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Effect of Amylolytic and Cellulolytic Enzymes on Whole Plant Corn Silage: Characteristics of Silage and Animal Digestion

Research Article

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ABSTRACT

The aim of this study was to evaluate effects of adding exogenous enzymes to silage on fermentative losses and profile, aerobic stability, chemical composition, in vitro degradation, microbial quality, and nutrients intake and digestibility. Treatments were control (CON); addition of exo-1,4-α-glycosidase glucoamylase (GLU); addition of β-glucan 4-glucanhydrolase (CEL); and GLU + CEL. CEL increased (P≤0.038) gas losses and effluents production, CEL and GLU decreased (P=0.039) DM recovery compared to control but not differ from GLU + CEL. CEL silage had higher (P≤0.021) starch and crude protein and *in vitro* digestibility of dry matter (DM) and neutral detergent fiber (NDF) (P≤0.032), while GLU had higher (P=0.001) acid detergent fiber (ADF). CEL showed lower (P=0.012) ethanol content and higher (P=0.02) anaerobic bacteria counts, while GLU showed higher (P=0.012) lactate concentration and lower (P≤0.002) counts of bacteria and fungi. Lambs fed with CEL presented higher (P≤0.012) digestibility coefficients for DM, organic matter (OM), crude protein (CP) and NDF. Decrease on DM recovery indicates no improvements on the nutritive value of silage. On the other hand, cellulolytic enzyme positively affected animal digestion.

KEY WORDS aerobic stability, fermentative losses, microbial quality, nutrient digestibility.

INTRODUCTION

A variety of enzyme additives have been added to forage at ensiling to improve fermentation and the nutritive value of silage. Inclusion of enzyme additives to forage aims to break down plant cell walls at ensiling, which can improve silage fermentation once provide sugars for homofermentative lactic acid bacteria. Besides that, enzymes may also increase the digestibility of cell walls, enhancing the nutritive value of silage (Muck *et al*. 2018).

Cellulase is an enzyme that breaks down cellulose into beta-glucose and short-chain polysaccharides. Cellulase is made up of a complex of several different enzymes, including exoglucanases (also called cellobiohydrolases), endoglucanases, and beta-glucosidases. Fibrolytic enzymes added to silages can increase silage digestibility and decrease aerobic stability, as released sugars are rapidly used by spoilage yeasts and molds (Kung and Muck, 2015). Cellulolytic enzymes may act on the more-digestible components of NDF, leaving indigestible components intact what

reduces the overall digestibility of consumed NDF (Nadeau *et al*. 2000; Dehghani *et al*. 2012; Jin *et al*. 2015).

Glucoamylases are amylolytic enzymes considered exoamylases, which cleave 1,4-α-glycosidic bonds from the nonreducing end of the glycosidic chains releasing d-glucose. Thus, these enzymes can increase the content of fermentable carbohydrates and reduce the nonfermentable dextrins (Oliveira *et al*. 2019). Glucoamylase (1,4-α-D-glucan glucohydrolase) is extensively used to hydrolyze starch solubilized, being particularly important in cereal silages, mainly of rehydrated corn grain (Gandra *et al*. 2019). The addition of amylolytic and cellulolytic enzymes to silage with a high content of starch and NDF, such as corn whole plant, can favor the fermentation process, increasing the digestibility of starch and fiber. We hypothesized that the inclusion of amylolytic and cellulolytic enzymes simultaneously in whole plant corn silage improves the fermentation process and animal digestion. This trial aimed to evaluate the effects of amylolytic, and cellulolytic enzymes added to whole-plant corn silage on fermentative losses, aerobic stability, nutritional value, fermentative profile, microbiological population, and animal intake and digestion.

MATERIALS AND METHODS

This experiment was carried out between May and August 2018 at the Department of Animal Science of the Federal University of Grande Dourados, located at 22° 14′S, 54° 49′W and 450 m of altitude.

Harvesting, treatments and ensiling

The soil was classified as dystroferric Red Latosol, with a very clayey texture (EMBRAPA, 1997): clay content (61 g/kg); organic matter (10.46 g/dm^3); pH (5.54), P (0.71 mg/dm^3); k (0.08 cmol/dm^3); Ca (0.75 cmol/dm^3); Ca + Mg (1.15 cmol/dm^3); H + Al (4.02 cmol/dm^3); SB (1.23 cmol/dm^3); T (5.25 cmol/dm^3) and V (23.43%). Whole plant corn silage (hybrid corn DKB 353 DEKALB^{TM}) was produced in a 5ha experimental field divided in 20 locations, until reaching at 105 d. Corn was harvested between stages R4 to R5. Approximately 100 kg of whole corn plant from each location was manually harvested (ground level) and chopped to a theoretical cut of 10 mm using a stationary cutter. Samples (1000 g) of chopped corn plant were assessed for contents of DM (method 950.15), ash (method 942.05), OM (DM−ash), crude protein (CP=N×6.25; method 984.13), and ether extract (EE; method 920.39) according to AOAC International (AOAC, 2000; Table 1).

Neutral detergent fiber, acid detergent fiber, and lignin (sulfuric acid method) were determined according to Van Soest *et al*. (1991).

Net energy content of lactation was calculated according with NRC (2001).

Table 1 Chemical composition of the whole corn plant before the ensiling process (g/kg DM, unless stated)

Item	Diet
Dry matter, g/kg as-fed	254
Organic matter	939
Neutral detergent fiber	556
Acid detergente fiber	327
Starch	274
Crude Protein	101
Lignin	56.2
Ether extract	25.4
Net energy[1], Mcal/kg DM	1.72
Buffering capacity, mEq/kg of DM	213

[1] Calculated according with NRC (2001).

Four treatments in a factorial arrangement were randomly assigned to 40 experimental silos (plastic buckets, 30 cm height, and 30 cm diameter) equipped with Bunsen valves. Two kilograms of sand was placed in the bottom of the buckets and covered with a nylon mesh screen (500 µm) to drain effluents. Inoculant and chitosan were applied individually to forage assigned for each bucket to generate true replications. Forage was added to the buckets at a compaction rate of 600 kg/m^3 and silos were sealed, weighed, and stored at room temperature (24.6±2.7 ˚C; mean±SD) for 60 d. Treatments consisted of no enzymes (control; CON); 300 ml of fresh forage of exo-1,4-α-glycosidase glucoamylase, obtained from a selected strain of *Aspergillus niger*, enzymatic activity 300 U/mL (GLU; Kerazyme 3035, Kera Nutrição Animal, Bento Gonçalves, Brazil); 300 mL of fresh forage of β-glucan 4-glucanhydrolase, obtained from a selected strain of *Trichoderma reesei* (CEL; Kerazyme 3035, Kera Nutrição Animal, Bento Gonçalves, Brazil); and GLU + CEL. All treatments were inoculated with microbial additive (4 g/ton *Lactobacillus plantarum*: 4 × 10^{10} cfu/g + *Pediococcus acidilactici*: 4 × 10^{10} cfu/g; KERAsil, Kera Nutrição Animal, Bento Gonçalves, Brazil). Microbial inoculant was diluted in water (2 g/L) and sprayed on the forage, according to manufacturer's information (https://www.kerabrasil.com.br/laminas/Kerasil.pdf).

Fermentative losses

After 70 days of fermentation, mini silos were weighed to calculated gas losses. Effluent losses were calculated based on the difference between weight of silo assembly (plastic bucket, nylon screen, and sand layer) before the storage and weight of silo assembly (plastic bucket, nylon screen, and sand layer containing silage effluent) after 60 d. The gas losses, effluent losses and dry matter recovery were calculated according to Jobim *et al*. (2007), as follows:

GL (g DM/kg)= SWE (g) – SWO (g) / DME (kg)

Where:
SWE: silo weight at the ensiling.
SWO: silo weight at the opening.
DME: total DM ensiled.

EP (g DM/kg)= WSAO (g) –WSAE (g) / DME (kg)

Where:
WSAO: weight of silo assembly after the opening (g).
WSAE: weight of silo before the ensiling (g).

DMR (g/kg)= DMO (g) / DME (kg)
Where:
DMO: total DM after the opening of silo (kg).
DME: total DM before the ensiling (kg).

Silage aerobic stability

Aerobic stability was considered as the period (h) in which corn silage temperature remained less than 1 ˚C above the room temperature (Driehuis *et al*. 2001). During the 5 days period of aerobic stability evaluation, silos were maintained at room temperature (28.55±4.27, mean±SD), and temperature of silage was measured every 12 h after oxygen exposure using an infrared thermometer (MS6530, Wiltronics Research Pty. Ltd., Victoria, Australia). In addition, samples (100 g) from silos of each treatment were collected every 24 h to determine pH (Kung *et al*. 1984).

Chemical composition and *in vitro* degradation

Forage samples (500 g) from each experimental silo were collected to assess DM, OM, NFC, CP, EE, NDF, ADF, lignin, ash, NE_L and macro minerals as previously described. Dry matter and NDF *in vitro* digestibility were determined using filter bags and artificial rumen incubator (TE-150, Tecnal, Piracicaba, Brazil) according to Tilley and Terry (1963) and adapted by Holden (1999). Briefly, filter bags with samples were incubated for 48 h at 39 ˚C in a buffer-inoculum solution (1600 mL of buffer solution and 400 mL of rumen inoculum). Jars containing the buffer-inoculum solution were purged with CO_2 and lids had gas relief valves. After the incubation period, the buffer-inoculum was drained from the jars and the filter bags were gently squeezed against the sides of jar to remove the gas trapped in inflated bags. Afterward, bags were rinsed in jars with 3 changes of warm tap water.

Fermentative profile

Silage liquid was extracted from forage samples using a hydraulic press and pH was measured using a digital potentiometer (MB-10, Marte, Santa Rita do Sapucai, Brazil). Silage liquid aliquots (2 mL) were mixed with 1 mL of sulfuric acid (1 *N*) for determination of ammonia nitrogen concentration through the colorimetric method described by Foldager (1977).

Volatile fatty acids, ethanol, and lactic acid concentrations in silage juice were determined at the Department of Applied Chemistry of Federal University of Sao Carlos (Araras, Brazil) according to the methods described by Rodrigues *et al*. (2012). Briefly, aliquots (1 mL) of silage juice were mixed with formic acid (0.2 mL) in amber glass bottles and frozen until analysis. Volatile fatty acids and ethanol concentrations were determined in a gas chromatograph (Focus GC, Thermo Fisher Scientific Inc., Waltham, MA) equipped with an automatic sample injector (model AS-3000, Thermo Fisher Scientific Inc.), a glass column (2.0 m×0.5 cm 80/120 Carbopack B-DA/4% Carbowax 20M phase; Sigma-Aldrich, St. Louis, MO), 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 flowing at 30 mL/min. The lactic acid concentration was measured by HPLC (LC-10ADVP Shimadzu HPLC system, Shimadzu Inc., Kyoto, Japan) according to Ding *et al*. (1995).

Microbiological quality and enzymatic activity

Samples (200 g) from the middle layer within each mini silo were collected at the opening for microbiological population counts. Ten grams from samples were diluted in sterilized sodium chloride solution (0.9%, 90 mL) and a serial dilution was performed. Microorganism counts were carried out in triplicate through decimal dilution series in plates with De Man, Rogosa, Sharpe agar for LAB (Briceño and Martinez, 1995), 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 mold and yeast as described by Rabie *et al*. (1997). The absolute values were obtained as colony-forming units and then log-transformed. For enzymatic activity evaluation, samples (5 g) were constantly shaked at 100 rpm for 1 h with distilled water (40 mL). Then, it was filtered through nylon cloth and centrifuged (3000×g for 5 min at 5 ˚C). The enzymatic activity was determined by adding 0.1 mL of enzymatic suspension (supernatant) to 0.9 mL of sodium acetate buffer (0.1 M and pH 5.0). The measurement for glucoamylase activity is in accordance with Gandra *et al*. (2019) and cellulase activity according to Nidetsky and Claeyssens (1994).

***In vivo* nutrients intake and digestibility**

Twelve castrated lambs (32.4±2.86 kg body weight and 6.1±0.4mo) were assigned to three contemporary 4 × 4 Latin square design trial, consisting of 19-d periods, with the last 5 d for data record and sampling. Diet was formulated for 200 g average daily gain, using Small Ruminants Nutritional System (SRNS) (Table 2).

Table 2 Ingredients and chemical composition of diets (g/kg DM, unless stated)

Item	Diet
Ingredients	
Corn silage	750
Corn meal	120
Whole raw soybean	100
Mineral mix[1]	30.0
Chemical	
Dry matter, g/kg as-fed	427
Organic matter	918
Neutral detergent fiber	467
Acid detergent fiber	257
Crude protein	112
Lignin	65.6
Ether extract	43.0
Net energy[2], Mcal/kg	1.67

[1] Contained per kilogram: Ca: 134 g; P: 60 g; Mg: 10 g; Na: 110 g; S: 12 g; Se: 30 mg; I: 60 mg; Co: 150 mg; Zn: 6000 mg; Fe: 2500 mg and Mn: 4500 mg.

[2] Calculated according to NRC (2001).

Lambs within each square were randomly assigned to diets CON, GLU, CEL, and GLU + CEL. Silage was produced in 200 L tubs (3 tubs per treatment). Silages were produced as previously described, microbial inoculant was individually weighted, diluted in water, and manually mixed with whole-plant corn silage. Animals were housed in metabolic cages and fed twice daily, at 07:00 and 13:00 h, targeting refusals between 10 to 15%. Samples of feeds and refusals were collected daily during the sampling period and pooled in a composite sample for chemical analyses.

On days 15–17 of each experimental period, total fecal collections were performed through a metabolic cage. The feces were weighed every 24 h of collection and a 10% aliquot of each day collection was destined to further analysis of digestibility of dry matter, crude and neutral detergent fiber.

Samples of silages, dietary ingredients, orts, and feces were analyzed for DM (method 950.15) and crude protein (CP, N×6.25; Kjeldahl method 984.13) according to AOAC (2000), and for neutral detergent fiber (without sodium sulfite) according to Van Soest *et al*. (1991). Nutrient digestibility (NuD) was estimated as:

$$NuD\ (g/kg) = Nu_{intake}\ (g) - Nu_{fecal}\ (g) / Nu_{intake}\ (kg)$$

Where:
Nu_{intake}: nutrient intake.
Nu_{fecal}: nutrient fecal excretion.

Statistical analysis

Statistical analysis of silage evaluations were performed using PROC MIXED of SAS (SAS, 2001). Data from the silo experiment were analyzed using the following model:

$$Y_{ijl} = \mu + G_i + C_j + G_i \times C_j + e_{ijl}$$

with $e_{ij} \approx N(0, \sigma^2_e)$,

Where:
Y_{ij}: observed value.
μ: overall mean.
G_i: fixed effect of glucoamylase (i=1 and 2).
C_j: fixed effect of cellulase (j=1 and 2).
$G_i \times C_j$: interaction effect of glucoamylase by cellulase and
e_{ijl}: random residual error (l =1 to 10).
N: stands for Gaussian deviation.
σ^2_e: variance of error.

The treatment effect was evaluated by analysis of variance with 5% significance.

Data of nutrients intake and digestibility were analyzed according to the following model:

$$Y_{ijklm} = \mu + S_i + a_{j:i} + G_k + C_l + G_k \times C_l + P_m + e_{ijklm}$$

with $a_{j:i} \approx N(0, \sigma^2_a)$; $e_{ijklm} \approx N(0, \sigma^2_e)$

Where:
Y_{ijkl}: value of the dependent variable.
μ: overall mean.
S_i : fixed effect of Latin Square (i=1, 2 and 3).
$a_{j:i}$: random effect of j^{th} animal within the i^{th} Latin Square (j=1 to 12).
G_k: fixed effect of glucoamylase (k=1 and 2).
C_l: fixed effect of cellulase (l=1 and 2).
$G_k \times C_l$: interaction effect of glucoamylase by cellulose.
P_m: fixed effect of experimental period.
e_{ijklm}: random experimental error.
N: stands for Gaussian deviation.
σ^2_a: variance of animals.

σ^2_e: variance of error.

The significance level of 5% was considered for all statistical analyses.

RESULTS AND DISCUSSION

Experiment 1

Cellulases increased (P≤0.038) gas losses and effluents production (Table 3). Interaction effect (P≤0.039) was observed on losses by gases (DM) and total (DM), which was greater for silages treated with cellulases and glucoamylases compared with CON but not differ from GLU + CEL. At the same way, recovery DM was smaller for CEL and GLU compared with CON but not differ from GLU + CEL (P=0.039).

Table 3 Amylolytic and cellulolytic enzymes effects on corn silage fermentation losses and aerobic stability

Item	Treatments[1]				SEM	P-value[2]		
	CON	GLU	CEL	GLU + CEL		GLU	CEL	INT
				Losses (g/kg)				
Gases (fresh)	12.0	17.4	39.4	23.6	0.29	0.296	0.019	0.101
Effluents (kg/ton)	11.6	14.3	23.9	21.0	1.16	0.969	0.035	0.192
Gases (DM)	97.5^{a}	170^{b}	165^{b}	148^{ab}	0.61	0.085	0.117	0.035
Effluents (DM)	10.6	12.2	21.8	18.7	0.11	0.638	0.038	0.223
Total (DM)	108^{a}	183^{b}	187^{b}	167^{ab}	0.65	0.087	0.068	0.031
Recovery (DM)	913^{a}	869^{b}	845^{b}	873^{ab}	0.52	0.047	0.377	0.039
				Aerobic stability temperature (°C)				
Sum (5 d)	674	667	666	684	2.30	0.101	0.410	0.224
Maximum	32.0	32.6	30.8	31.9	0.25	0.432	0.326	0.157
Stability	28.9	31.1	28.6	29.1	3.51	0.321	0.741	0.321
				Hours				
Stability	95.0	105	112	108	0.35	0.654	0.765	0.321

[1] CON: control; GLU: exo-1,4-α-glycosidase glucoamylase (Kerazyme 3035, enzymatic activity 300 U/mL) and CEL: β-glucan 4-glucanhydrolase (Kerazyme 5052 enzymatic activity 700 U/mL).
[2] GLU: amyloglucosidase effect; CEL: cellulase effect and INT: amyloglucosidase × cellulase interaction.
SEM: standard error of the means.

After aerobic exposure, no differences were observed between silages to measure temperature of all treatments (Table 3). There was no difference in pH between silages in the first 24 h of aerobic exposure. Control and GLU silages had higher pH value, since 48 h until the end of evaluation period and silages treated with CEL and GLU + CEL showed lower values until the end of oxygen exposure (Figure 1). Control silages showed lower activities of glucoamylase and cellulases enzymes, as GLU silage for cellulase activity. Silages treated with GLU + CEL showed intermediate activity of both enzyme complexes (Figure 2).

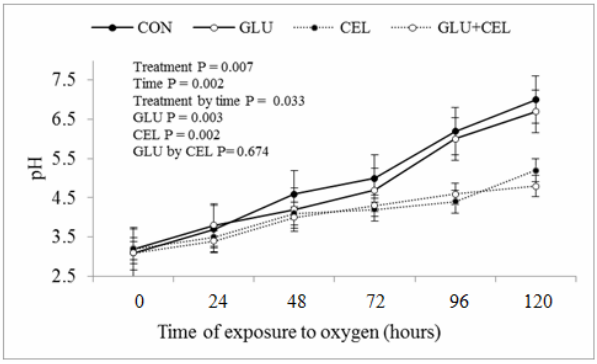

Figure 1 Amylolytic and cellulolytic enzymes effects on corn silage pH after aerobic exposure

Silages treated with cellulases showed higher (P≤0.021) starch and crude protein and lower (P=0.001) ADF content, besides higher (P≤0.032) *in vitro* digestibility of DM and NDF (Table 4). However, GLU silages presented higher (P=0.001) ADF and intermediate (P=0.003) starch content. An interaction effect was observed (P≤0.007) for DM, NDF, NFC and NEL content. CEL and GLU silage showed lower DM and NFC content than CON, but not differ from GLU + CEL silages.

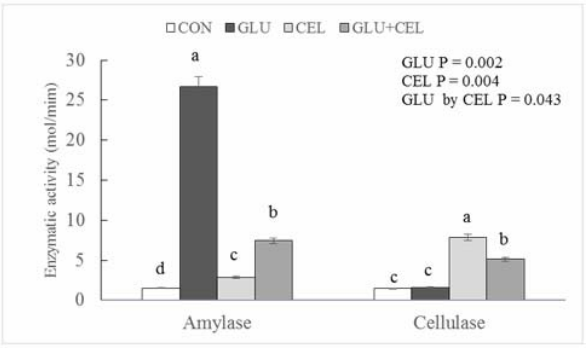

Figure 2 Amylolytic and cellulolytic enzymes effects on corn enzymatic activity

Unlike CON silages presented lower NDF content compared with GLU and CEL silages, not differing from GLU + CEL. Additionally, silages treated with cellulases demonstrated higher levels of NEL compared to CON, but not differ to GLU and GLU + CEL. Corn silages treated with cellulases presented lower (P=0.012) ethanol content and GLU silages showed higher (P=0.012) lactate concentration (Table 5) and lower (P≤0.002) counts of anaerobic, aerobic, total bacteria, and fungi (Table 6). However, CEL silage presented higher anaerobic bacteria counts (P=0.02). An interaction effect (P=0.003) was observed for lactic acid bacteria. GLU + CEL silage showed greater counts than GLU silage, but not differ from CON and CEL.

Experiment 2

In the intake and digestion trial, an interaction effect (P≤0.043) was observed for feed intake. Lambs fed CEL silage showed greater intake of DM, OM, CP and NDF than those in the GLU + CEL group, but not differ from animals fed CON and GLU silages.

Table 4 Amylolytic and cellulolytic enzymes effects on corn silage chemical composition and *in vitro* degradation

Item	Treatments[1]				SEM	P-value[2]		
	CON	GLU	CEL	GLU + CEL		GLU	CEL	INT
Dry matter	285[a]	264[b]	272[b]	279[ab]	0.16	0.001	0.267	0.001
Organic matter	946	941	941	940	0.05	0.303	0.231	0.909
Neutral detergent fiber	576[b]	589[a]	600[a]	582[ab]	0.29	0.681	0.111	0.007
Acid detergent fiber	437	457	379	419.5	0.53	0.001	0.001	0.531
Non-fiber carbohydrate	264[a]	249[b]	227[b]	247[ab]	0.36	0.705	0.002	0.002
Starch	207	222	258	241	0.31	0.003	0.021	0.761
Crude protein	81.3	83.9	88.2	86.7	0.09	0.727	0.007	0.245
Lignin	64.4	67.4	68.8	62.6	0.14	0.547	0.388	0.839
Fat	24.9	23.8	25.8	24.2	0.04	0.121	0.434	0.774
Net energy (Mcal/kg)	1.48[b]	1.52[ab]	1.57[a]	1.51[ab]	1.01	0.432	0.001	0.006
In vitro degradation (g/kg)								
Dry matter	487	503	565	556	0.87	0.849	0.024	0.654
Neutral detergent fiber	468	497	511	507	0.76	0.543	0.032	0.653

[1] CON: control; GLU: exo-1,4-α-glycosidase glucoamylase (Kerazyme 3035, enzymatic activity 300 U/mL) and CEL: β-glucan 4-glucanhydrolase (Kerazyme 5052 enzymatic activity 700 U/mL).
[2] GLU: amyloglucosidase effect; CEL: cellulase effect and INT: amyloglucosidase × cellulase interaction.
SEM: standard error of the means.

Table 5 Amylolytic and cellulolytic enzymes effects on corn silage fermentation profile

Item	Treatments[1]				SEM	P-value[2]		
	CON	GLU	CEL	GLU + CEL		GLU	CEL	INT
pH	3.26	3.14	3.11	3.09	0.02	0.543	0.661	0.871
N-NH_3 (% TN)	3.87	3.67	3.52	3.62	0.12	0.213	0.554	0.441
				mmol/kgDM				
Ethanol	2.95	2.02	1.95	2.12	0.03	0.125	0.012	0.546
Acetate	6.56	6.04	6.01	6.13	0.15	0.554	0.554	0.441
Propionate	0.005	0.008	0.003	0.006	0.02	0.443	0.541	0.564
Butyrate	1.02	1.08	1.00	1.02	0.01	0.441	0.442	0.551
Lactate	6.02	7.44	6.12	6.09	0.21	0.012	0.681	0.429

[1] CON: control; GLU: exo-1,4-α-glycosidase glucoamylase (Kerazyme 3035, enzymatic activity 300 U/mL) and CEL: β-glucan 4-glucanhydrolase (Kerazyme 5052 enzymatic activity 700 U/mL).
[2] GLU: amyloglucosidase effect; CEL: cellulase effect and INT: amyloglucosidase × cellulase interaction.
SEM: standard error of the means.

Table 6 Amylolytic and cellulolytic enzymes effects on corn silage microbial profile

Item	Treatments[1]				SEM	P-value[2]		
	CON	GLU	CEL	GLU + CEL		GLU	CEL	INT
			log_{10}					
Lactics	7.23[ab]	6.60[b]	7.41[ab]	8.26[a]	0.02	0.001	0.432	0.003
Anaerobics	5.45	5.15	8.00	7.28	0.02	0.002	0.002	0.422
Aerobics	7.72[a]	4.00[c]	6.82[ab]	5.00[b]	0.01	0.001	0.434	0.021
Total	7.84	6.62	7.75	8.45	0.02	0.001	0.111	0.116
Fungi and molds	5.26	4.80	6.08	6.08	0.03	0.001	0.881	0.431

[1] CON: control; GLU: exo-1,4-α-glycosidase glucoamylase (Kerazyme 3035, enzymatic activity 300 U/mL) and CEL: β-glucan 4-glucanhydrolase (Kerazyme 5052 enzymatic activity 700 U/mL).
[2] GLU: amyloglucosidase effect; CEL: cellulase effect and INT: amyloglucosidase × cellulase interaction.
SEM: standard error of the means.

For nutrient digestibility, lambs fed CEL silages presented higher (P≤0.012) digestibility coefficients for DM, OM, CP and NDF. This study hypothesized that inclusion of amylolytic and cellulolytic enzymes simultaneously in whole plant corn silage improves the fermentation process and animal digestion. Enzymes additives showed a significative response on fermentation, mainly because glucoamylase increased lactic acid concentration. But the decrease on DM recovery indicates no improvements on the nutritive value of corn silage. Besides that, cellulolytic enzyme positively affected animal digestion trough an improvement on digestibility of DM, OM, CP and NDF, while amylolytic had no effects. Enzymes incorporation increased gas and total losses (DM) resulting in 6, 31% drop in DM recovery. CEL increased gas and effluents losses, probably due to enhances on anaerobic bacteria count, as a greater microbial activity in silages treated with enzymes is likely related to increases on the fermentative losses observed in this study.

Table 7 Amylolytic and cellulolytic enzymes effects on sheep dry matter and nutrients intake and digestibility

Item	Treatments[1]				SEM	P-value[2]		
	CON	GLU	CEL	GLU + CEL		GLU	CEL	INT
Intake (kg/day)								
Dry matter	1.54[a]	1.42[ab]	1.65[a]	1.38[b]	0.69	0.765	0.632	0.034
Organic matter	1.45[a]	1.33[ab]	1.54[a]	1.22[b]	0.96	0.732	0.564	0.033
NDF	1.23[a]	1.06[ab]	1.25[a]	1.01[b]	0.51	0.675	0.342	0.039
Crude protein	0.123[ab]	0.125[ab]	0.169[a]	0.106[b]	0.05	0.732	0.498	0.043
Digestibility (g/kg)								
Dry matter	684	757	772	747	0.8	0.223	0.012	0.451
Organic matter	704	775	790	765	0.9	0.534	0.007	0.561
NDF	687	742	762	742	0.9	0.431	0.009	0.453
Crude protein	564	730	754	747	1.0	0.341	0.011	0.548

[1] CON: control; GLU: exo-1,4-α-glycosidase glucoamylase (Kerazyme 3035, enzymatic activity 300 U/mL) and CEL: β-glucan 4-glucanhydrolase (Kerazyme 5052 enzymatic activity 700 U/mL).
[2] GLU: amyloglucosidase effect; CEL: cellulase effect and INT: amyloglucosidase × cellulase interaction.
SEM: standard error of the means.

In contrast, despite of greater total losses in GLU treatment, corn silage with GLU showed lower counts of anaerobic, aerobic, total bacteria and fungi. Using enzyme-based silage additives has been shown to reduce neutral detergent fiber (NDF) in a number of studies. NDF decreases have been seen. Grass silages have been more consistent than other types of silage. Silage made from alfalfa data from studies also implies that hemicellulases and pectinases are more powerful than cellulases. Cellulases are effective at lowering fiber content (Beauchemin *et al.* 2003).

Enzymes can decrease aerobic stability because of excessive release of wet corn starch (WCS), increasing available sugars that can be quickly used by undesirable microorganisms, such as spoilage yeasts and molds (Kung and Muck, 2015). According to Higginbotham *et al.* (1998) yeasts usually initiate aerobic deterioration, and molds continue the deterioration process, because yeasts grow faster but tolerate less heat than molds. In this study, fungi counts were reduced in GLU and no altered in CEL treatment, consequently no effects on aerobic stability were observed.

Cellulolytic enzyme added to corn silage increased starch and crude protein content and reduced ADF. The last can be related to the increase in the degradation of fiber fractions, which is also confirmed for improvements on *in vitro* degradation of DM and NDF by CEL. Amylolytic enzyme increased ADF and starch content, with no effects on *in vitro* degradation. Dry matter content was greater in CON, compared to GLU and CEL, but not differ from GLU + CEL. CEL probably showed a lower dry matter content because of greater effluents losses, but the same was not observed in GLU. This is also observed by Lynch *et al.* (2015) when adding cellulase and xylanase to corn forage before ensiling alone, causing a decrease on DM recovery in the enzyme-treated silage.

Exogenous enzymes hydrolyze complex carbohydrates into different products (malto-, cello-, and xylo-oligosaccharides), supporting growth of fibrolytic microorganisms, which was called cross-feeding mechanism and could cause a synergistic effect between fibrolytic and amylolytic enzymes (Zilio *et al.* 2019). However, in the present study the combination treatments resulted in no further beneficial effects, which agrees with the low cellulase activity observed on GLU treatment.

GLU caused an improvement on lactic acid concentration, despite of reduced bacteria count had been observed. According to Ning *et al.* (2017), amylolytic enzymes can contributes to starch hydrolysis during the ensiling processes, which can explain the increase on lactic acid concentration. In addition, no effects on silage pH were observed when adding enzymes to silage, differently from the observed by Lynch *et al.* (2015) who added cellulase and xylanase to corn forage and showed lower pH and higher WSC after 70 d of ensiling.

The overwhelming majority of studies with enzymes have applied cellulases and hemicellulases for improve the release of plant cell wall carbohydrates, increasing its availability for LAB to ferment to lactic acid (Muck *et al.* 2018). However, different than expected, in the present study CEL did not affect LAB count but increased anaerobic bacteria count and reduced ethanol concentration. Eun *et al.* (2007) demonstrated that fibrolytic enzymes products could greatly improve forage utilization, but the optimum doses and the activities supplied are critical for achieving this response.

Exogenous fibrolytic enzyme products can greatly improve forage utilization (Muck *et al.* 2018). In fact, lambs fed silages containing CEL had greater total tract digestibility. CEL positive effects on DM and NDF digestibility were somewhat expected, as demonstrated by the *in vitro* assay. When the substrate (e.g., sugars) is limited, enzymes can

help boost silage fermentation. Soluble sugars are necessary for bacteria to make lactic acid, which is needed to reduce the pH of the silage for effective fermentation. In general, adding enzymes to silages improves fermentation by a minor amount.

Despite of increases on NDF degradability, which could allow greater voluntary intake by reducing physical fill in the rumen (Dado and Allen, 1995), feed intake was not influenced by adding none of the enzymes. On the other hand, increased NDF degradability could also enhance the energy density of diets and stimulates microbial N production (Oba and Allen, 2000) being economically viable. Thus, the increases in NDF degradation observed in our study have the potential to substantially improve the performance of animals fed diets containing corn silage.

Despite of amylolytic enzymes have potential to increase nutrients digestibility by acting on starch-protein matrix, which could enhance microbial attachment and enzymatic digestion of starch granules (Giuberti *et al*. 2014), no beneficial responses were observed on nutrient intake and digestibility. The same was observed by Lara *et al*. (2018), evaluating lambs fed corn silage with inoculant alone or in combination with amylolytic enzymes.

Cellulases, hemicellulases, xylanases, amylases, and pectinases are all common enzyme-based silage additions. Enzymes that digest the fiber part of forages include cellulases, hemicellulases, and pectinases. Amylase is a digestive enzyme that breaks down starch (amylose), hence it's best used on starch-rich silages like corn silage (Gandra *et al*. 2018). Enzyme additives are mostly applied in combination with bacterial inoculants (Muck *et al*. 2018), as observed in this study, where we added to all treatments microbial additive composed by *Lactobacillus plantarum* and *Pediococcus acidilactici*. These bacteria are common facultative heterofermentative strains, which are commonly associated with reduction on pH and acetic and butyric acid contents and increases on lactic acid contents and DM recovery (Muck and Kung, 1997).

CONCLUSION

The addition of cellulolytic and amylolytic enzymes in the whole plant maize silage positively influenced the characteristics of silage and animal digestion.

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