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Microbial inoculant and an extract of *Trichoderma longibrachiatum* with xylanase activity effect on chemical composition, fermentative profile and aerobic stability of guinea grass (*Pancium maximum* Jacq.) silage

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ABSTRACT. The objective of this study was to determine the effects of increasing dose of a microbial inoculant alone or in combination with a *Trichoderma longibrachiatum* extract with xylanase activity on total losses, chemical composition, fermentative profile, microbiological quality and aerobic stability of guinea grass (*Panicum maximum* Jacq. cv. Mombasa) silage. Sixty mini-silos (0.022 m^3) were used in a 3 × 2 factorial experiment, composed by three levels (0, 4 or 8 g · t^{-1} of fresh forage) of microbial inoculant (INO) and two levels (0 or 1 IU · g^{-1} of fresh forage) of enzyme product (ENZ). INO consisted of *Lactobacillus plantarum* at 4 × 10^{10} cfu · g^{-1} and *Pediococcus acidilactici* at 4 × 10^{10} cfu · g^{-1}. Silos were opened after 60 days. The combination of INO8 with ENZ caused the lowest gas losses. ENZ increased silage crude protein content, as well as the dry matter and neutral detergent fibre (NDF) *in vitro* digestibility. INO doses exerted a positive quadratic effect on NDF *in vitro* digestibility. ENZ addition increased acetic acid concentration, while INO treatments linearly decreased acetic and butyric acid concentrations and linearly increased lactic and propionic acid concentrations in silage. INO exhibited a negative quadratic effect on pH and NH_3-N concentration of guinea grass silage and positive linear increase in the counts of anaerobic bacteria. Combinations of ENZ and INO8 decreased silage aerobic stability. Although there was observed no combined effect of ENZ and INO on silage chemical composition and fermentative profile, they exerted positive influence on NDF *in vitro* digestibility of the guinea grass silage when added alone (ENZ and INO at a dose of 4 g · t^{-1}).

Introduction

Tropical grasses, such as guinea grass (*Panicum maximum* Jacq), are relatively high yielding, exhibit perennial growth, and may be conserved for periods of forage shortage. However, tropical grasses have low contents of DM and soluble carbohydrates, high buffering capacity, and can contain large air volumes trapped within the silo. These features result in prolonged plant respiration and aerobic microbial

activity during the initial stage of the ensiling process, further reducing availability of water-soluble carbohydrate required for fermentation (Shao et al., 2004). In addition, tropical grasses have low counts of lactic acid bacteria (Pholsen et al., 2016) to promote silage pH decrease and prevent growth of undesirable microorganisms (Pang et al., 2012).

In order to improve fermentative characteristics and decrease process-related losses, microbial inoculants have been extensively recommended when ensiling forages (Kung et al., 2003). *Lactobacillus plantarum* strains produce relatively high amounts of lactic acid which leads to a rapid decrease of silage pH. However, *L. plantarum* species produce lactic acid slowly under pH above 5 and grasses have an average pH of 6 (McDonald, 1981). It was shown that *Pediococcus acidilactici* predominate and promote pH decrease in the initial stage of silage fermentation (Fitzsimons et al., 1992). Thus, it is reasonable to hypothesize that an inoculant consisting of *P. acidilactici* and *L. plantarum* should accelerate silage fermentation, and consequently decrease nutrient losses during ensiling.

The addition of fibrolytic enzymes to silages has received considerable attention over the past decades. A primary function of fibrolytic enzymes is to break down forage fibre during fermentation, making silage more digestible at the feed-out phase. Breakdown of fibre into soluble sugars also helps bacteria producing lactic acid, lowering silage pH (Dean et al., 2005). Although there are several studies evaluating microbial inoculants or enzyme addition alone, data on their combined effect in tropical grass silages is spare in the literature.

This study was designed to evaluate the effect of increasing dose of a commercial microbial inoculant alone or in combination with an extract of *Trichoderma longibrachiatum* with xylanase (fibrolytic enzyme) activity on total losses, chemical composition, fermentative profile, microbiological quality and aerobic stability of guinea grass (*Panicum maximum* Jacq. cv. Mombasa) silage. It was hypothesized that the combination of microbial inoculant with fibrolytic enzyme would exhibit positive synergistic effect on total losses and chemical composition of grass silage.

Material and methods

Treatments and ensiling

The study was conducted at the Federal University of Grande Dourados, Department of Animal Science School of Agrarian Sciences, Dourados (Brazil; 22º14´S latitude, 54º49´W longitude and 450 m altitude). The guinea grass (*Panicum maximum* Jacq. cv. Mombasa) was manually harvested at ground level 45 days after planting from 10 locations within one 4-ha plot. Approximately 100 kg of grass tillers from each location was separately chopped in a stationary cutter to a theoretical cut length of 10 mm.

Samples (1000 g) of grass tillers were frozen for further analyses of contents of DM (method 950.15), ash (method (942.05), organic matter (DM − ash), crude protein (CP, N × 6.25; method 984.13) and ether extract (EE, method 920.39) according to AOAC International (2000). Amylase treated neutral detergent fibre (aNDF, without sodium sulphite), acid detergent fibre and lignin (sulphuric acid method) were determined according to Van Soest et al. (1991). Net energy of lactation was estimated according to NRC (2001). The chemical composition of guinea grass after harvesting is shown in Table 1.

Table 1. Chemical composition of guinea grass (*Panicum maximum*) before ensiling

Indices	Amount
Dry matter, g · kg^{-1} fresh matter	235
g · kg^{-1} DM	
neutral detergent fibre	622
acid detergent fibre	403
lignin	52.2
crude protein	133
non-fibre carbohydrate[1]	126
ash	106
ether extract	14.1
Net energy of lactation[2], Mcal · kg^{-1} DM	1.23

[1] non-fibre carbohydrate = 1000 − (crude protein + neutral detergent fibre + ether extract + ash), all values expressed in g · kg^{-1} DM; [2] calculated according to NRC (2001)

Sixty mini-silos (0.022 m^3) were used in a 3 × 2 experimental design, composed by three levels (0, 4 or 8 g · t^{-1} of fresh forage) of microbial inoculant (INO; Kera SIL®, Kera Nutrição Animal, Bento Gonçalves, Brazil) and two levels (0 or 1 IU · g^{-1} of fresh forage) of enzyme product (ENZ; Fibrozyme, Alltech Inc., Nicholasville, KY). INO consisted of *L. plantarum* at 4×10^{10} cfu · g^{-1} and *P. acidilactici* at 4×10^{10} cfu · g^{-1}, and diluted in water (2 g · t^{-1}) and sprayed separately onto the forage assigned to each mini-silo. ENZ was an extract from *Trichoderma longibrachiatum* fermentation (dry mixture of inactivated yeast, dry brewery yeast and yucca extract) containing a min. 100 IU of xylanase activity per g of the product. ENZ was top dressed and mixed with the forage assigned to each mini-silo and cane molasses (100 g) was added to all mini-silos per kg of fresh forage.

Sand (2 kg) was added to plastic buckets (30 cm height and 30 cm diameter), and a nylon screen was placed on the sand. Fresh forage was added to achieve a density of 650 kg · m^{-3}, then silos were sealed, weighed and stored at room temperature (28.5 ± 2.3 °C) for 60 days. All mini-silos contained Bunsen valves to allow gas release. Silo density was achieved after silo volume calculation: $V = \pi r^2 \times h$, where r – radius, h – height of the plastic bucket.

Gas and effluent losses

Mini-silos were weighed at day 60 to determine gas losses, then they were opened and silage content and silo assembly (plastic bucket, sand layer and nylon screen) were weighed to determine effluent production. Gas losses were determined as follows:

$$GL = \frac{SWE - SWO}{DME \times 100}$$

where: GL – gas losses (% DM), SWE – silo weight at ensiling (kg), SWO – silo weight at opening (kg), DME – forage ensiled on DM basis (amounts of forage in kg × % DM).

Effluent losses were calculated as:

$$EL = \frac{SAA - SAB}{FE \times 1000}$$

where: EL – effluent production (kg of effluent produced/t of forage ensiled), SSA weight of silo assembly after the opening (kg), SAB – weight of silo assembly before ensiling, FE – amount of forage ensiled (kg).

Dry matter recovery (DMR) was estimated as:

$$DMR = \frac{FDM}{IDM} \times 100$$

where: FDM – forage dry matter after the silos opening (kg), IDM – forage dry matter before ensiling (kg).

Changes in the DM content were calculated as the difference in module of DM percentage at the ensiling moment and the DM percentage at the opening.

Chemical composition and volatile fatty acids (VFA) concentration

Samples from each silo homogenized and subsampled (500 g from each silo) to extract silage juice using a hydraulic press. Silage juice pH was immediately measured after extraction by a digital potentiometer (MB-10, Marte, Santa Rita do Sapucaí, Brazil). Two ml of silage juice were mixed with 1 ml of sulphuric acid (1N) and analysed for NH_3-N concentration by colorimetric method described by Kulasek (1972) and adapted by Foldager (1977).

A separate sample (500 g) from each mini-silo was collected to assess contents of DM, organic matter, CP, EE, aNDF, ADF, lignin, ash and net energy of lactation, as previously described. Non-fibre carbohydrate (NFC) was calculated as: NFC = 1000 − (NDF + CP + EE + ash), being all values expressed as g · kg^{-1} DM. DM and NDF *in vitro* digestibility were determined using filter bags and an artificial rumen incubator (TE-150, Tecnal, Piracicaba, Brazil) according to Tilley and Terry (1963) and adapted by Holden (1999).

VFA analysis was carried out at the Department of Animal Nutrition and Production of the University of São Paulo, Pirassununga (Brazil), according to Rodrigues et al. (2012). Briefly, 1 ml of silage juice was mixed with 0.2 ml of formic acid in amber glass bottles. VFA peaks were identified by a gas chromatography. The gas chromatograph (Focus GC, Thermo Fisher Inc., Waltham, MA, USA) was equipped with an automatic injector (model AS-3000, Thermo Electron Corporation®, Waltham, MA, USA), a glass packed column (2.0 m × 1/5″, 80/120 Carbopack® B-DA/4% Carbowax® 20M phase) and a flame ionization detector set at 270 °C. The chromatograph oven and injector temperatures were set to 190 °C and 220 °C, respectively. Hydrogen was used as the carrier gas at 30 ml · min^{-1}.

Microbiological quality

Pooled samples (100 g) from different locations, subtracting the surface layer and the effluent layer, within each silo were assessed to determine microbiological profile. In short, 10 g from the pooled samples were diluted in sterilized sodium chloride solution (0.9%, 90 ml), then a serial dilution was done. Microorganism counts were carried out in triplicate through decimal dilution series in plates with: de Man, Rogosa and Sharpe (MRS) agar for lactic acid bacteria (Briceño and Martínez, 1995), spread-plate and pour-plate nutrient agar for aerobic and anaerobic bacteria (48 h of incubation at 30 °C), and potato dextrose agar (120 h of incubation at 26 °C) for mould and yeast as described by Rabie et al. (1997). The absolute values were obtained as colony-forming units and results were log transformed.

Aerobic stability

Aerobic stability was defined as the period (h) during which silage temperature remained below 1 °C above room temperature (Driehuis et al., 2001).

Table 2. Microbial inoculant and an extract of *Trichoderma longibrachiatum* with xylanase activity effect on total losses and dry matter recovery of guinea grass (*Panicum maximum* Jacq.) silage

Indices	Treatment[1]						SEM	*P*-value[2]			
	no ENZ			ENZ				ENZ	INO[3]		INT
	INO0	INO4	INO8	INO0	INO4	INO8			L	Q	
Gas losses, g · kg^{-1} fresh matter	1.99	0.55	1.95	0.63	0.89	0.40	0.13	0.005	0.629	0.004	0.004
Gas losses, g · kg^{-1} DM	7.79	7.09	10.5	11.3	9.33	10.20	1.18	0.479	0.069	0.035	0.087
Effluent losses[4], kg · t^{-1} fresh matter	24.3	22.6	23.4	21.2	23.4	22.40	0.44	0.247	0.882	0.883	0.282
Effluent losses[4], g · kg^{-1} DM	2.28	2.11	1.86	1.89	2.14	2.02	0.04	0.419	0.139	0.276	0.826
Total losses, g · kg^{-1} DM	10.1	9.2	12.36	13.2	11.5	12.20	1.16	0.453	0.073	0.036	0.098
Dry matter recovery, g · kg^{-1} DM	89.9	90.8	87.64	86.8	88.5	87.8	1.03	0.432	0.070	0.032	0.076

[1] microbial inoculant (*L. plantarum* at 4 × 10^{10} cfu · g^{-1} and *P. acidilactici* at 4 × 10^{10} cfu · g^{-1}) added at 0, 4 or 8 g · t^{-1} of fresh forage (INO0, INO4, and INO8, respectively), and an extract of *Trichoderma longibrachiatum* with xylanase activity added (ENZ) or not (no ENZ) at a dose of 1 IU · g^{-1} of fresh forage; [2] effects of enzyme product (ENZ), microbial inoculant dose (INO), and ENZ by INO interaction (INT); [3] linear (L) and quadratic (Q) effect of INO; [4] effluent losses were calculated by the difference between the silo assembly (plastic bucket, nylon screen and sand) weight before the ensiling and the weight of silo assembly (plastic bucket, nylon screen and sand with effluent) after the ensiling

Temperature after opening of mini-silos was measured every 8 h during 5 days using an infrared digital thermometer (MS6530, Wiltronics Research Pty. Ltd., Ballarat, Australia). One bucket was randomly selected to collect samples (200 g) every 24 h to determine DM and pH after silo content was exposed to oxygen (Kung et al., 1984).

Statistical analysis

Data were submitted to analysis of variance using the PROC MIXED (version 9.3 of SAS, SAS Institute, Cary, NC, USA) verifying the normality of residuals and homogeneity of variances using PROC UNIVARIATE, according to the following model:

$$Y_{ij} = \mu + I_i + E_j + I_i \times E_j + e_{ij}$$

where: Y_{ij} – dependent variable, μ – overall mean, I_i – fixed effect of microbial inoculant, E_j – fixed effect of enzyme, $I_i \times E_j$ – microbial inoculant by enzyme interaction fixed effect, and e_{ij} – residual. The degrees of freedom (DDFM) were calculated as Kenward and Rogers DDFM (option DDFM = kenwardroger in SAS Software). Polynomial regression was used to analyse dose effect of microbial inoculant.

Data of pH and DM losses during the aerobic stability evaluation were analysed as repeated measures through the MIXED procedure of SAS, adding the fixed effect of time and interactions between time and INO or ENZ treatments to the previous model. Significance level was set at 0.05.

Results

Total losses and DM recovery

INO doses had a negative quadratic effect ($P \leq 0.036$) on gas losses (g · kg^{-1} DM) ($P = 0.035$) and total losses (g · kg^{-1} DM) ($P = 0.036$) of guinea grass silage, and consequently exhibited a positive quadratic effect ($P = 0.032$) on DM recovery (Table 2). An interaction effect of INO by ENZ was detected ($P = 0.004$) on gas losses (g · kg^{-1} fresh matter), when there was no ENZ added to the silage. Group INO4 was characterized by the lowest gas losses but when ENZ was added the effect was opposite. Moreover the combination of INO8 and ENZ caused the lowest gas losses.

Chemical composition and *in vitro* digestibility

ENZ increased contents of CP ($P = 0.004$), lignin ($P = 0.025$), and DM and NDF *in vitro* digestibility ($P = 0.001$ and $P = 0.006$, respectively) of guinea grass silage (Table 3). The addition of INO linearly increased ($P = 0.001$) ADF concentration, and exerted a positive quadratic effect on DM and lignin contents ($P = 0.049$ and $P = 0.015$, respectively), as well as on NDF *in vitro* digestibility ($P = 0.033$). No interaction between ENZ and INO was detected on chemical composition and *in vitro* digestibility.

Fermentative profile

Addition of ENZ increased ($P = 0.007$) acetate concentration in the silage juice (Table 4). The INO linearly decreased acetate ($P = 0.002$) and butyrate ($P = 0.033$) concentrations while linearly increased lactate ($P = 0.043$), propionate ($P = 0.001$) and branched-chain fatty acids ($P = 0.002$) concentrations in the silage. Further, INO exhibited a negative quadratic effect on silage pH ($P = 0.003$) and NH_3-N ($P = 0.017$) concentration. No interaction effect was observed on silage fermentative profile.

Table 3. Microbial inoculant and an extract of *Trichoderma longibrachiatum* with xylanase activity effect on chemical composition and *in vitro* digestibility of guinea grass (*Panicum maximum* Jacq.) silage

Indices	Treatment[1]						SEM	*P*-value[2]			
	no ENZ			ENZ				ENZ	INO[3]		INT
	INO0	INO4	INO8	INO0	INO4	INO8			L	Q	
Chemical composition, g · kg^{-1} DM											
dry matter, g · kg^{-1} fresh matter	195	224	168	185	222	187	0.25	0.622	0.076	0.049	0.081
organic matter	894	897	901	890	899	890	0.14	0.124	0.074	0.295	0.210
crude protein	143	137	133	159	174	184	0.31	0.001	0.113	0.859	0.542
ether extract	14.5	13.8	14.3	14.8	14.4	14.5	0.03	0.984	0.796	0.555	0.966
neutral detergent fibre	599	601	619	580	563	550	0.73	0.004	0.765	0.755	0.366
acid detergent fibre	387	405	411	348	371	441	0.72	0.268	0.001	0.940	0.144
non-fibre carbohydrate	137	145	135	136	147	142	0.52	0.268	0.861	0.940	0.512
lignin	45.8	50.3	51.1	50.0	61.8	53.6	0.04	0.025	0.252	0.015	0.245
ash	105	103	98.9	110	101	109	0.14	0.124	0.074	0.295	0.210
Net energy of lactation, MJ · kg^{-1} DM	5.73	5.75	5.75	5.73	5.83	5.78	0.12	0.417	0.610	0.761	0.541
In vitro digestibility, g · kg^{-1} DM											
dry matter	645	668	649	700	712	691	0.75	0.001	0.889	0.169	0.872
neutral detergent fibre	608	647	611	659	671	656	0.72	0.006	0.985	0.033	0.503

[1,2,3] see Table 2

Table 4. Microbial inoculant and an extract of *Trichoderma longibrachiatum* with xylanase activity effect on fermentative profile of guinea grass (*Panicum maximum* Jacq.) silage

Indices	Treatment[1]						SEM	*P*-value[2]			
	no ENZ			ENZ				ENZ	INO[3]		INT
	INO0	INO4	INO8	INO0	INO4	INO8			L	Q	
pH	4.08	3.81	3.94	4.54	3.88	3.97	0.05	0.419	0.103	0.003	0.219
NH_3-N, % N	26.1	19.5	22.8	24.2	18.8	28.4	0.76	0.349	0.354	0.017	0.387
Lactate, g · kg^{-1}	3.92	6.79	7.60	3.76	5.52	6.77	0.02	0.776	0.043	0.338	0.327
Acetate, g · kg^{-1}	5.42	4.98	6.83	13.7	11.4	9.77	0.04	0.007	0.002	0.453	0.762
Propionate, g · kg^{-1}	1.08	1.50	2.07	1.32	1.48	2.02	0.01	0.229	0.001	0.696	0.926
Butyrate, g · kg^{-1}	3.59	2.34	1.45	2.78	1.47	1.22	0.06	0.498	0.033	0.562	0.456
Branched-chain fatty acids, g · kg^{-1}	3.95	6.81	7.61	3.87	5.54	6.79	0.07	0.572	0.002	0.348	0.432

[1,2,3] see Table 2

Microbiological quality and aerobic stability

ENZ treatments increased (P = 0.001) counts of both aerobic and anaerobic bacteria in mini-silos (Table 5) and maintained a lower pH (P = 0.004) after silage was exposed to oxygen (Table 6). The INO linearly decreased (P = 0.028 counts of mould and yeast in silage (Table 5). The addition of ENZ caused lower pH whereas INO linearly decreased (P = 0.006) silage pH during the aerobic stability trial. The INO addition linearly increased silage stability (h); however when ENZ was added the increasing effect of INO8 was inhibited (INO × ENZ interaction, P = 0.008).

Time effect was noticed for pH and DM content after the silage exposure on oxygen (Figure 1 and 2, respectively).

Table 5. Microbial inoculant and an extract of *Trichoderma longibrachiatum* with xylanase activity effect on microbiological quality of guinea grass (*Panicum maximum* Jacq.) silage, cfu · g^{-1} $\log_{10}$

Indices	Treatment[1]						SEM	*P*-value[2]			
	no ENZ			ENZ				ENZ	INO[3]		INT
	INO0	INO4	INO8	INO0	INO4	INO8			L	Q	
Bacteria											
lactic	4.73	7.57	7.63	4.53	7.07	7.15	0.19	0.541	0.001	0.454	0.074
aerobic	9.08	2.80	5.39	6.50	6.81	5.31	0.28	0.001	0.001	0.001	0.121
anaerobic	2.87	4.75	7.75	4.60	6.94	7.70	0.26	0.001	0.001	0.111	0.143
Total	9.08	9.25	9.75	9.50	9.18	9.70	0.19	0.519	0.881	0.562	0.321
Mould and yeast	4.32	2.99	2.93	4.03	3.57	3.00	0.09	0.219	0.028	0.342	0.983

[1,2,3] see Table 2

Table 6. Microbial inoculant and an extract of *Trichoderma longibrachiatum* with xylanase activity effect on aerobic stability of guinea grass (*Panicum maximum* Jacq.) silage

Indices	Treatment[1]						SEM	*P*-value[2]			
	no ENZ			ENZ				ENZ	INO[3]		INT
	INO0	INO4	INO8	INO0	INO4	INO8			L	Q	
Temperature, °C											
maximum	28.0	30.5	30.3	37.5	35.0	34.2	0.61	0.001	0.586	0.812	0.003
accumulated (5 d)	516	547	542	583	567	556	5.24	0.008	0.948	0.481	0.021
stability	26.3	24.8	29.5	27.0	30.5	30.7	0.43	0.402	0.003	0.244	0.003
stability, h	30.0	94.0	120	72.0	94.0	100	5.03	0.306	0.001	0.053	0.008
pH	6.52	5.49	5.22	5.79	4.84	4.52	0.12	0.004	0.006	0.078	0.658
Dry matter, g · kg^{-1}	227	217	228	222	213	244	0.43	0.513	0.687	0.043	0.543
Dry matter losses, g · kg^{-1} DM	88.8	44.7	76.8	52.3	65.0	74.9	0.06	0.312	0.412	0.005	0.513

[1,2,3] see Table 2

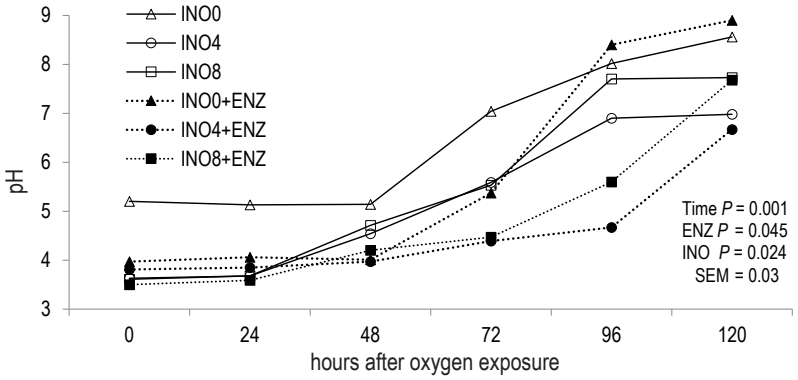

Figure 1. Effect of microbial inoculant and an extract of *Trichoderma longibrachiatum* with xylanase activity on pH after guinea grass (*Panicum maximum* Jacq.) silage was exposure to oxygen
Microbial inoculant (*L. plantarum* at 4 × 10^{10} cfu · g^{-1} and *P. acidilactici* at 4 × 10^{10} cfu · g^{-1}) added at 0, 4 or 8 g · t^{-1} of fresh forage (INO0, INO4, and INO8, respectively), and an extract of *Trichoderma longibrachiatum* with xylanase activity (ENZ) added at a dose of 1 IU · g^{-1} of fresh forage

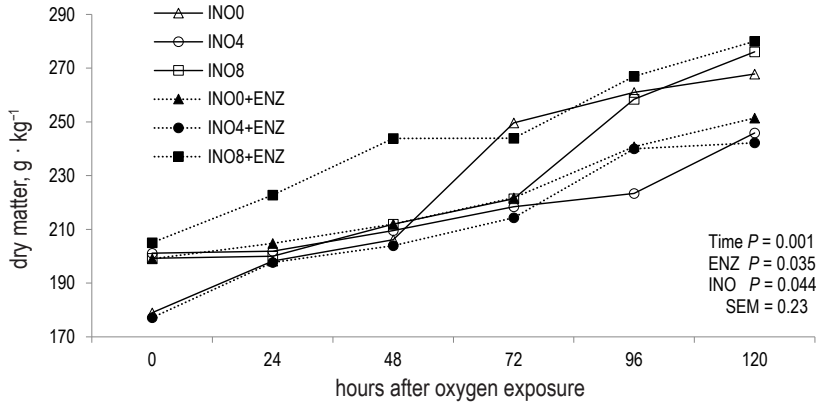

Figure 2. Effect of microbial inoculant and an extract of *Trichoderma longibrachiatum* with xylanase activity on dry matter content after guinea grass (*Panicum maximum* Jacq.) silage was exposed on oxygen
Microbial inoculant (*L. plantarum* at 4 × 10^{10} cfu · g^{-1} and P. acidilactici at 4 × 10^{10} cfu · g^{-1}) added at 0, 4 or 8 g · t^{-1} of fresh forage (INO0, INO4, and INO8, respectively), and an extract of *Trichoderma longibrachiatum* with xylanase activity (ENZ) added at a dose of 1 IU · g^{-1} of fresh forage

Discussion

Relatively low DM and soluble carbohydrates contents, and an inadequate cutting time impair grass forage fermentation and chemical quality (McDonald et al., 1991). In the current study, the material used to silage production averaged 235 g · kg^{-1} of DM. However, grass silages need at least 250 g · kg^{-1} of DM content to avoid excessive losses (McDonald et al., 1991). To avoid

unsuitable fermentation, 200 g · kg^{-1} fresh matter of cane molasses was added into all experimental silos, and therefore achieving adequate contents of DM and soluble carbohydrate for grass silage fermentation was possible.

In general, the enzymes levels used had minimum effects on losses during silage fermentation and most effects on gas and total losses were attributed to INO which added at 4 g · t^{-1} exhibited the lowest values of total losses and consequently had the highest DM recovery. Gas losses are related to secondary fermentation, especially from clostridium bacteria and aerobic microorganisms (Muck, 1996). Secondary fermentation frequently happens when there is a slow drop of pH in ensiled mass associated with a low production of lactic acid. In the current study, INO addition decreased pH, linearly increased the amounts of lactic acid bacteria and linearly decreased counts of mould and yeast in the silage, which likely prevented secondary fermentation processes. Santos et al. (2014) inoculated guinea grass with a mixture of homofermentative lactic acid bacteria (LAB; containing *L. plantarum*, *P. acidilactici* and *Enterococcus faecium*) and reported a decrease in gas losses with a consequent increase of silage DM recovery. In addition, a synergetic effect of ENZ and INO8 combination on gas losses was noticed. Fibrolytic enzyme products can increase cell wall degradation and availability of water-soluble carbohydrate for LAB fermentation, leading to a more rapid drop of pH, especially in forages with a high content of structural carbohydrates (Dehghani et al., 2012; Sun et al., 2012). Desta et al. (2016) observed a dramatic increase (> 3 folds) of lactic acid concentration at day 7, 30, 60 or 90 after ensiling grass silage containing an exogenous fibrolytic enzyme. Desta et al. (2016) also reported lower silage pH at day 30, 60 or 90 after ensiling with enzyme treatment in comparison to control one.

Despite no interaction effect of ENZ and INO was found, both ENZ and INO alone exerted positive effects on grass silage chemical composition and *in vitro* digestibility. Addition of products with enzyme activity during ensiling directly promotes fibre degradation (Dehghani et al., 2012). For instance, xylanase catalyzes the hydrolysis of xylanes, increasing fibre solubility of forages (Sunna and Antranikian, 1997; Polizeli et al., 2005). Desta et al. (2016) observed a decrease of NDF content of grass ensiled with fibrolytic enzyme product, and this was also confirmed in our study. In addition, Sheperd and Kung (1996) showed that enzyme treatment of maize silage reduced NDF content and improved *in vitro* NDF digestion. The addition of INO at 4 g · t^{-1} of fresh matter increased the values of DM content and NDF *in vitro* digestibility of silage, and these effects are likely related to INO positive effects on counts of lactic acid bacteria and silage pH. Microbial inoculants also improve lactic acid production and inhibit sugar degradation, which are negatively associated with gas production and DM losses (Muck, 1996). Tian et al. (2014) found similar results of DM content and NDF digestibility of grass (*Leymus chinensis*) silage inoculated with the same bacterial species as used in the current experiment.

Although ENZ had minimal effects on VFA profile, INO treatments had broad effects on silage pH, NH_3-N and fermentative profile. However, results of grass silage fermentative profile after treatment with fibrolytic enzymes have been inconsistent. In the same experiment, Dean et al. (2005) reported either a linear increase or a linear decrease of fibrolytic enzyme treatment on acetic acid concentration of bermudagrass silage using products with xylanase activity of 5190 and 7025 μmol · min^{-1} · ml^{-1}, respectively. Different results can be expected, since each enzyme requires specific water activity, temperature, pH, enzyme and substrate concentration to achieve maximum benefit (Aehle, 2004). In the current experiment, the increase of acetic acid concentration is partially related to the linear increase of aerobic bacteria in silo, notably of acetic acid bacteria which may grow at low pH (Muck, 2010).

Microbial inoculant treatments reduced pH and NH_3-N, especially in the INO4-treated silages. Decreased pH was related to the positive effect of INO treatments on counts of LAB and concomitant increase in lactic acid production. Decrease in silage pH minimizes *Clostridium* spp. growth and ammonia production (Xing et al., 2009; Tian et al., 2014). INO treatments decreased acetate and butyrate concentrations and were consistent with the results of Santos et al. (2014). Low pH values often imply an increased lactic acid production, which reduces the production of other VFA and proteolysis in the silo (Muck, 1996).

INO linearly decreased counts of mould and yeast in silage which is primarily related to higher production of acetic acid in INO treated mini-silos. It is well known that acetic acid has antimycotic properties (Woolford, 1990) and can inhibit yeast development (Moon, 1983). This effect can also partially explain why INO-treated silos had lover pH increases and lower DM losses after silage exposure to oxygen. Yeasts are usually the first

microorganisms to grow after oxygen exposure. Yeast development is based on lactic acid utilization in aerobic environment, increasing silage pH and allowing the growth of spoilage microorganisms (Muck, 2010).

Conclusions

The combination of an extract of *Trichoderma longibrachiatum* with xylanase activity (ENZ) and microbial inoculant added at 8 g · t^{-1} of fresh forage (INO8) caused the lowest gas losses, but showed negative effect on silage aerobic stability. ENZ treatment decreased neutral detergent fibre (NDF) content and increased its digestibility of guinea grass silage regardless INO addition. The INO4 treatment exhibited the highest values of dry matter recovery and NDF digestibility, and the lowest values of pH and NH_3-N content in the silos regardless ENZ addition. The combination of ENZ and INO has a minimal positive synergetic effect on silage quality, but may be a strategy to increase NDF digestibility and decrease proteolysis in guinea grass silage.

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References

Aehle W. (Editor), 2004. Enzymes in Industry: Production and Applications. 2nd Edition. Wiley-VCH Verlag GmbH & Co. KGaA, Weiheim (Germany), https://doi.org/10.1002/3527602135

AOAC International, 2000. Official Methods of Analysis of AOAC International. 17th Edition. Gaithersburg, MD (USA)

Briceño A.G., Martínez R., 1995. Comparison of methods for the detection and enumeration of lactic acid bacteria in yogurt (in Spanish). Arch. Latinoam. Nutr. 45, 207–212

Dean D.B., Adesogan A.T., Krueger N., Littell R.C., 2005. Effect of fibrolytic enzymes on the fermentation characteristics, aerobic stability, and digestibility of bermudagrass silage. J. Dairy Sci., 994–1003, https://doi.org/10.3168/jds.S0022-0302(05)72767-3

Dehghani M.R., Weisbjerg M.R., Hvelpund, T., Kristensen N.B., 2012. Effect of enzyme addition to forage at ensiling on silage chemical composition and NDF degradation characteristics. Livest. Sci. 150, 51–58, https://doi.org/10.1016/j.livsci.2012.07.031

Desta S.T., Yuan X., Li J., Shao T., 2016. Ensiling characteristics, structural and nonstructural carbohydrate composition and enzymatic digestibility of Napier grass ensiled with additives. Bioresour. Technol. 221, 447–454, https://doi.org/10.1016/j.biortech.2016.09.068

Driehuis F., Oude Elferink S.J.W.H., Van Wikselaar P.G., 2001. Fermentation characteristics and aerobic stability of grass silage inoculated with *Lactobacillus buchneri,* with or without homofermentative lactic acid bacteria. Grass Forage Sci. 56, 330–343, https://doi.org/10.1046/j.1365-2494.2001.00282.x

Fitzsimons A., Duffner F., Curtin D., Brophy G., O`Kiely P., O`Connell M., 1992. Assessment of *Pediococcus acidilactici* as a potential silage inoculant. Appl. Environ. Microbiol. 58, 3047–3052

Foldager J., 1977. Protein requirement and non-protein nitrogen for high producing cow in early lactation. PhD Thesis. Michigan State University, East Lasing, MI, (USA)

Holden L.A., 1999. Comparison of methods of in vitro dry matter digestibility for ten feeds. J. Dairy Sci. 82, 1791–1794, https://doi.org/10.3168/jds.S0022-0302(99)75409-3

Kulasek G.A., 1972. A micromethod for determination of urea in plasma, whole blood and blood cells using urease and phenol reagent. Pol. Arch. Wet. 15, 801–810

Kung Jr. L., Grieve D.B., Thomas J.W., Huber J.T., 1984. Added ammonia or microbial inocula for fermentation and nitrogenous compounds of alfalfa ensiled at various percents of dry matter. J. Dairy Sci. 67, 299–306, https://doi.org/10.3168/jds.S0022-0302(84)81302-8

Kung Jr. L., Stokes M.R., Lin C.J., 2003. Silage additives. In: D.R. Buxton, R.E. Muck, J.H. Harrison (Editors). Silage science and technology. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America. Madison, WI (USA), pp. 305–360

McDonald I., 1981. A revised method for the estimation of protein degradability in the rumen. J. Agric. Sci. 96, 251–252, https://doi.org/10.1017/S0021859600032081

McDonald P., Henderson A.R., Heron S.J.E. (Editors), 1991. The Biochemistry of Silage. 2nd Edition. Chalcombe Publications. Mallow, Bucks (UK), https://doi.org/10.1017/S0014479700023115

Moon N.J., 1983. Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. J. Appl. Bacteriol. 55, 453–460, https://doi.org/10.1111/j.1365-2672.1983.tb01685.x

Muck, R., 1996. Inoculant of silage and its effects on silage quality. In: Informational Conference with Dairy and Forage Industries. Proceedings…US Dairy Forage Research, 43-52.

Muck R.E., 2010. Silage microbiology and its control through additives. Rev. Bras. Zootec. 39, 183–191, https://doi.org/10.1590/S1516-35982010001300021

NRC, 2001. Nutrient Requirements of Dairy Cattle. 7th Revised Edition. The National Academics Press. Washington, DC (USA), https://doi.org/10.17226/9825

Pang H., Tan Z., Qin G., Wang Y., Li Z., Jin Q., Cai Y., 2012. Phenotypic and phylogenetic analysis of lactic acid bacteria isolated from forage crops and grasses in the Tibetan Plateau. J. Microbiol. 50, 63–71, https://doi.org/10.1007/s12275-012-1284-5

Pholsen S., Khota W., Pang H., Higgs D., Cai Y., 2016. Characterization and application of lactic acid bacteria for tropical silage preparation. Anim. Sci. J. 87, 1202–1211, https://doi.org/10.1111/asj.12534

Polizeli M.L.T.M., Rizzatti A.C.S., Monti R., Terenzi H.F., Jorge J.A., Amorim D.S., 2005. Xylanases from fungi: properties and industrial applications. Appl. Microbiol. Biotechnol. 67, 577–591, https://doi.org/10.1007/s00253-005-1904-7

Rabie C.J., Lübben A., Marais G.J., Jansen Van Vuuren H., 1997. Enumeration of fungi in barley. Int. J. Food Microbiol. 35, 117–127, https://doi.org/10.1016/S0168-1605(96)01210-X

Rodrigues P.H.M., Gomes R.C., Meyer P.M., Borgatti L.M.O., Franco F.M.J., de Godoy G.L.A., 2012. Effects of microbial inoculants and amino acid production by-product on fermentation and chemical composition of sugarcane silages. Rev. Bras. Zootecn. 41, 1394–1400, https://doi.org/10.1590/S1516-35982012000600011

Santos E.M., Pereira O.G., Garcia R., Ferreira C.L.L.F., Oliveira J.S., Silva T.C., 2014. Effect of regrowth interval and a microbial inoculant on the fermentation profile and dry matter recovery of guinea grass silages. J. Dairy Sci. 97, 4423–4432, https://doi.org/10.3168/jds.2013-7634

Shao T., Ohba N., Shimojo M., Masuda Y., 2004. Effects of adding glucose, sorbic acid and pre-fermented juices on the fermentation quality of guineagrass (*Panicum maximum* Jacq.) silages. Asian Australas. J. Anim. Sci. 17, 808–813, https://doi.org/10.5713/ajas.2004.808

Sheperd A.C., Kung L. Jr., 1996. An enzyme additive for corn silage: effects on silage composition and animal performance. J. Dairy Sci. 79, 1760–1766, https://doi.org/10.3168/jds.S0022-0302(96)76543-8

Sun Q., Gao F., Yu Z., Tao Y., Zhao S., Cai Y., 2012. Fermentation quality and chemical composition of shrub silage treated with lactic acid bacteria inoculants and cellulase additives. Anim. Sci. J. 83, 305–309, https://doi.org/10.1111/j.1740-0929.2011.00962.x

Sunna A., Antranikian G., 1997. Xylanolytic enzymes from fungi and bacteria. Crit. Rev. Biotechnol. 17, 39–67, https://doi.org/10.3109/07388559709146606

Tian J., Yu Y., Yu Z., Shao T., Na R., Zhao M., 2014. Effects of lactic acid bacteria inoculants and cellulase on fermentation quality and *in vitro* digestibility of *Leymus chinensis* silage. Grassland Sci. 60, 199–205, https://doi.org/10.1111/grs.12059

Tilley J.M.A., Terry R.A., 1963. A two-stage technique for the *in vitro* digestion of forage crops. Grass Forage Sci. 18, 104–111, https://doi.org/10.1111/j.1365-2494.1963.tb00335.x

Van Soest P.J., Robertson J.B., Lewis B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597, https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Woolford M.K., 1990. The detrimental effects of air on silage. J. Appl. Bacteriol. 68, 101–116, https://doi.org/10.1111/j.1365-2672.1990.tb02554.x

Xing L., Chen L.J., Han L.J. 2009. The effect of an inoculant and enzymes on fermentation and nutritive value of sorghum straw silages. Bioresour. Technol. 100, 488–491, https://doi.org/10.1016/j.biortech.2008.06.017