The effects of compound treatment of *Aspergillus oryzae* and fibrolytic enzyme on *in vitro* degradation, gas production and fermentative profile of maize silage and sugarcane silage

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Abstract

The present study was conducted to evaluate the effect of a live culture of *Aspergillus oryzae* (A; CCT4359) and fibrolytic enzyme (E; Fibrozyme Alltech Inc.) on fibre digestibility by a gas production bioassay and *in vitro* degradation of maize silage and sugarcane silage. A completely randomized design trial was performed to evaluate: A doses (0, 20, 60 and 100 mg/l), E doses (0, 160, 320 and 480 mg/l) and roughage source (R; maize and sugarcane silage) in a 4 × 4 × 2 factorial arrangement. The inclusion of increasing doses of A and E increased dry matter and neutral detergent fibre *in vitro* digestibility linearly, but for E this effect occurred only in maize silage. There was a linear increase in the potential for gas production at the highest dose of A only in sugarcane silage, with no effect on *lag time* (L). Increasing doses of E increased the volume of gases produced linearly, and a trend of linear reduction of L, regardless of the roughage. There was a linear reduction in ammonia-nitrogen concentration in response to increasing doses of A and E, and an increase in acetic acid concentration at the highest dose of A, regardless of roughage. The additives had no synergistic effect on gas production and digestibility, but were efficient in altering the fermentative pattern, demonstrating the potential to increase fibre degradation.

Introduction

The fibre of ruminant diets promotes ruminal health via stimulation of rumination and tamponade, as well as it is used as a substrate for ruminal fermentation (Mertens, 2000). Feed additive strategies have been evaluated to improve fibre digestibility and feed efficiency. Exogenous fibrolytic enzyme increases the dry matter (DM) and neutral detergent fibre(NDF) *in vitro* degradation (Gandra *et al*., 2017; Zayed *et al*., 2020), fermentative kinetics (Elghandour *et al*., 2016) and beef cattle average daily gain (Tirado-González *et al*., 2018). However, the efficiency of exogenous enzyme is highly variable (Meale *et al*., 2014; Abid *et al*., 2019). The beneficial impact of the exogenous fibrolytic enzymes on fibre digestibility depends on several factors, such as the basal diet composition, enzymatic preparation, methods of application and doses (Mendoza *et al*., 2014; Elghandour *et al*., 2016), ruminal retention time and pH, which affects the ruminal activity and enzymatic stability (Meale *et al*., 2014).

Microbial feed additive, such as the fungus *Aspergillus oryzae*, may enhance the utilization of fibre through the improvement of microbial activity (Giraldo *et al*., 2008; Sun *et al*., 2017), due to the mechanical action on the fibre, increasing bacterial access and adherence of microorganisms to fibre fraction, increasing the fibre digestibility (Sosa *et al*., 2011; Sun *et al*., 2014). The use of *A. oryzae* increases the feed intake (Latif *et al*., 2014), average daily gain (Tricarico *et al*., 2007) and milk yield (Kim *et al*., 2006; Sun *et al*., 2017).

Both additives, fibrolytic enzyme and *A. oryzae*, have the potential to modulate rumen fermentation and increase fibre digestibility. To the authors' knowledge, there are no studies evaluating the effect of these additives combination on *in vitro* digestibility and gas production. It was hypothesized that *A. oryzae* and exogenous fibrolytic enzyme would synergistically improve gas production and *in vitro* degradation of different forages. The present study was conducted to evaluate the effect of *A. oryzae* and fibrolytic enzyme levels on maize and sugarcane silages degradation, fermentative profile and *in vitro* gas production.

Material and methods

All experimental procedures were in agreement with the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999), with all animal procedures approved by the University of São Paulo Animal Bioethics Committee (protocol number 7551070817). The experiment was carried out at the Ruminal Fermentation Laboratory (LFR), in the Faculty of Animal Science and Food Engineering (FZEA), the University of São Paulo (Pirassununga, SP, Brazil, 21°57′S, 47°27′E, 630 m a.s.1.).

Treatments and experimental design

In vitro gas production bioassay was performed as a completely randomized design with three repetitions (inoculum) per treatments. Treatments were obtained from a $4 \times 4 \times 2$ factorial arrangement,in which were evaluated: (1) doses of *A. oryzae* (A): 0, 20, 60 and 100 mg/l (A0, A1, A2 and A3, respectively); (2) fibrolytic enzyme (E, Fibrozyme Alltech Inc., Nicholasville, KY, USA): 0, 160, 320 and 480 mg/l (E0, E1, E2 and E3, respectively); and (3) roughage: maize and sugarcane silage (Table 1); totalling 32 treatments.

Aspergillus oryzae (CCT 4359) was cultivated in Petri plates with Sabouraud dextrose agar 4% (Acumidia) and kept in a BOD Incubator (MarqLabor) at 28°C for fungal growth. Then, a morphological analysis was performed by microculture to confirm the cultivated fungus. Large-scale production was carried out at the Department of Food Engineering and Technology of São Paulo State University (UNESP, São José do Rio Preto/SP – Brazil). The fungus was grown in Erlenmeyer (1 litre) containing Sabouraud dextrose agar 4% (Acumidia) and incubated at 28°C to increase the number of viable spores. Subsequently, the vegetative portion along with the spores was collected and incorporated into a nutrient solution containing $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, KH_2PO_4, $FeSO_4.7H_2O$, $ZnSO_4$, e $MnSO_4$. This solution was incorporated into a mixture of sugarcane bagasse and wheat bran (3:1 ratio) and transferred to a bioreactor. The bioreactor was composed of ten jacketed modules, previously autoclaved, made of aluminium. Each module was 20 cm in diameter and 20 cm in length (wide bioreactor), vertically connected. The tem- perature was monitored at different heights with T-type thermo- couples placed between consecutive modules. Water was circulated through the jacket in order to keep the wall temperatureconstant (Cunha *et al*., 2020; Frassatto *et al*., 2020).

After 7 days, the bioreactor was opened and a sample was collected for colony-forming unit (CFU) counting, and all fermented content was removed and frozen (−20°C). The culture medium (sugarcane bagasse and wheat bran) overgrown with fungi *A. oryzae* was preserved by lyophilization. Freeze-drying was performed with a lyophilizer (Semi-Industrial Freeze Dryer LJI010) at an external manifold with a pressure of 0.2 mbar for 20 h (Grzegorczyk *et al*., 2018). The CFU counting was 6×10^8/g of dried *A. oryzae*.

The doses of the additives used in the present study were defined considering a daily supplementation for cattle with a ruminal capacity of 50 litres. Thus, the doses of *A. oryzae* were equivalent to 0, 1, 3 and 5 g, and for fibrolytic enzyme were 0, 8, 16 and 24 g.

Animals, substrate and inoculum preparation

Six Holstein cows (*Bos taurus taurus*) were used as inoculum donors for *in vitro* assays. Animals were cannulated, kept in free-stall pens with free access to water, receiving a diet with 600 g/kg maize silage and 400 g/kg concentrate for 21 days.

At 6:00 h, before the first feeding, samples of the solid and liquid phases of the ruminal content of each animal were collected separately and individually. The ruminal content was manually filtered with cotton cloth, to obtain solid and liquid fractions. Then, samples of the solid phase were stored in plastic bags,kept in a heated box at 39°C. The liquid phase was stored in pre-warmed thermal bottles, previously flushed with CO_2. After sampling, the material was immediately sent to the laboratory. One inoculum was prepared with two donor animals, totalling three repetitions. For each inoculum, equal proportions of liquid and solid were homogenized in a blender for 10 s, previously inflated with CO_2, and filtered through three layers of cotton cloth, according to Bueno *et al*. (2005). The inocula were kept in an *in vitro* incubator at 39°C (TE−150 Tecnal®, Piracicaba, Brazil) and constantly saturated with CO_2 until use.

In vitro *bioassay and treatments*

The fermentative kinetics bioassays were performed according to Theodorou *et al*. (1994) method, adapted by Mauricio *et al*.(1999) and Bueno *et al*. (2005). Two gas production bioassayswere performed simultaneously: a short incubation test (24 h)defined as a methanogenesis bioassay; and another long-term incu-bation period (96 h) defined as fermentative kinetics bioassay. Blanks were used for each inoculum and all samples were used in triplicate. For the methanogenesis bioassay, approximately 500 mg of ground sample (1 mm sieve) of roughages were weighed and placed in bags (Ankom, F57), and then placed in fermentationflasks (160 ml). The doses of A and E were added within 100 µlof saline solution per vial, in order to standardize the *headspace* for gas production in all vials.

After the additives were added to the vials, 25 ml of the ruminal inoculum was diluted with 75 ml of nutrient solution (Menke's buffered medium) as described by Onodera andHenderson (1980), continuously saturated with CO_2 and kept at 39°C until use, and immediately transferred to each vial. All vials were sealed with 20 mm butyl rubber septum stoppers (Bellco Glass, Vineland, NY, USA), manually shaken, and kept in a forced-ventilation oven at 39°C.

After incubation for 4, 8, 12, 16 and 24 h, the headspace gas pressure was measured with a pressure transducer and a datalogger (PressDATA 800®, LFR, FZEA-USP, Pirassununga, Brazil), and the values obtained were used to estimate the gas volumes produced, employing the equation defined for the test laboratory conditions: $V = p \times 6.4278$, where V is gas volume (ml) and p is gas pressure (psi) (Santos *et al*., 2020). After each pressure reading, a 2 ml sample of the gases produced inside the vials was collected with a syringe to measure the methane concentration. Samples were stored in 10 ml Vacutainer tubes, and cooled until quantitative analyses. After measuring the pressure and collecting the gas samples, the internal pressure of each vial was released at each incubation period with the aid of a syringe, equalizing the vial and atmosphere pressures, in order to not overestimate the gas production of subsequent collection. Then, the vials were sha-ken for content homogenization before returning to the incubator.At the end of the bioassay (24 h), the vials were opened and bags removed for the determination of *in vitro* degradation of DM (IVDMD24) and NDF after 24 h of incubation (IVNDF24).

For the fermentative kinetics bioassay, 1 g of ground sample (1 mm sieve) was placed in bags (Ankom, F57), and transferred to

Table 1. Chemical composition of roughages used as substrates in *in vitro* degradability and gas production bioassay

Ítem[a]	Maize silage	Sugarcane silage
Chemical composition, g/kg DM		
Dry matter, g/kg as-fed	318	300
Organic matter	948	949
Neutral detergent fibre (NDF)	486	516
Acid detergent fibre (ADF)	306	472
Non-fibre carbohydrate (NFC)[a]	352	389
Crude protein (CP)	73.9	31.0
Acid detergent lignin (ADL)	49.4	53.7
Ether extract (EE)	36.4	12.9

DM, dry matter.
[a]NFC = 1000 – (NDF + CP + EE + ash) (Balieiro Neto *et al*., 2009).

fermentation flasks (160 ml), submitted to the same treatments used in the methanogenesis bioassay. Then, 10 ml of the inoculum was diluted in 90 ml of nutrient solution, following the procedures described for the methanogenesis bioassay. The internal gas pressure in the flasks was measured after incubation for 4, 8, 12, 18, 24, 30, 36, 48, 60, 72 and 96 h, and the data were transformed into volume employing the following equation: $V = p \times 4.6788$ (Santos *et al*., 2020).

At the end of the bioassay (96 h), each vial was opened and the liquid phase was sampled (2 ml) and transferred to a vessel containing 0.4 ml of formic acid for the determination of short-chain fatty acids (SCFA) and N-ammoniacal concentration ($N\text{-}NH_3$). The bags were also removed from the bottles for the determination of *in vitro* digestibility of DM (IVDMD96) and NDF after 24 h of incubation (IVNDF96).

Laboratory analysis and procedures

The roughage samples were characterized for their chemical composition according to AOAC (2000): DM content (ID 950.15), ash (ID 942.05), crude protein (ID 984.13), ether extract (ID 920.39). The NDF (using amylase, without sodium sulphite) and acid detergent fibre analysis was performed according to Mertens (2002). Lignin analysis was performed according to Van Soest and Robertson (1985), whose measurement occurred after 12 M sulphuric acid cellulose hydrolysis in the sample residue.

The samples collected in the methanogenesis bioassay were submitted to methane measurement by gas chromatography (Model 2014; Shimadzu, Tokyo), according to the methodology described by Sallam *et al*. (2010), representing a cumulative 24 h of fermentation. For NH_3-N assay, 2 ml of supernatant was mixed with 1 ml of 1 N H_2SO_4, and analysis was performed using the phenol-hypochlorite method (Broderick and Kang, 1980). The concentration of SCFA was quantified by gas chroma-tography, with column Stabilwax, according to Erwin *et al*. (1961), adapted by Getachew *et al*. (2002).

For *in vitro* DM degradation (IVDMD) and *in vitro* NDF degradation (IVNDFD) evaluation, the bags were washed in running water until fully bleached, and transferred to a forced ventilation oven at 55°C and kept for 72 h. Sequentially, they were dried in a non-ventilated oven at 105°C for 45 min. They were then placed in a desiccator and weighed to obtain undigested DM. Subsequently, the bags were destined for NDF analysis in a fibre analyser (Ankom®) at 90°C for 1 h and sequentially washed with hot water and acetone, dried at 60°C for 72 h and then weighed, according to the previous methodology (Detmann *et al*., 2001; Casali *et al*., 2008).

The cumulative gas production curves were adjusted by using the model proposed by France *et al*. (1993):

$$V_t = V_f \times \left\{1 - \exp^{(-b \times (t-L) - c \times (\sqrt{t} - \sqrt{L}))}\right\}$$

where V_t is the accumulated volume (ml) of gases produced after the period of incubation, V_f is the final volume or maximum potential for gas production (asymptotic value; ml/g DM), b (1/h) and c (1/2 h) are constant fractional rates, L is *lag time*, and t is the time (h) of incubation.

Statistical analysis

Data were analysed using SAS® 9.4 software (Statistical Analysis System Inst. Inc., Cary, NC) according to the following statistical model:

$$Y_{ijkl} = m + E_i + R_j + A_k + E \times R_{ij} + E \times A_{ik} + R \times A_{jk} + E \times R \times A_{ijk} + e_{ijkl}$$

with $e_{ijkl} \approx N(0, s^2_e)$; where Y_{ijkl} is the observed value of the dependent variable; μ is the general mean; E_i is the fixed effect of the fibrolytic enzyme level (i = 1–4); R_j is the fixed effect of roughage (j = 1 and 2); A_k is the fixed effect of *A. oryzae* level (k = 1–4); $E \times R_{ij}$, $E \times A_{ik}$, $R \times A_{jk}$ and $E \times R \times A_{ijk}$ are interaction effects between previously defined fixed effects; e_{ijkl} is the random residual error (l = 1–3); N stands for Gaussian distribution; and s^2 is the residual variance. The degrees of freedom were adjusted using the Kenward–Roger method. Treatment averages were compared with the Fischer means test (LSD; $\alpha = 0.05$). Interaction effects were declared at $P \leq 0.10$. Fibrolytic enzyme and *A. oryzae* level effects were decomposed using polynomial regression method.

Results

There was no ($P \geq 0.163$) three-way interaction among fibrolytic enzyme, *A. oryzae* and roughage on studied variables (Table 2). There was no ($P \geq 0.131$) two-way interaction among fibrolytic enzyme and *A. oryzae*. As we had a great roughage effect, the results obtained in response to doses of *A. oryzae* and fibrolytic enzyme were presented for each roughage separately.

For the digestibility parameters, it was observed that the inclusion of *A. oryzae* increased the IVDMD96 independently of the roughage (P = 0.031) and the effect was linear (P = 0.010) as the dose increased (0.516, 0.518, 0.519 and 0.526 for A0, A1, A2 and A3, respectively; Table 3). Likewise, increasing doses of *A. oryzae* promoted an increase in IVNDFD96, regardless of the roughage (P = 0.001), whose highest value was also for the highest dose of *A. oryzae* (0.316, 0.319, 0.324 and 0.335 for A0, A1, A2 and A3, respectively). For the degradability parameters obtained from the France model, there was a significant two-way interaction between *A. oryzae* and roughage (P = 0.010; Table 3), where the effect of treatment with increasing doses of *A. oryzae* over the V_f was different according to the roughage source. In sugarcane silage, *A. oryzae* increased the potential for gas

Table 2. ANOVA table showing *P* values (probability of non-significant effects) for the main treatment factors (roughage, fibrolytic enzyme and *Aspergillus oryzae*), and their interactions on digestibility, gas production parameters, total gas production, methanogenesis and fermentative profile

	Main factors[a]			Interactions[b]				Regression[c]			
Item	E	A	R	E × R	A × R	E × A	E × A × R	E(L)	E(Q)	A(L)	A(Q)
Digestibility[d]											
IVDMD24	0.993	0.687	0.001	0.222	0.120	0.582	0.431	0.988	0.961	0.911	0.949
IVNDFD24	1.000	0.994	0.001	1.000	0.991	0.131	0.393	0.987	1.000	0.972	1.000
IVDMD96	0.122	0.031	0.001	0.095	0.750	0.170	0.163	0.031	0.412	0.010	0.629
IVNDFD96	0.082	0.001	0.001	0.091	0.816	0.160	0.274	0.010	0.210	0.104	0.655
GP parameters[e]											
V_f	0.011	0.001	0.001	0.388	0.010	0.534	0.901	0.050	0.141	0.001	0.073
b	0.165	0.143	0.001	0.404	0.551	0.073	0.772	0.472	0.946	0.391	0.201
c	0.111	0.001	0.001	0.342	0.124	0.040	0.720	0.231	0.135	0.010	0.106
L	0.071	0.111	0.001	0.996	0.451	0.181	0.574	0.323	0.634	0.455	0.691
Total GP[f] (h)											
4	0.001	0.589	0.001	0.803	0.121	0.841	0.989	0.001	0.331	0.509	0.581
8	0.001	0.001	0.001	0.964	0.001	0.942	0.865	0.001	0.084	0.001	0.041
12	0.001	0.001	0.001	0.620	0.001	0.664	0.886	0.021	0.334	0.001	0.081
18	0.001	0.001	0.001	0.531	0.001	0.739	0.981	0.179	0.763	0.001	0.251
24	0.011	0.001	0.001	0.473	0.001	0.828	0.940	0.051	0.902	0.011	0.310
48	0.044	0.001	0.001	0.444	0.042	0.666	0.791	0.071	0.321	0.021	0.323
96	0.052	0.001	0.001	0.579	0.363	0.921	0.872	0.082	0.841	0.033	0.281
Methanogenesis											
Gas production	0.478	0.241	0.447	0.985	0.501	0.891	0.921	0.121	0.601	0.041	0.582
CH_4 content	0.666	0.604	0.511	0.112	0.123	0.672	0.473	0.831	0.233	0.752	0.833
Adjusted CH_4	0.700	0.312	0.242	0.340	0.184	1.000	0.774	0.261	0.612	0.212	0.122
Net CH_4	0.541	0.293	0.001	0.151	0.112	0.911	0.602	0.461	0.291	0.821	0.461
CH_4 efficiency	0.601	0.461	0.364	0.113	0.140	0.791	0.581	0.610	0.221	0.870	0.111
Fermentative profile											
$N\text{-}NH_3$[g]	0.271	0.049	0.682	0.087	1.000	0.200	0.988	0.100	0.555	0.081	0.262
Acetic acid	0.071	0.031	0.001	0.522	0.391	0.781	0.852	0.111	0.152	0.032	0.092
Propionic acid	0.482	0.971	0.001	0.964	0.394	0.481	0.483	0.220	0.631	0.821	0.906
Butyric acid	0.573	0.533	0.001	0.800	0.243	0.671	0.881	0.402	0.910	0.530	0.733
Isobutyric acid	0.921	0.982	0.010	0.861	0.960	0.991	1.000	0.713	0.531	0.912	0.612
Valeric acid	0.305	0.599	0.001	0.901	0.871	0.911	0.791	0.851	0.653	0.853	0.561
Isovaleric acid	0.960	0.999	0.001	1.000	0.900	1.000	1.000	0.601	0.922	0.983	0.864

[a]E: fibrolytic enzyme; A: *A. oryzae*; R: roughage.
[b]Two-way and three-way interaction among main factors.
[c]L: linear; Q: quadratic.
[d]IVDMD: *in vitro* dry matter digestibility (24 and 96 h of incubation); IVNDFD: *in vitro* NDF digestibility (24 and 96 h).
[e]Gas production parameters, obtained by the model of France *et al*. (1993); V_f: potential for gas production; *b* and *c*: constants fractional rates; *L*: *lag time*.
[f]Cumulative gas production at different times of incubation. CH_4, methane.
[g]$N\text{-}NH_3$: ammoniacal nitrogen.

production when compared to the control ($P = 0.001$), whereas the higher dosage resulted in the highest observed value. In maize silage, this effect was significant only in the highest dose of *A. oryzae*.

There was a trend of a significant two-way interaction among fibrolytic enzyme and roughage source for IVDMD96 and IVDFD96 ($P = 0.095$ and $P = 0.091$, respectively; Table 4), where the inclusion of fibrolytic enzyme increased the IVDMD96 only when the substrate was the maize silage, with no effect on sugarcane silage ($P = 0.952$). Similarly, there was an increase in IVNDFD96 only in response to the highest dose of fibrolytic enzyme when the substrate was the maize silage. Furthermore,

Table 3. Digestibility coefficient of dry matter and neutral detergent fibre, at 96 h of incubation, and gas production parameters, in response to doses of *A. oryzae* and fibrolytic enzyme, with different roughage sources

	A. oryzae				Fibrolytic enzyme					Probabilities (*P*)[2]						
Item	A0	A1	A2	A3	E0	E1	E2	E3	S.E.M.[1]	E	A	R	E × R	A × R	E × A	E× A × R
Digestibility[3]																
IVDMD96	*0.52*	*0.52*	*0.52*	*0.53*	*0.52*	*0.52*	*0.52*	*0.52*	0.004	0.122	0.031	0.001	0.095	0.750	0.170	0.163
Maize silage	0.59	0.59	0.59	0.60	0.59^{b}	0.59^{ab}	0.59^{a}	0.60^{a}		–	–	–	–	–	–	–
Sugarcane silage	0.44	0.44	0.45	0.46	0.45^{b}	0.45^{b}	0.45^{b}	0.45^{b}		–	–	–	–	–	–	–
IVNDFD96	*0.32*	*0.32*	*0.32*	*0.34*	*0.32*	*0.32*	*0.27*	*0.27*	0.005	0.082	0.001	0.001	0.091	0.816	0.160	0.274
Maize silage	0.37	0.37	0.38	0.39	0.37^{b}	0.37^{b}	0.37^{b}	0.39^{a}		–	–	–	–	–	–	–
Sugarcane silage	0.26	0.27	0.27	0.28	0.27^{b}	0.27^{b}	0.27^{b}	0.27^{b}		–	–	–	–	–	–	–
GP parameters[4]																
V_f (ml/g DM)	*176*	*179*	*175*	*201*	*173*	*185*	*186*	*187*	3.3	0.011	0.001	0.001	0.388	0.010	0.534	0.901
Maize silage	191^{b}	190^{b}	189^{b}	202^{a}	187	194	195	195		–	–	–	–	–	–	–
Sugarcane silage	161^{c}	168^{b}	162^{b}	200^{a}	159	175	178	178		–	–	–	–	–	–	–
b (1/h)	*0.02*	*0.02*	*0.02*	*0.02*	*0.02*	*0.02*	*0.02*	*0.02*	0.001	0.165	0.143	0.001	0.404	0.551	0.073	0.772
Maize silage	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02		–	–	–	–	–	–	–
Sugarcane silage	0.02	0.02	0.02	0.01	0.02	0.02	0.02	0.02		–	–	–	–	–	–	–
c (1/2 h)	*−0.02*	*−0.01*	*−0.02*	*0.01*	*−0.02*	*−0.01*	*−0.01*	*−0.01*	0.005	0.111	0.001	0.001	0.342	0.124	0.040	0.720
Maize silage	0.01	−0.01	−0.01	0.01	−0.01	0.01	0.01	0.01		–	–	–	–	–	–	–
Sugarcane silage	−0.04	−0.03	−0.03	0.01	−0.04	−0.01	−0.02	−0.02		–	–	–	–	–	–	–
L (h)	*1.9*	*2.0*	*1.8*	*2.2*	*2.3*	*1.9*	*1.9*	*1.9*	0.14	0.071	0.111	0.001	0.996	0.451	0.181	0.574
Maize silage	2.9	3.2	3.0	3.3	3.3	3.0	3.0	3.0		–	–	–	–	–	–	–
Sugarcane silage	1.0	0.8	0.7	1.2	1.2	0.8	0.8	0.8		–	–	–	–	–	–	–

Means within a row with different superscript letters differ.
Means highlighted in italics are the combination of roughage X additive.
[1]Standard error mean.
[2]E: fibrolytic enzyme; A: *A. oryzae*; R: roughage.
[3]IVDMD: *in vitro* dry matter digestibility coefficient (96 h of incubation); IVNDFD: *in vitro* NDF digestibility coefficient (96 h).
[4]Gas production parameters, obtained by the model of France *et al*. (1993); V_f: potential for gas production; *b* and *c*: constants fractional rates; *L*: lag time.

there was a linear increase in V_f in response to increasing enzyme doses ($P = 0.011$), regardless of the roughage (173.1, 184.8, 186.4 and 186.7 ml/g of DM, for E0, E1, E2 and E3, respectively). In addition, there was a trend of reduction of *lag time* in response to enzymatic treatment in both roughages (2.23, 1.90, 1.90 and 1.90 h for E0, E1, E2 and E3, respectively; $P = 0.071$).

For the cumulative gas production, it was observed that treatment with increasing doses of *A. oryzae* also increased gas production, but this effect was different according to the roughage (Table 4). In sugarcane silage, *A. oryzae* increased the gas production when compared to the control ($P = 0.001$), whereas the higher dosage resulted in the highest observed gas production value in all evaluated times, however there was no effect on gas production after 4 h of incubation. In maize silage, this effect was significant only in the highest dose of *A. oryzae*. Similarly, it was observed that enzymatic treatment increased gas production in both roughages ($P < 0.051$, Table 4).

In the methanogenesis bioassay, there was no significant effect of treatments on methane production parameters ($P > 0.241$; Table 5). Similarly, there was no two-way interaction effect($P \geq 0.120$) among treatments on IVDMD24 and IVNDFD24. Although there was no effect of enzyme and *A. oryzae* on IVD24 of DM and NDF, the degradation was higher ($P = 0.001$; Table 2) in maize than sugarcane silage (Table 5; 0.454 *v.* 0.262 for IVDMD; and 0.196 *v.* 0.127 for IVNDFD, respectively).

A reduction in N-NH_3 concentration was observed in response to *A. oryzae* treatment ($P = 0.049$; Table 6), regardless of roughage (15.4, 14.7, 14.6 and 14.64 mg/dl, for A0, A1, A2 and A3, respectively). In addition, the highest dose of *A. oryzae* (A3) increased the concentration of acetic acid, independently of the roughage (23.38, 23.36, 23.18 and 25.02, for A0, A1, A2 and A3, respectively; $P = 0.031$). No significant effect of the *A. oryzae* on the other fermentative parameters was observed ($P > 0.305$).

There was a trend ($P = 0.087$; Table 6) for N-NH_3 concentration reduction in response to fibrolytic enzyme treatment, however this effect was significant only for maize silage. In addition, there was a trend of increase in acetic acid concentration in response to the enzymatic treatment ($P = 0.071$), where the highest dose (E3) presented the highest value for this variable, regardless of the roughage (23.6, 22.9, 23.4 and 25.0 μM, for E0, E1, E2 and E3, respectively).

Discussion

The results of the current study indicate that live cultures of *A. oryzae* and fibrolytic enzyme are able to modulate the *in vitro* ruminal fermentation, decreasing N-NH_3 and improving acetic acid concentration and *in vitro* degradation of DM and NDF. However, there is no synergic effect among them, and the positive response to these additives was dependent on doses and type of roughage used. To our knowledge, this is the first study on the use of live cultures of *A. oryzae* in combination with fibrolytic enzyme in the modulation of *in vitro* ruminal fermentation, although previous studies have used these additives separately (Meale *et al*., 2014; Sun *et al*., 2014).

The potential for gas production in an *in vitro* assay is directly related to the chemical composition, especially fibrous content and structural polysaccharides (Musco *et al*., 2016). In this con- text, the degradability characteristics of roughage should be considered when evaluating the effect of additives on ruminal fibre digestibility. In the current study, additives increased gas production at all incubation times, regardless of roughage. This result observed for the *A. oryzae* is similar to others reported in the literature (Morgavi *et al*., 2004; Sun *et al*., 2014), where the microbial additive may increase fibrolytic activity by stimulating the growth of the rumen microorganisms. *Aspergillus oryzae* has a physical action on fibre due to the growth of mycelium and hyphae through the roughage, increasing bacterial access and colonization of the fibre fraction, increasing fibre digestibility (Giraldo *et al*., 2008). Furthermore, fungi can produce a range of enzymes, fibrolytic and amylolytic, and phytohormones that stimulate the activities of microorganisms, which can also promote improvements in fibre digestibility (Sher *et al*., 2017; Zayed, 2018). Similarly, the addition of fibrolytic enzyme may increase fibrolytic activity by stimulating the growth of ruminal microorganisms through the release of cell wall compounds (López-Aguirre *et al*., 2016).

Despite the increase in gas production observed in all incubation times, *A. oryzae* increased the *in vitro* NDF and DM digestibility, regardless of the type of roughage. The possible explanation for this could be the chemical composition of roughage, since the proportion of rumen potentially digestible NDF may affect the response to the addition of fibrolytic enzyme (Mendoza *et al*., 2014). Mendoza *et al*. (2014) describe that the addition of exogenous enzymes promotes improvements in the digestibility parameters of roughages that present a high propor- tion of potentially digestible fibre fraction. In the current study, the fibrolytic enzyme presents high xylanase activity, which may have acted in the most digestible fractions of sugarcane, altering or weakening the cell wall structure, without reflecting on the significant effect on *in vitro* NDF digestibility (Giraldo *et al*., 2008; Sakita *et al*., 2020).

As mentioned earlier, in the current study, our initial approach was that *A. oryzae* and exogenous fibrolytic enzyme would synergistically improve gas production and *in vitro* fibre degradation. That approach was based on the fact that the xylanases can hydrolyse xylan, increasing cellulose accessibility to the cellulase enzymes through increasing fibre swelling and fibre porosity (Gonçalves *et al*., 2015). When using these additives together,the presence of a diversity of enzymes together could reach a var-iety of substrates, increasing the fibre digestibility (Srinivas *et al*., 2008). So, as mentioned earlier, *A. oryzae* can produce a range of enzymes, including cellulose, and the supplementation with fibrolytic enzyme would substantially increase fibre degradability, once that xylan is one of the major mechanisms that limited the accessibility of the cellulase enzymes to the cellulose. However, in the current study, there is no synergic effect among them. A possible explanation would be related to the type of exogenous enzyme used. The supplementation of cellulases with xylanase can increase the rate and extent of cellulose hydrolysis (Hu *et al*., 2011). However, it appears that the type of interaction between xylanase and cellulase enzymes is dependent on several factors, such as enzyme ratio and total enzyme loading (Jeoh *et al*., 2006; Zerva *et al*., 2020). Addressing this, Hu *et al*. (2011) observed a strong synergistic effect at low cellulase loading and when a high xylanase to cellulase ratio was used. Furthermore, Zayed *et al*. (2020) in an *in vitro* study reported that the simultan-eous use of inoculants containing fungal and bacterial strains, as asource of exogenous cellulolytic enzymes, increased the fibre digestibility of rice straw.

In this context, other factors may influence the results when using fibrolytic enzyme in order to modulate rumen fermentation, such as dose–response effect, as well as the types of enzymes. The main fibrolytic enzymes are cellulases and xylanases, which act

Table 4. Cumulative gas production (GP expressed in ml/g DM) in response to doses of *A. oryzae* and fibrolytic enzyme

	A. oryzae				Fibrolytic enzyme					Probabilities (*P*)[2]						
Item	A0	A1	A2	A3	E0	E1	E2	E3	S.E.M.[1]	E	A	R	E × R	A × R	E × A	E × A × R
4 h	*7.0*	*6.7*	*7.1*	*7.2*	*5.9*	*7.2*	*7.1*	*7.7*	0.38	0.001	0.589	0.001	0.803	0.121	0.841	0.989
Maize silage	6.5	5.4	6.0	5.9	4.8	6.3	6.2	6.5		–	–	–	–	–	–	–
Sugarcane silage	7.4	7.9	8.2	8.5	7.0	8.0	8.1	9.0		–	–	–	–	–	–	–
8 h	*16.9*	*16.8*	*17.2*	*19.5*	*15.5*	*17.8*	*18.4*	*18.7*	0.70	0.001	0.001	0.001	0.964	0.001	0.942	0.865
Maize silage	17.6^{b}	15.3^{b}	16.1^{b}	17.6^{a}	14.6	16.8	17.3	17.9		–	–	–	–	–	–	–
Sugarcane silage	16.2^{c}	18.3^{b}	18.2^{b}	21.4^{a}	16.4	18.8	19.5	19.5		–	–	–	–	–	–	–
12 h	*28.7*	*29.4*	*30.1*	*35.4*	*28.2*	*31.3*	*31.6*	*32.4*	0.74	0.001	0.001	0.001	0.620	0.001	0.664	0.886
Maize silage	35.2^{b}	33.4^{b}	34.0^{b}	37.8^{a}	32.8	35.6	35.3	36.8		–	–	–	–	–	–	–
Sugarcane silage	22.2^{c}	25.4^{b}	26.1^{b}	32.9^{a}	23.5	27.1	27.9	28.1		–	–	–	–	–	–	–
18 h	*43*	*45*	*46*	*54*	*44*	*48*	*47*	*49*	1.2	0.001	0.001	0.001	0.531	0.001	0.739	0.981
Maize silage	56^{b}	56^{b}	57^{b}	63^{a}	55	59	57	61		–	–	–	–	–	–	–
Sugarcane silage	29^{c}	34^{b}	36^{b}	46^{a}	33	36	37	38		–	–	–	–	–	–	–
24 h	*54*	*57*	*58*	*67*	*56*	*60*	*59*	*62*	1.6	0.011	0.001	0.001	0.473	0.001	0.828	0.940
Maize silage	72^{b}	72^{b}	73^{b}	79^{a}	71	75	72	77		–	–	–	–	–	–	–
Sugarcane silage	37^{c}	41^{b}	44^{b}	56^{a}	41	44	46	46		–	–	–	–	–	–	–
48 h	*97*	*100*	*102*	*113*	*99*	*103*	*103*	*106*	2.4	0.044	0.001	0.001	0.444	0.042	0.666	0.791
Maize silage	120^{b}	120^{b}	122^{b}	129^{a}	118	124	121	128		–	–	–	–	–	–	–
Sugarcane silage	74^{c}	79^{bc}	81^{b}	97^{a}	80	82	85	85		–	–	–	–	–	–	–
96 h	*145*	*147*	*147*	*160*	*145*	*150*	*150*	*153*	2.8	0.052	0.001	0.001	0.579	0.363	0.921	0.872
Maize silage	166	167	168	176	164	171	168	174		–	–	–	–	–	–	–
Sugarcane silage	123	126	127	143	126	129	133	132		–	–	–	–	–	–	–

Means within a row with different superscript letters differ.
Means highlighted in italics are the combination of roughage X additive.
[1]Standard error mean.
[2]E: fibrolytic enzyme; A: *A. oryzae*; R: roughage.

Table 5. Effect of *A. oryzae* and fibrolytic enzyme on digestibility and methane emission evaluated by *in vitro* methanogenesis bioassay

	A. oryzae				Fibrolytic enzyme					Probabilities (*P*)[b]						
Item	A0	A1	A2	A3	E0	E1	E2	E3	S.E.M.[a]	E	A	R	E × R	A × R	E × A	E × A × R
Digestibility[c]																
IVDMD24	*0.36*	*0.36*	*0.36*	*0.36*	*0.36*	*0.36*	*0.36*	*0.36*	0.004	0.993	0.687	0.001	0.222	0.120	0.582	0.431
Maize silage	0.46	0.45	0.45	0.46	0.45	0.46	0.45	0.45		–	–	–	–	–	–	–
Sugarcane silage	0.26	0.27	0.26	0.26	0.26	0.26	0.27	0.26		–	–	–	–	–	–	–
IVNDFD24	*0.16*	*0.16*	*0.16*	*0.17*	*0.16*	*0.16*	*0.16*	*0.16*	0.003	1.000	0.994	0.001	1.000	0.991	0.131	0.393
Maize silage	0.20	0.20	0.20	0.20	0.19	0.20	0.20	0.20		–	–	–	–	–	–	–
Sugarcane silage	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13		–	–	–	–	–	–	–
Methanogenesis[d]																
GP24 (ml/g IVDMD)	*259*	*260*	*274*	*284*	*262*	*263*	*269*	*282*	14.1	0.478	0.241	0.447	0.985	0.501	0.891	0.921
Maize silage	268	252	268	272	260	258	268	275		–	–	–	–	–	–	–
Sugarcane silage	249	267	279	295	263	268	271	289		–	–	–	–	–	–	–
CH_4 content (%)	*5.9*	*5.8*	*5.7*	*5.9*	*5.7*	*5.9*	*5.9*	*5.7*	0.19	0.666	0.604	0.511	0.112	0.123	0.672	0.473
Maize silage	6.1	5.8	5.4	5.8	5.5	5.9	6.1	5.5		–	–	–	–	–	–	–
Sugarcane silage	5.8	5.7	6.0	6.0	5.9	5.8	5.7	5.9		–	–	–	–	–	–	–
Adjusted CH_4 (ml/g IVDMD)	*13*	*13*	*13*	*15*	*13*	*13*	*14*	*14*	1.0	0.700	0.312	0.242	0.340	0.184	1.000	0.774
Maize silage	14	12	12	14	12	13	14	13		–	–	–	–	–	–	–
Sugarcane silage	12	13	14	15	13	14	13	15		–	–	–	–	–	–	–
Net CH_4 (ml)	*5.7*	*5.1*	*5.2*	*5.9*	*5.1*	*5.6*	*5.7*	*5.5*	0.46	0.541	0.293	0.001	0.151	0.112	0.911	0.602
Maize silage	7.3	6.1	5.6	6.7	5.7	6.7	7.3	6.1		–	–	–	–	–	–	–
Sugarcane silage	4.1	4.1	4.7	5.0	4.4	4.5	4.2	4.9		–	–	–	–	–	–	–
CH_4 efficiency (ml/100 GP)	*5.1*	*4.9*	*4.8*	*5.1*	*4.8*	*5.1*	*5.1*	*4.9*	0.25	0.601	0.461	0.364	0.113	0.140	0.791	0.581
Maize silage	5.3	4.9	4.4	5.0	4.6	5.1	5.3	4.6		–	–	–	–	–	–	–
Sugarcane silage	5.0	4.8	5.2	5.2	5.1	5.1	4.8	5.2		–	–	–	–	–	–	–

Means highlighted in italics are the combination of roughage X additive.
[a]Standard error of the mean.
[b]E: fibrolytic enzyme; A: *A. oryzae*; R: roughage.
[c]IVDMD: *in vitro* dry matter digestibility coefficient (24 h of incubation); IVNDFD: *in vitro* NDF digestibility coefficient (24 h).
[d]GP24: gas production per gram of degraded dry matter, after 24 h of incubation; methane (CH_4) efficiency = [100 × (adjusted CH_4/GP)].

Table 6. Fermentative profile in response to the treatment with *Aspergillus oryzae* and fibrolytic enzyme

	A. oryzae				Fibrolytic enzyme					Probabilities (P)[2]						
Item	A0	A1	A2	A3	E0	E1	E2	E3	S.E.M.[1]	E	A	R	E × R	A × R	E × A	E × A × R
N-NH_3 (mg/dl)[3]	*15.4*	*14.7*	*14.6*	*14.6*	*15.2*	*14.8*	*14.6*	*14.8*	0.45	0.271	0.049	0.682	0.087	1.000	0.200	0.988
Maize silage	15.4	14.7	14.7	14.7	15.4[a]	14.8[b]	14.7[b]	14.7[b]		–	–	–	–	–	–	–
Sugarcane silage	15.3	14.7	14.5	14.5	15.0[b]	14.7[b]	14.5[b]	14.8[b]		–	–	–	–	–	–	–
Acetic acid (mM)	*23.4*	*23.3*	*23.2*	*25.0*	*23.5*	*23.0*	*23.4*	*25.0*	0.87	0.071	0.031	0.001	0.522	0.391	0.781	0.852
Maize silage	24.8	25.1	24.9	25.7	24.3	24.6	25.0	26.6		–	–	–	–	–	–	–
Sugarcane silage	21.9	21.5	21.4	24.3	22.7	21.3	21.7	23.3		–	–	–	–	–	–	–
Propionic acid (mM)	*16.2*	*16.2*	*16.5*	*16.3*	*16.0*	*16.0*	*16.4*	*16.8*	0.57	0.482	0.971	0.001	0.964	0.394	0.481	0.483
Maize silage	17.2	17.4	18.3	17.3	17.3	17.3	17.4	18.1		–	–	–	–	–	–	–
Sugarcane silage	15.2	14.9	14.6	15.2	14.7	14.6	15.3	15.4		–	–	–	–	–	–	–
Butyric acid (mM)	*5.8*	*5.7*	*5.8*	*5.6*	*5.6*	*5.7*	*5.7*	*5.9*	0.21	0.573	0.533	0.001	0.800	0.243	0.671	0.881
Maize silage	6.7	6.5	7.0	6.3	6.5	6.7	6.5	6.8		–	–	–	–	–	–	–
Sugarcane silage	4.9	4.9	4.7	4.8	4.7	4.7	5.0	5.0		–	–	–	–	–	–	–
Isobutyric acid (mM)	*0.6*	*0.6*	*0.6*	*0.6*	*0.6*	*0.6*	*0.6*	*0.6*	0.08	0.921	0.982	0.010	0.861	0.960	0.991	1.000
Maize silage	0.7	0.7	0.7	0.7	0.6	0.7	0.7	0.7		–	–	–	–	–	–	–
Sugarcane silage	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.5		–	–	–	–	–	–	–
Valeric acid (mM)	*1.3*	*1.3*	*1.3*	*1.2*	*1.2*	*1.3*	*1.3*	*1.3*	0.06	0.305	0.599	0.001	0.901	0.871	0.911	0.791
Maize silage	1.7	1.6	1.7	1.6	1.5	1.6	1.7	1.7		–	–	–	–	–	–	–
Sugarcane silage	1.0	0.9	0.9	0.9	0.9	0.9	0.9	1.0		–	–	–	–	–	–	–
Isovaleric acid (mM)	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	0.06	0.960	0.999	0.001	1.000	0.900	1.000	1.000
Maize silage	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1		–	–	–	–	–	–	–
Sugarcane silage	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9		–	–	–	–	–	–	–

Means within a row with different superscript letters differ.
Means highlighted in italics are the combination of roughage X additive.
[1]Standard error mean.
[2]E: fibrolytic enzyme; A: *A. oryzae*; R: roughage.
[3]N-NH3: ammoniacal nitrogen.

mainly in the degradation of cellulose and hemicellulose, respectively. Considering the high xylanase activity of the exogenous enzyme used in the present study, this could explain the result observed for IVDMD96 in response to the enzymatic treatment, which was significant only for maize silage, due to the fact that sugarcane silage has a lower hemicellulose content, justifying the lesser effect of the fibrolytic enzyme on this roughage when compared to maize silage. López-Aguirre *et al*. (2016), when evaluating different types of fibrolytic enzymes (cellulase, xylanase and the combination of both) at different dosages, observed that cellulase presented the best fermentative kinetics parameters for gas production, since the highest dose of fibrolytic enzyme with xylanase activity reduced gas production at all incubation times. Thus, the high dosage of xylanase may have affected the binding of enzymes to receptors present in the substrate, and consequently reducing the binding of fibrolytic microorganisms to fibre (Beauchemin *et al*., 2001). However, Togtokhbayar *et al*. (2015) described that the addition of fibrolytic enzyme with high xyla- nase activity at different doses in an *in vitro* assay using wheat straw as a substrate promoted increased gas production at all eval-uated incubation times. These results demonstrate that the chem-ical composition of roughage affects exogenous enzyme responses,as well as the dose and type of enzyme used.

There are indications that enzymes produced by fungi, such as the *A. oryzae*, may act on the degradation of lignocellulosic materials, but a longer ruminal retention time is required for the effective action of fibrolytic microorganisms (Kumar *et al*., 2009). Thus, it is assumed that the addition of exogenous fibrolytic enzymes may promote *lag time* reduction, since enzymes can degrade substrate complex carbohydrates to simple forms at an early stage of fermentation, thus allowing rapid growth of bacterial population, and consequent colonization and fibre fermentation (López-Aguirre *et al*., 2016). In the current study, both additives increased the V_f. However, for *A. oryzae*, this effect was dependent on the roughage source. In addition, *A. oryzae* showed higher *lag time*, in both roughages, when compared to the fibrolytic enzyme (3.15 *v.* 2.96 h for maize silage, and 0.89 *v.* 0.84 h for sugarcane silage), which indicates that *A. oryzae* needs more time for its development to promote significant improvementsin fibre digestibility. Moreover, the lack of results from the *A. ory-zae* on gas production after 4 h of incubation may reinforce this hypothesis.

In this context, a way to potentiate the effect of the fungus would be to increase the number of viable spores, by increasing the doses of *A. oryzae*, which could mitigate the effect of *lag time*. It is assumed that the increase in the number of viablespores would result in greater adherence to the fibrous particle, increasing the surface area available for fibrolytic activity and, consequently, enhancing the fibre digestibility over the incubationtime (Sjaastad *et al*., 2010). This could justify the better results of gas production potential, as well as for digestibility parameters, for treatments with the highest dose of *A. oryzae*.

In the current study, *A. oryzae* reduced ammonia-N content regardless of roughage, whereas fibrolytic enzyme reduced ammonia-N only in maize silage. Moreover, either enzyme or *A. oryzae* increased acetate concentration. Acetate productionis related to the action of fibrolytic microorganisms that ferment carbohydrates to acetate. These microorganisms use ammonia-N as the source of nitrogen (Russell and Wilson, 1996), which may justify the effects of additives in both variables. Thus, the additives stimulated the fibrolytic microbiota, increasing the *in vitro* NDF digestibility, and increasing the acetate as a final fermentation product. In addition, this result can be correlated to the increase in gas production with the *A. oryzae* treatment, and the highest values were observed at the highest *A. oryzae* dose, which maybe explained by the higher number of spores in this treatment, resulting in a higher fibre degradation.

Conclusion

The additives did not have a synergistic effect on gas production and digestibility, contrary to the initial hypothesis, that the additives would synergistically enhance the fibre digestibility. However, the results of the current study indicate that doses of *A. oryzae* and fibrolytic enzyme are able to modulate the *in vitro* ruminal fermentation, decreasing $N\text{-}NH_3$ and improving acetic acid concentration and *in vitro* degradation of DM and NDF.

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Conflict of interest. None.

Ethical standards. All applicable international, national and/or institutional guidelines for the care and use of animals were followed (University of São Paulo Animal Bioethics Committee, protocol number 7551070817).

References

Abid K, Jabri J, Beckers Y, Yaich H, Malek A, Rekhis J and Kamoun M (2019) Influence of adding fibrolytic enzymes on the ruminal fermentationof date palm by-products. *Archives Animal Breeding* 62, 1–8.

AOAC Association of Official Analytical Chemists (2000) *Official Methods of Analysis of the Association of the Analytical Chemists*, 17th Edn. Arlington, VA, USA: AOAC Association of Official Analytical Chemists.

Balieiro Neto G, Ferrari Junior E, Nogueira JR, Possenti R, Valdinei Tadeu Paulino VT and Bueno MS (2009) Fermentation losses, chemical com- position, aerobic stability and apparent digestibility of sugarcane ensiling with chemical and microbial additives. *Pesquisa Agropecuária Brasileira* 44, 621–630.

Beauchemin KA, Morgavi DP, McAllister TA, Yang WZ and Rode LM (2001) The use of enzymes in ruminant diets. In Garnsworthy PC and Wiseman J (eds), *Recent Advances in Animal Nutrition*. Loughborough, UK: Nottingham University Press, pp. 297–322.

Broderick GA and Kang JH (1980) Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. *Journal of Dairy Science* 63, 64–75.

Bueno ICS, Cabral Filho SLS, Gobbo SP, Louvandini H, Vitti DMSS and Abdalla AL (2005) Influence of inoculum source in a gas production method. *Animal Feed Science and Technology* 123, 95–105.

Casali AO, Detmann E, Valadares Filho SC, Pereira JC, Henriques LT,Freitas SG and Paulino MF (2008) Influence of incubation time and par- ticles size on indigestible compounds contents in cattle feeds and feces obtained by *in situ* procedures. *Revista Brasileira de Zootecnia* 37, 335–342. Cunha LP, Casciatori FP, Vicente IV, Garcia RL and Thoméo JC (2020)*Metarhizium anisopliae* conidia production in packed-bed bioreactor using rice as substrate in successive cultivations. *Process Biochemistry* 97, 104–111.

Detmann E, Paulino MF, Zervoudakis JT, Valadares Filho SC, Euclydes RF, Lana RP and Queiroz DS (2001) Chromium and internal markers in intake determination by crossbred steers, supplemented at pasture. *Revista Brasileira de Zootecnia* 30, 1600–1609.

Elghandour MMY, Kholif AE, Hernandez J, Mariezcurrena MD, Lopez S, Camacho LM, Marquez O and Salem AZM (2016) Influence of the add- ition of exogenous xylanase with or without pre-incubation on the in vitro ruminal fermentation of three fibrous feeds, Czech. *Journal of Animal Science* 61, 262–272.

79, 321–330. Meale SJ, Beauchemin KA, Hristov AN, Chaves AV and Mcallister TA (2014) Board-invited review: opportunities and challenges in using exogenous enzymes to improve ruminant production. *Journal of Animal Science* 92, 427–442.

Mendoza GD, Loera-Corral O, Plata-Pérez FX, Hernández-García PA and Ramírez-Mella M (2014) Considerations on the use of exogenous fibrolytic enzymes to improve forage utilization. *The Scientific World Journal* 2014, 247437.

Erwin ES, Marco GJ and Emery EM (1961) Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. *Journal of Dairy Science* 44, 1768–1771.

FASS (1999) *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching*. Savoy, IL, USA: Federation of Animal Sciences Societies.

France J, Dhanoa MS and Theodorou MK (1993) A model to interpret gas accumulation profiles with *in vitro* degradation of ruminants feeds. *Journal of Theoretical Biology* 163, 99–111.

Frassatto PAC, Casciatori FP, Thoméo JC, Gomes E, Boscolo M and Silva R (2020) Fungal cellulases: production by solid-state cultivation in packedbed bioreactor using solid agro-industrial by-products as substrates and applica-tion for hydrolysis of sugarcane bagasse. *Semina: Ciências Agrárias* 41, 2097–2116.

Gandra JR, Miranda JA, Goes RHTB, Takiya CS, Del Valle TA, Oliveira ER, Freitas-Junior JE, Gandra ERS, Araki HMC and Santos ALAV (2017) Fibrolytic enzyme supplementation through ruminal bolus on eating behav-ior, nutrient digestibility and ruminal fermentation in Jersey heifers fed either corn silage- or sugarcane silage-based diets. *Animal Feed Scienceand Technology* 231, 29–37.

Getachew G, Makkar HPS and Becker K (2002) Tropical browses contents of phenolics compounds, *in vitro* gas production and stoichiometric relation-ship between short chain fatty acid an *in vitro* gas production. *Journal of Agricultural Science* 139, 341–352.

Giraldo LA, Tejido ML, Ranilla MJ and Carro MD (2008) Effects of exogen- ous fibrolytic enzymes on *in vitro* ruminal fermentation of substrates with different forage:concentrate ratios. *Animal Feed Science and Technology* 141, 306–325.

Gonçalves GAL, Takasugia Y, Jiaa L, Moria Y, Nodab S, Tanakac T, Ichinosed H and Kamiyaa N (2015) Synergistic effect and application of xylanases as accessory enzymes to enhance the hydrolysis of pretreated bagasse. *Enzyme and Microbial Technology* 72, 16–24.

Grzegorczyk M, Kancelista A, Łaba W, Piegza M and Witkowska D (2018)The effect of lyophilization and storage time on the survival rate and hydro-lytic activity of *Trichoderma* strains. *Folia Microbiologica* 63, 433–441.

Hu J, Arantes V and Saddler JN (2011) The enhancement of enzymatic hydroly-sis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? *Biotechnology for Biofuels* 4, 36. Jeoh T, Wilson DB and Walker LP (2006) Effect of cellulase mole fractionand cellulose recalcitrance on synergism in cellulose hydrolysis and binding. *Biotechnology Progress* 22, 270–277.

Kim HS, Ahn BS, Chung SG, Moon YH, Ha JK, Seo IJ, Ahn BH and Lee SS (2006) Effect of yeast culture, fungal fermentation extract and non-ionic surfactant on performance of Holstein cows during transition period. *Animal Feed Science and Technology* 126, 23–29.

Kumar P, Barrett DM, Delwiche MJ and Stroeve P (2009) Methods for pre- treatment of lignocellulosic biomass for efficient hydrolysis and biofuel pro-duction. *Industrial and Engineering Chemistry Research* 48, 3713–3729.

Latif MR, Zahran SM, Ahmed MH, Zeweil HS and Sallam SMA (2014) Effect of feeding *Saccharomyces cerevisiae* and/or *Aspergillus oryzae* on nutrient utilization and rumen fermentation characteristics of sheep. *Alexandria Journal of Agricultural Research* 59, 121–127.

López-Aguirre D, Hernández-Meléndez J, Rojo R, Sánchez-Dávila F, López-Villalobos N, Salem Abdel-Fattah ZM, Vázquez-Armijo JF, RuízS and Joaquin S (2016) *In vitro* gas production kinetics and degradability of a diet for growing lambs: effect of fibrolytic enzyme products at differentdose levels. *Italian Journal of Animal Science* 15, 453–460.

Mauricio RM, Mould FL, Dhanoa MS, Owen E, Channa KS and TheodorouMK (1999) A semi-automated *in vitro* gas production technique for rumin- ant feedstuff evaluation. *Animal Feed Science and Technology*

Mertens DR (2000) Physical effective NDF and its use in formulating dairy rations. In Hall MB, Harris Jr B and Haskins B (eds), *Florida Ruminant Nutrition Symposium*. Best Western, Gainesville, FL: University of Florida, pp. 142–160.

Mertens DR (2002) Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beaker or crucibles: collaborative study. *Journal of AOAC International* 85, 1217–1240.

Morgavi DP, Beauchemin KA, Nsereko VL, Rode LM, McAllister TA and Wang Y (2004) *Trichoderma* enzymes promote *Fibrobacter succinogenes* S85 adhesion to, and degradation of, complex substrates but not pure cellu-lose. *Journal of the Science of Food and Agriculture* 84, 1083–1090.

Musco N, Koura IB, Tudisco R, Awadjihe G, Adjolohoun S, Cutrignelli MI, Mollica MP, Houinato M, Infascelli F and Calabro S (2016) Nutritional characteristics of forage grown in south of Benin. *Asian-Australasian Journal of Animal Sciences* 29, 51–61.

Onodera R and Henderson C (1980) Growth factors of bacterial origin for the culture of the rumen oligotrichprotozoon, *Entodinium caudatum*. *Journal ofApplied Bacteriology* 48, 125–134.

Russell JB and Wilson DB (1996) Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? *Journal of Dairy Science* 79, 1503–1509.

Sakita GZ, Bompadre TFV, Dineshkumar D, Lima PMT, Abdalla Filho AL, Campioni TS, Oliva Neto P, Bremer Neto H, Louvandini H and AbdallaAL (2020) Fibrolytic enzymes improving *in vitro* rumen degradability of tropical forages. *Journal of Animal Physiology and Animal Nutrition* 104, 1267–1276.

Sallam S, Bueno I, Nasser ME and Abdalla AL (2010) Effect of eucalyptus (*Eucalyptus citriodora*) fresh or residue leaves on methane emission *in vitro*. *Italian Journal of Animal Science* 9, 299–303.

Santos KC, Carvalho FFR, Carriero MM, Magalhães ALR, Batista AMV, Fagundes GM and Bueno ICS (2020) Use of different carbohydrate sources associated with urea and implications for in vitro fermentation and rumen microbial populations. *Animal Production Science* 60, 1028–1038.

Sher H, Faheem M, Ghani A, Mehmood R, Rehman H and Bokhari SAI (2017) Optimization of cellulase enzyme production from *Aspergillus ory- zae* for industrial applications. *World Journal of Biology and Biotechnology* 2, 155–158.

Sjaastad ØV, Hove K and Sand O (2010) *Physiology of Domestic Animals*. Oslo, Norway: Scandinavian Veterinary Press.

Sosa A, Galindo J, Tejido ML, Díaz A, Martínez ME, Saro C, Carro MD and Ranilla MJ (2011) Effects of *Aspergillus oryzae* on in vitro ruminal fermen- tation. In Ranilla MJ, Carro MD, Ben Salem H and Morand-Fehr P (eds), *Challenging Strategies to Promote the Sheep and Goat Sector in the Current Global Context*. Zaragoza, ESP: CIHEAM/CSIC, Universidad de León, FAO, Options Méditerranéennes, pp. 175–179.

Srinivas B, Chaturvedi OH, Malik R and Asgar M (2008) Effect of enzyme to substrate ratio of exogenous fibrolytic and protease enzymes on *in vitro* gasproduction kinetics. *Indian Journal of Small Ruminants* 14, 181–190.

Sun H, Wu YM, Wang YM, Liu JX and Myung KH (2014) Effects of *Aspergillus oryzae* culture and 2-hydroxy-4-(methylthio)-butanoic acid on *in vitro* rumen fermentation and microbial populations between different roughage sources. *Asian-Australasian Journal of Animal Sciences* 27, 1285–1292.

Sun H, Wu Y, Wang Y, Wang C and Liu J (2017) Effects of addition of *Aspergillus oryzae* culture and 2-hydroxyl-4-(methylthio) butanoic acid on milk performance and rumen fermentation of dairy cows. *Animal Science Journal* 88, 602–609.

Theodorou MK, Williams BA, Dhanoa MS, McAllan AB and France J (1994). A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Animal Feed Science and Technology* 48, 185–197.

Tirado-González DN, Miranda-Romero LA, Ruíz-Flores A, Medina-Cuéllar SE, Ramírez-Valverde R and Tirado-Estrada G (2018) Meta-analysis: effects of exogenous fibrolytic enzymes in ruminant diets. *Journal of Applied Animal Research* 46, 771–783.

Togtokhbayar N, Cerrillo MA, Rodriguez GB, Elghadour MMY, Salem AZM, Urankhaich C, Jigjidpurev S, Odongo NE and Kholif AD (2015)

Effect of exogenous xylanase on rumen *in vitro* gas production and degradability of wheat straw. *Animal Science Journal* 86, 765–771.

Tricarico JM, Abney MD, Galyean ML, Rivera JD, Hanson KC, Mcleod KRand Harmon DL (2007) Effects of a dietary *Aspergillus oryzae* extract con- taining alpha-amylase activity on performance and carcass characteristics offinishing beef cattle. *Journal of Animal Science* 85, 802–811.

Van Soest PJ and Robertson JB (1985) *Analysis of Forages and Fibrous Foods*.Ithaca, USA: Cornell University.

Zayed MS (2018) Enhancement the feeding value of rice straw as animal fodder through microbial inoculants and physical treatments. *International Journal of Recycling of Organic Waste in Agriculture* 7, 117–124.

Zayed MS, Szumacher-Strabel M, El-Fattah DAA, Madkour MA, Gogulski M, Strompfová V, Cieślak A and El-Bordeny NE (2020). Evaluation of cel- lulolytic exogenous enzyme-containing microbial inoculants as feed addi- tives for ruminant rations composed of low-quality roughage. *The Journal of Agricultural Science* 158, 326–338.

Zerva A, Pentari C, Grisel S, Berrin JG and Topakas E (2020) A new syner- gistic relationship between xylan-active LPMO and xylobiohydrolase to tackle recalcitrant xylan. *Biotechnology for Biofuels* 13, 142.