4.50 ^{b}	15.3	9.67
E150	0.092	1194 ^{ab}	166 ^{d}	2.50 ^{b}	15.5	14.0
E200	0.055	1873 ^{a}	188 ^{d}	4.25 ^{b}	14.5	11.5
60 d						
Control	0.065	1111 ^{ab}	169 ^{d}	27.8 ^{b}	16.5	5.00
E100	0.042	1429 ^{ab}	241 ^{bcd}	30.0 ^{b}	14.3	6.00
E150	0.045	1587 ^{ab}	347 ^{abc}	83.8 ^{a}	14.8	6.75
E200	0.010	1474 ^{ab}	229 ^{cd}	19.5 ^{b}	5.00	1.50
90 d						
Control	0.042	1281 ^{ab}	496 ^{a}	100 ^{a}	12.5	9.00
E100	0.010	1480 ^{ab}	424 ^{a}	30.5 ^{b}	6.50	5.25
E150	0.005	796 ^{b}	384 ^{ab}	12.0 ^{b}	3.50	3.75
E200	0.002	1010 ^{ab}	333 ^{abc}	13.3 ^{b}	3.00	3.75
SEM [1]	0.0104	199.2	28.9	7.796	1.885	1.455
EFE doses						
Control	0.082 ^{a}	1027	265	43.5	15.9 ^{a}	10.6 ^{a}
E100	0.047 ^{b}	1354	285	21.7	12.0 ^{ab}	6.97 ^{ab}
E150	0.047 ^{b}	1192	299	32.8	11.3 ^{bc}	8.17 ^{ab}
E200	0.022 ^{c}	1452	250	12.3	7.50 ^{c}	5.58 ^{b}
SEM [1]	0.0061	115.0	19.9	2.526	1.149	0.931
Length of storage						
30 d	0.094 ^{a}	1227	168	3.50	16.0 ^{a}	13.2 ^{a}
60 d	0.041 ^{b}	1400	247	40.3	12.6 ^{b}	4.81 ^{b}
90 d	0.015 ^{c}	1142	409	39.0	6.38 ^{c}	5.44 ^{b}
SEM [1]	0.0053	99.6	17.1	2.194	0.995	0.803
p-value						
Dose	<0.01	0.10	0.18	<0.01	<0.01	0.01
Storage length	<0.01	0.16	<0.01	<0.01	<0.01	<0.01
D × L [2]	0.44	0.01	0.01	<0.01	0.12	0.16

$^{a–d}$ Means in a column with different superscripts differ ($p < 0.05$).[1] SEM—Standard error of means. [2] D × L—Interaction between dose and storage length.

4. Discussion

The length of storage might affect the nutrient composition and digestibility of maize silages. Hence, in this study, EFE doses were examined factorially with length of storage. Der Bedrosian et al. [14], found that the DM content of maize silages, harvested at two different maturities (32% and 41% of DM), tended to increase over storage time. Conversely, Weinberg and Chen [15] observed that the DM content of maize silages decreased by 10% from fresh herbage to 12 months of storage. Weinberg and Chen [15] attributed the reduction of DM content to the hydrolysis of NDF, which did not occur in the current study. In the present study, the reduction of the DM content from 30 to 90 days of storage, although statistically significant, was small (3.83%). There was also an EFE dose effect on the DM content, which decreased as the dose was increased. Spoelstra et al., Sheperd and

Kung, and Ying et al. [26–28] also showed a decrease in the DM content in maize silages treated with fibrolytic enzymes. In contrast, other authors did not observe any effect of fibrolytic enzymes on the DM content when applied to maize silages at ensiling [9,29,30].

We observed that as the DM content declined with time in the silo, the DM losses increased, as was previously observed by Weinberg and Chen [15]. The increase in DM losses with time can be associated with the production of acetic acid and carbon dioxide by the action of heterofermentative bacteria [31], as will be discussed later.

The data on the impact of the length of storage on the NDF content is not consistent. The NDF content increased from 30 to 60 days in the current study. Similarly, Sanderson [32] reported that the NDF concentration of sorghum silages was greater at 160 than at 30 days of ensiling. Weinberg and Chen [15] found the same pattern of the NDF content increasing over time for wheat silages at the milk stage, but not for maize silages. Der Bedrosian et al. [14] did not find any changes in the NDF content over a year of storage for two maize hybrids. Moreover, a reduction in the NDF content by the application of exogenous fibrolytic enzymes at ensiling was previously observed in several studies [9,13,26–30], in which the NDF content reduction was attributed to the degradation of the cell wall carbohydrates. However, in the present study, the effects of EFE treatment on the NDF content were not clear.

The tendency toward increased NDF digestibility at 90 days observed in the present study when silages were treated with E150 and E200 was similar to the results reported by Sheperd and Kung and Ying et al. [27,28]. The optimal ratio between endoglucanase and xylanase should be more than 0.4:1 to improve the digestibility [12], and in the current study, the ratio was 0.69:1.

Regarding the NDF digestibility, Sanderson [32] reported an increase in the NDF digestibility of sorghum silages with the progression of ensiling, but Weinberg and Chen [15] showed that the NDF digestibility of maize and wheat silages at the flowering stage decreased as the length of storage was increased. The NDF digestibility was also reduced after 70 days of ensiling in the study by Lynch et al. [30]. The authors claimed that the easily digestible components of the fiber fraction are more susceptible to hydrolysis during silage fermentation.

Der Bedrosian et al. [14] observed that in the brown mid-rib hybrid, the starch content remained constant during ensiling, but for the conventional hybrid, the starch content decreased slightly over time. In the present study, a conventional hybrid was used and we did not observe any effect of the length of ensiling on the starch content. In the same manner, the starch digestibility after 48 h of ruminal in situ incubation was not affected between 30 and 90 days of storage in the present study, but Der Bedrosian et al. [14] showed that it increased for different hybrids at different maturity stages. However, it might be influenced by the method used, since the previous authors performed an in vitro assay. Ruminal incubation time would at least partially justify those differences between studies, with longer incubation times be less likely to show differences of starch digestibility among treatments. The explanation for the increase in starch digestibility at 60 days by E100 in comparison to the control could be related to the hydrolysis of the cell wall in the kernel endosperm [6], which could facilitate access to the starch granules by the microbes and enzymes in the gastrointestinal tract of ruminants [33]. Although Nsereko et al. [34] showed that increased concentrations of applied enzymes to silage have beneficial effects, Morgavi et al. [35] suggested that EFEs compete for available binding sites of rumen bacteria, where low concentrations of enzymes could stimulate the adherence of the bacteria to the substrate, whereas higher concentrations of enzymes can cause competition.

As expected, increasing the length of storage increased ammonia-N. Proteolysis in silages can occur because of plant and microbial enzymes, but deamination depends on microbial metabolism. Furthermore, proteases can survive at very low pH [36].

The length of ensiling affected the fermentative profile of the maize silages. Although the length of ensiling did not affect the WSC content, the pH of the maize silages dropped from 30 to 60 days and increased again at 90 days. Because the main factor responsible for

maintaining the low pH of silage is lactic acid (pK_a = 3.86), the reduced concentration of this acid at 90 days resulted in a higher pH than at 60 days.

Regarding the effect of EFE dose on the pH, at 30 days of storage, the increase in the lactic acid content was accompanied by a decrease in pH as the doses increased. At 60 days, the same pattern of increased lactic acid content with higher doses was also observed, but the pH remained equally low for all the treatments. Usually, changes in pH or in the lactic acid are not observed because the substrate in maize silages might not be a limiting factor to fermentation [27]. Higginbotham et al. [9], evaluating the addition of cellulase and xylanase to maize silage at ensiling, found that at 30 days of storage, there was an increase in lactic acid content (4.14% × 3.64%) and no differences in pH between treated and untreated silages. Because the authors did not observe differences in WSC content, they justified that the increase in the lactic acid content was a consequence of the NDF solubilization.

The higher concentration of acetic acid in silages stored for long periods of time is a consequence of the conversion of lactic acid to equimolar parts of acetic acid and 1,2-propanediol [37]. The present study showed that the percentage of lactic acid reduction (55%) from 60 to 90 days is in accordance with the increase in the percentage of acetic acid (39%). The 1,2-propanediol concentration did not increase over time, and this could be explained by its metabolization to other components, such as propionic acid and 1-propanol, by strains of *Lactobacillus diolivorans* [38], which was shown by an increase in propionic acid over time in the current study.

Similar to storage length, the application of EFEs also increased the acetic acid content, which has been shown to increase aerobic stability of maize silage [39] because of its antifungal capacity [38]. The main active compound of the product applied in the silages was xylanase; therefore, it was expected that more xylose monomers would be released by the hydrolysis of the hemicellulose. The fermentation of xylose in the silo by anaerobic facultative bacteria produces acetic acid and lactic acid as the main products [40]. Supporting this idea, Dehghani et al. [41] found that a mixture of glucanase and xylanase increased the acetic acid concentration when the enzymes were applied to maize stover silage. Furthermore, Del Valle et al. [42] found the highest acetic acid concentration when applying xylanase at a dose of 185 mg/kg DM in sugarcane silages. Since the fermentation of xylose produces equimolar amounts of acetic acid and lactic acid [43], the further increase in the acetic acid content might be a consequence of the lactic acid conversion into acetic acid by the heterofermentative lactic acid bacteria [44].

We observed that by increasing the length of storage and by applying EFEs, there was a higher concentration of acetic acid and a lower concentration of ethanol. The negative correlation (R^2 = 0.82; $p < 0.01$) between the concentrations of acetic acid and ethanol can probably be explained by the antifungal properties of this acid [45,46]. Although statistically significant, such differences may not be relevant biologically, due to the relatively low concentrations of ethanol in all treatments.

The increase in acetic acid along the length of storage was accompanied by increased DM losses. The DM losses are linked with the production of acetic acid and carbon dioxide [31] by the anaerobic degradation of lactic acid [37]. Even though a release of carbon dioxide occurs during the production of acetic acid by xylose or lactic acid fermentation in silages [37,47], the EFE application did not result in an increase in DM losses in the silages.

The decrease in the concentrations of ethyl acetate and ethyl lactate produced by the combination of ethanol with acetic or lactic acid, respectively, was probably caused by the reduction of the ethanol concentration over time, because ethanol, rather than carboxylic acid, is more likely to be the limiting factor for the esterification process [48]. However, the magnitude of the reduction of the ester content was below the value proposed by the equation of Weiss and Auerbach [49], in which a decline of 0.5% ethanol of DM would result in a reduction of 100 mg/kg in the concentration of ethyl esters.

Regarding the concentrations of ethanol, ethyl lactate, and ethyl acetate, the present study has shown typical values in comparison to other published work evaluating maize silage produced in tropical areas. For ethanol concentrations, the values found in the litera-

ture were mostly below 0.7% of DM, and the ethyl acetate and ethyl lactate concentrations ranged, roughly, from 10–60 mg/kg DM, and 20–300 mg/kg DM, respectively [50–53]. Such values are lower than typical values reported for maize silage produced under cold areas [48,54–58] perhaps due to the more heterolactic fermentation found in tropical silages [59], cultail ethanol formation and, in turn, ethyl ester accumulation in silage.

The increases in the butyric acid content from 30 to 90 days of storage suggest a possible action of proteolytic microorganisms, such as clostridia [60,61]. However, the butyric acid concentration was far below the threshold limit (0.3% of DM) to consider that clostridial fermentation in the silages occurred.

5. Conclusions

The EFE application in maize silage did not increase the digestibility of nutrients, but increased the acetic acid concentration (1.87 vs. 1.18% DM) and decreased the contents of ethanol (0.02 vs. 0.08% DM), ethyl lactate (7.50 vs. 15.9 mg/DM) and ethyl acetate (5.58 vs. 10.6 mg/DM). The increase in the length of storage also increased the acetic acid content (2.19 vs 1.03% DM) and decreased the ethanol content (0.02 vs. 0.09% DM), but DM losses increased over time (7.05 vs. 2.32%). Even though they are statistically significant, some differences in this work may not be relevant biologically.

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