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Effect of Exogenous Enzymes on Feed Digestion and Anaerobic Digestion of Holstein Cow Faeces

Research Article

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ABSTRACT

This study aimed to evaluate the effect of exogenous enzymes (ExE) on feeding behaviour, feed intake, nutrient digestibility and rumen disappearance rate of Holstein cows, as well as methane production from faeces of these cows by means of anaerobic digestion. Five cannulated Holstein cows were distributed in a 5 × 5 Latin square design (5 periods of 21 days each) and received five treatments which differed in inclusion of different ExE in the diet (control: diet without enzymes; amylase: basal diet with 7.5 g of amylase/cow/day; xylanase: basal diet with 15 g of xylanase/cow/day; cellulase + protease: basal diet with 7.5 g cellulase + protease/cow/day; and pool: basal diet with 30 g enzyme mixture (all enzymes added at the same dose of individual treatments). Therefore, feeding behaviour, dry matter intake (DMI), nutrient digestibility and rumen disappearance rate were evaluated. Representative pools of faeces from each cow were collected in each period to perform anaerobic digestion. Afterwards, 25 experimental batch-type biodigesters were filled with faece substrates and were subsequently arranged in a completely randomised design of 5 treatments with 5 replicates. Then, evaluations of total gas, methane production, total solid (TS) and volatile solid (VS) removal efficiency were performed. No effect of ExE was observed (P>0.05) on feeding behaviour (number of daily meals; total daily time spent eating, ruminating or masticating), DMI, nutrient digestibility (although enzyme pool and cellulase + protease tended to increase crude protein (CP), neutral detergent fiber (NDF) and acid detergent fiber (ADF) digestibility) or on rumen disappearance rate. No effect of ExE was observed on total gas and methane production or on the efficiency of removal of TS and VS from faeces. Exogenous enzymes did not increase efficiency of nutrient utilisation by the animals and, accordingly, did not affect the potential of methane emission from faeces of Holstein cows by means of anaerobic digestion.

KEY WORDS anaerobic digestion, digestibility, exogenous enzymes, methane, rumen disappearance rate.

INTRODUCTION

Dry matter intake (DMI) is fundamentally important in nutrition by establishing the amount of nutrients for health and production. Many factors may affect DMI, either by rumen fill or by metabolic-feedback. Therefore, feeds of low digestibility place constraints on DMI because of slow clearance from rumen (NRC, 2016). In over three decades the increasing trend on use of feed additives gained force owing to a ban on antibiotic growth promoters (Sujani and

Seresinhe, 2015). Feed additives, such as exogenous enzymes (ExE), may affect feeding behaviour and consequently alter DMI. The exogenous enzymes used in ruminant diets can be characterised in to main categories as fibrolytic, amylolytic and proteolytic based on specific substrate on which their enzyme activity can perform (Sujani and Seresinhe, 2015). The ultimate function of these ExE is to supply maximum nutrients from the digestible, potentially digestible and the indigestible fractions of cell walls (Mocherla *et al.* 2017). In dairy cows fed high-forage the supplementation of primarily mixtures of cellulases and xylanases may increase milk production and milk composition of legume-based diets, and primarily xylanases may improve those variables of grass-based diets (Tirado-González *et al.* 2017). In grass-based diets, these enzymes may also improve the average daily gain and feed conversion, as well as improving DMI (Tirado-González *et al.* 2017). Besides, according to Neumann *et al.* (2018), ExE can also be an interesting additive in high energy diets for feedlot cattle; in their study, using xylanase in bulls finished in feedlot, these authors observed that animals receiving enzymes increased carcass yield and were more efficient in conversion of dry matter (DM) consumed into carcass gain. These effects may be attributed to the fact that ExE enhance DM and nutrient digestibility, degradability and disappearance rate (Devant *et al.* 2020; Mocherla *et al.* 2017; Tirado-González *et al.* 2017). Therefore, inclusion of ExE in ruminant feeding can be crucial in reducing enteric methane (CH_4) production, considered as a greenhouse gas (GHG). It is widely known that the more productive the animal, the less is CH_4 emission. Methane production from enteric fermentation of ruminants generates feed gross energy losses ranging from 2 to 15% (Johnson and Johnson, 1995; Wanapat *et al.* 2015). Thus, the effect of ExE on reducing CH_4 production contributes to enhance feed energy efficiency. Then, in tropical countries where most of feed is from fibrous resources, addition of ExE is crucial since production of CH_4 is maximum with low yielding animals (Mocherla *et al.* 2017). A mini-review by Thammiah *et al.* (2017) mentions that ExE improve not only the utilisation of lignocellulosic biomass, but also have had a positive impact on the quality of environment through reduced output of excreta and pollutants. Other important factor is that there has been an intensification of animal production all over the world. This intensification and increased size of animal production units represent a considerable pollution hazard through accumulation of high amounts of animal waste (Holm-Nielsen *et al.* 2009). The main emissions within the farms include enteric CH_4 (as mentioned above) and CH_4 from housing facilities during long-term storage (Rotz, 2017). Although the concentration of CH_4 in the atmosphere is lower than that of carbon dioxide (CO_2), CH_4 has a heating potential 25 times more than that of CO_2 (IPCC, 2007). The global emission of GHG from manure grew between 1961 and 2010 from 0.57 to 0.99 gigatonnes of carbon dioxide equivalent ($GtCO_2eq$) per year. On average, emissions grew by 1.10% per year (IPCC, 2014), but despite these data, Lynch (2019) concluded that there are still insufficient data available to fully address important questions regarding the climate impacts of agricultural production. The handling and use of manure on livestock farms contribute to emissions of GHG (Petersen, 2018). Comparing gas emissions from two typical manure handling options at cattle feedlots (composting and static stockpile storage), Bai *et al.* (2020) found that composting inhibits CH_4 emissions but promotes NH_3 and N_2O emissions. Certainly, the efficient treatment of animal waste can support environmental protection in addition to bioenergy management (Achinas *et al.* 2018). Anaerobic digestion is a biological process that can convert organic substrates to biogas (Zhang *et al.* 2016). It is characterised by reactions in which biogas is produced from biodegradable products in the absence of oxygen (Neshat *et al.* 2017). Anaerobic digestion is increasingly used worldwide to generate energy from biogas and brings significant economic and environmental benefits (Scarlat *et al.* 2018) by being an efficient alternative technology that combines biofuel production with waste management (Achinas *et al.* 2017).

Although much is known about the effects of ExE on rumen fermentation, studies reporting their effects on fermentation of waste from cows (or other kind of ruminants) that have been fed these enzymes were not found. Hence, the overriding question was whether the effect of these enzymes on enhancing DM and nutrient digestibility and reduction of enteric CH_4 production incrases or reduces CH_4 emission conditions from faeces. Given the above, the hypothesis tested in this study was that the use of ExE in cows' feeding would increase nutrient utilisation efficiency by increasing DM and nutrient digestibility and, accordingly, reduce the potential of faeces on CH_4 production. Therefore, the study aimed to evaluate the effects of ExE on feeding behaviour, DMI, DM and nutrient digestibility, rumen disappearance rate, as well as CH_4 production from faeces by means of anaerobic digestion.

MATERIALS AND METHODS

Ethical issue and place of experimentation

The experiment followed the guidelines established in accordance with the ethical principles of animal experimentation of the Commission of Ethics in the Use of Animals of the College of Veterinary Medicine and Animal Science of the University of Sao Paulo (USP-Brazil) under the protocol number CEUA 9296281113.

Experimental approach

The experiment was performed in two phases, the first phase comprised the animal feeding and evaluation of digestive parameters in cows fed ExE. The second phase comprised the evaluation of total gas and CH_4 emission from faeces of cows fed ExE (used in phase 1) by means of anaerobic digestion.

Treatments, experimental design and feeding management

Five Holstein cows, non-pregnant and non-lactating, carrying rumen cannula and having a mean body weigth of 923 kg (±86), were kept in a roofed shed in individual pen with free access to sand bedding. The experimental design used was the 5 × 5 Latin square design, using the animal within each period as the experimental unit. The animals were distributed on one of five experimental diets, which differed according to enzymes used, as described: (1) Control: diet without enzymes; (2) Amylase: basal diet with 7.5 g of amylase/cow/day (Amaize©, ALLTECH); (3) Xylanase: basal diet with 15 g of xylanase/cow/day (Fibrozyme©, ALLTECH); (4) Cellulase + protease: basal diet with 7.5 g cellulase + protease/cow/day (Allzyme VegPro PO©, ALLTECH); and (5) Pool: basal diet with 30 g enzyme mixture (7.5 g amylase, 15 g xylanase and 7.5 g cellulase + protease)/cow/day.

The feed was offered at 8 a.m. and 4 p.m. in form of total mixed ration in a ratio of 30% of corn silage and 70% of concentrate. The feed and water consumption was *ad libitum*. The proportions of ingredients and the chemical composition of the diet are shown in Table 1.

Experimental period

The experiment was divided into five periods of 21 days each. The first 15 days were for diet adaptation and the last six days for data collection. Therefore, evaluations were recorded at the following times: the DMI between days 16 and 21; digestibility between days 11 and 20 (using the chromium oxide marker, consisting of two phases, the first five days for adaptation to the marker and the last five for faeces collection); feeding behaviour on day 17 and, finally the rumen solid mass disapearance rate between days 20 and 21 (using the rumen emptying technique).

Feeding behaviour and feed intake

The feeding behaviour (performed according to Maekawa *et al.* (2002)) was assessed for 24 hours through observation every 5 minutes. Each parameter observed was considered to be executed during entire interval period (5 minutes) between observations and was called activity. In the study are presented data concerning the Eating, Ruminating and Masticating parameters, reporting the total number of events (NE) of eating, ruminating or masticating as well as the total time per day the cows spent eating, ruminating or masticating. An event was considered to be two or more consecutive activities interrupted by a different activity than the current one. The data concerning the masticating parameter were considered as the sum of respective data concerning eating and ruminating parameters.

The cows had a free access to feed 24 hours a day, but the management strategy was to ensure leftovers of approximately 5%. The DMI was evaluated in 6 days, during which, leftovers from each cow were collected and weighed for the quantification of intake which was obtained by the difference between the amount of feed supplied and the leftovers. On the same days, samples of silage and concentrate were collected to determine the content of DM, ash, CP, EE, calcium, phosphorus, NDF and ADF.

Evaluation of apparent total tract digestibility

The digestibilities of DM, CP, EE, non-fibrous carbohydrates (NFC), organic matter (OM), NDF, and ADF were determined by using the external marker, chromium oxide (Cr_2O_3), whereby Cr_2O_3 was administered (15 g/cow.day) directly into the rumen (through envelopes made of absorbent paper) during five (5) days for adaptation and five (5) days for faeces collection. The apparent digestibility coefficients (ADC) were calculated based on the Cr_2O_3 content of the diet and faeces according to Conceição *et al.* (2007), using the following equations:

$$DMD = 100 - 100 \times (Cr_2O_3\ (\%)\ \text{in diet}/Cr_2O_3\ (\%)\ \text{in faeces})$$

$$ND = 100 - 100 \times (\%\ Cr_2O_3 d\ \text{in diet}/\%\ Cr_2O_3\ f) \times (\%\ Nf/\%\ Nd)$$

Where:
DMD: DM digestibility.
ND: nutrient digestibility.
% Cr_2O_3 d: chromium oxide content in the diet.
% Cr_2O_3 f: chromium oxide content in faeces.
% Nd: nutrient content in the diet.
% Nf: nutrient content in faeces.

The DM content of feed and faeces was determined by drying using a forced air oven at 65 ˚C for 72 hours according to AOAC (1995). All analyses were corrected for the analytical DM content determined at 105 ˚C for 16 hours. The ash was obtained by calcination in a muffle furnace at 550 ºC for 4 hours. The OM was obtained by the difference between 100 and ash (AOAC, 1990). The CP was obtained by the total N content (N×6.25) using the micro-Kjeldahl technique (method 920.87; AOAC, 1990). The EE was obtained by using ANKOM XT15 Extractor® equipment (method Am 5-04; AOCS, 2005).

Table 1 Proportions of ingredients and chemical composition of basal diet

Ingredients (as dry matter (DM) %)	
Corn silage	30.00
Dry ground corn grain	61.67
Soya bean meal	5.14
Urea	0.88
White salt	0.44
Limestone	0.09
Mineral mixture[1]	1.77
Chemical composition	
Dry matter (DM) %	76.48
Crude protein (% DM)	13.24
Ruminally degradable protein[3] (% crude protein (CP))	65.10
Ruminally undegradable protein[3] (% CP)	34.90
Neutral detergent fibre[2] (% DM)	26.98
Effective neutral detergent fibre[3] (% DM)	22.60
Acid detergent fibre (% DM)	14.13
Non-fibre carbohydrates (% DM)	46.10
Starch[3] (% DM)	39.30
Ashes (% DM)	4.56
Calcium (% DM)	0.48
Phosphorus (% DM)	0.34
Ether extract (% DM)	3.16
Total digestible nutrients[3] (% DM)	67.30
Net energy for lactation[3] (Mcal/kg DM)	1.55

[1] Mineral mixture, quantity per kg of product: Calcium: 200 g; Phosphorus: 60 g; Sulfur: 20 g; Magnesium: 20 g; Sodium: 70 g; Cobalt: 15 mg; Copper: 700 mg; Iron: 700 mg; Iodine: 40 mg; Manganese: 1600 mg; Selenium: 19 mg; Zinc: 3200 mg; vitamin A: 200000 IU; vitamin D_3: 50000 IU and vitamin E: 1500 IU.
[2] Determined through chemical analysis.
[3] Estimated by the Spartan Dairy Ration Evaluator/Balancer software, version 3.0.3.

The NDF and ADF were obtained by the method of Van Soest *et al.* (1991). The diet NDF was obtained by using thermostable α-amylase. Calcium (Ca) was determined by titration (method 968.08, AOAC, 1995) and phosphorus (P) by colorimetry (method 965.17; AOAC, 1990). The NFC content was obtained by subtracting the amounts of CP, EE, ash and NDF (expressed in percentage of DM) from 100.

Rumen solid mass disappearance rate

Rumen solid mass disappearance rate was evaluated on days 20 and 21 of each experimental period. The disappearance rate (kt) was determined by rumen emptying, where the rumen content was manually removed through rumen cannula as described by Allen and Linton (2007). On 20th day, the emptying was performed at 11 a.m. (three hours after morning feed administration). On 21st day, the emptying was performed at 8 a.m. prior to feed administration. During the removal, the liquid and solid phases were separated by using a 2 mm mesh sieve, then weighed. Samples of each phase were collected for DM determination. Afterwards, both phases were reconstituted and returned to rumen. With the values of rumen content and DMI, the solid mass kt was calculated and expressed in %/h or kg/h, according to the following equations:

$$kt\ (\%/h) = 100 \times (DMI\ (kg/d)/rumen\ content\ DM\ (kg)) / 24 \quad (3)$$

$$kt\ (kg/h) = (rumen\ content\ DM\ (kg)) \times (kt\ (\%/h)/100) \quad (4)$$

Anaerobic digestion essay

In parallel with the collection of faeces for digestibility essay, collections of faeces for anaerobic digestion were taken. Representative pools of faeces from each cow were collected (and frozen at -20 ˚C) in each period. Treatments were determined based on faeces from cows fed different diets, i.e. 5 × 5 samples of faeces in total. For anaerobic digestion essay, the faeces were thawed and diluted in water, and finally, the inoculum was added to compose substrates. Hence, the substrate composition was as follows: 33.30% of faeces, 3.30% of inoculum and 63.30% of water.

The inoculum was a sewage sludge from waste treatment with 0.16% of total solids (TS). Accordingly, the substrates were prepared to ensure an estimation of 5.0% of TS as per Lucas Junior *et al.* (1993) who found better biogas production in batch-type biodigesters when the TS content of substrates was less than 8.0%.

Batch-type biodigesters (Figure 1) were used, and 3 kg of substrate were prepared, 2 kg of which were used to fill biodigesters and 1 kg to perform the characterisation analyses of substrate (Table 2).

Twenty-five (25) biodigesters were arranged in a completely randomised design comprised of 5 treatments and 5 replicates. After filling, biodigesters were conditioned in a climate chamber with controlled temperature (33±2 ˚C) by electric resistance heating system and digital temperature recorder to guarantee that the test occurred in mesophilic conditions, ideal for digestion kinetics (Metcalf and Eddy, 2014). The temperature was monitored through a digital thermometer (in ˚C), and the readings and records were made immediately before the biogas reading. The composition of substrates in the different biodigesters is shown in Table 2.

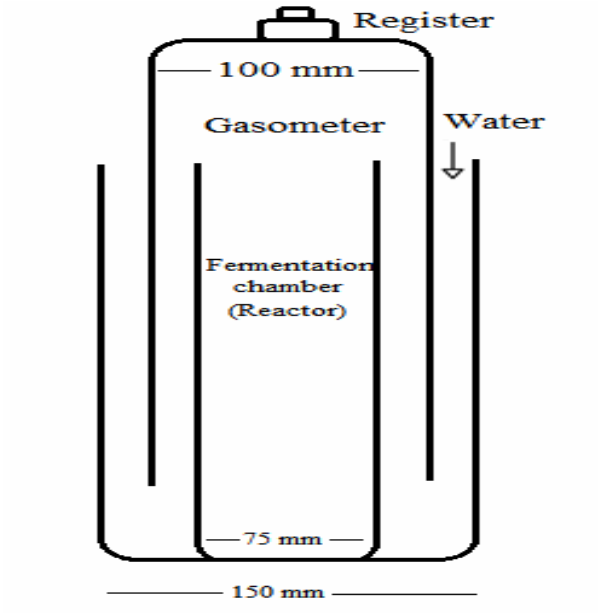

Figure 1 Diagram showing the batch-type biodigester design

Quantitative production of biogas through biodigesters

The batch-type biodigesters consisted of three straight cylinders with diameters of 15, 10 and 7.50 cm, with a mean capacity to ferment 2 litres of substrate each (Figure 1). The 15 and 7.50 cm cylinders were inserted one inside the other so that the space between the outer wall of the inner cylinder and the inner wall of the outer cylinder contained a volume of water (water seal) reaching the depth of 60 cm. The cylinder of intermediate diameter (gas meter) had one of the ends sealed to retain a record for biogas discharge while capsized in water seal to provide anaerobic conditions and to store produced gas.

The reading of biogas production was performed according to its accumulation in gas meter. It consisted of the height measured by the measuring tape attached to gas meter according to the vertical displacement. The reading value was multiplied by internal cross-sectional area of the gas meter.

After each reading, the gas meters were emptied by using biogas discharge register. The correction of biogas volume for the conditions of 1 atm at 20 ˚C was carried out according to the methodology described by Lucas Junior (1994). The correction of biogas volume was performed through the expression resulting from the combination of Boyle and Gay-Lussac laws:

$$(V_0P_0) / T_0 = (V_1P_1) / T_1 \qquad (5)$$

Where:
V_0: corrected biogas volume, m^3 or L.
P_0: corrected biogas pressure, 10322.27 mm H_2O.
T_0: corrected biogas temperature, 293.15 K.
V_1: biogas volume in the gas meter.
P_1: biogas pressure at the time of reading, 10344.11 mm H_2O.
T_1: biogas temperature, in K, at the time of reading.

Considering the average atmospheric pressure of Pirassununga (Sao Paulo-Brazil) equal to 10273.11 mm H_2O and the pressure conferred by the gas meters of 71 mm H_2O, the following expression was obtained to correct biogas volume:

$$V_0 = (V_1/T_1) \times 293.7703 \quad (6)$$

Biogas sampling was performed whenever biogas volume was measured. Samples were collected by using a 60 mL syringe connected to the gas register at the top of gas meter. Then 50 mL of biogas, for analysis, were injected in collecting flasks (glass flasks of 50 mL of capacity, Frascolex, Sao Paulo, Brazil). The gas meters were then emptied to allow a new accumulation of gas. The test was terminated when biogas production ceased, i.e. there was no significant displacement of gas meter.

The concentration of CH_4 was determined by gas chromatography (Trace 1300, Thermo Fisher Scientific®, Rodano, Milan, Italy) in controlled temperature (25 ˚C) according to Kaminski *et al.* (2003). Biogas samples were diluted in glass flasks, with a known volume, 16.78 times in atmospheric air. Then, 6 mL were injected into the chromatograph injector (split/splitless), 4 mL of which were used to wash the injection system and 2 mL were used for analysis. One (1) mL was also used for the system with a flame ionisation detector (FID), responsible for the measurement of CH_4.

The chromatograph was calibrated with 3.10% CH_4 that was diluted in atmospheric air. Gaseous mixture was used as a reference with 50% CH_4 in balance with helium (He) (mol/mol). Helium with a flow rate of 30 mL/min was used as the dragging gas.

Table 2 Characteristics of substrates used in biodigesters

Variable	Treatments					SEM	P-value
	Control	Amylase	Xylanase	C + P	Pool		
TS (%)	4.10	4.00	4.38	4.12	4.24	0.0810	NS
VS (%)	3.72	3.59	3.94	3.61	3.76	0.0762	NS
CP (% de TS)	13.27	12.62	13.14	13.21	12.70	0.2499	NS
OM (% de TS)	89.56	89.59	89.55	88.92	88.24	0.2682	NS
EE (% de TS)	2.50	2.21	2.01	2.38	1.89	0.1032	NS
NDF (% de TS)	38.66	38.74	35.00	34.69	35.35	0.8425	NS
ADF (% de TS)	27.21	25.24	26.14	26.24	24.50	0.5599	NS
Lignin (% de TS)	10.49	10.32	10.23	9.45	9.95	0.2499	NS
GE (kcal/kg TS)	4279	4269	4205	4251	4119	28.521	NS
pH	6.28	6.45	6.47	6.57	6.48	0.0579	NS

TS: total solids; VS: volatile solids; OM: organic matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fibre; ADF: acid detergent fibre; GE: gross energy; C + P: cellulase + protease and Pool: amylase + xylanase + (C + P).
SEM: standard error of the means.
NS: non significant.

The volume of CH_4 produced (m^3 or L) was calculated using the production data and biogas composition of each digester according to the equation:

$$Vol = (Vol_{BIOGAS} \times \% \text{ gas}) / 100 \quad (7)$$

Where:
Vol: volume (m^3 or L).
Vol_{BIOGAS}: volume of biogas produced (m^3 or L).
% Gas: content of gas of interest in biogas.

The production of CH_4 was calculated by dividing the total production of each gas by the amount of VS added or removed (the difference between VS added in the filling time of biodigesters and VS eliminated during the fermentation).

Nutrient removal

The substrates added (before biodigestion) and recovered residue (after biodigestion) in each biodigester were weighed and multiplied by their DM content in percentage to calculate the DM content in grams. The added or recovered nutrients, expressed in grams, were calculated by multiplying between the added or recovered, and expressed as grams of DM, then were expressed as a percentage and divided by 100 according to the following equation:

$$\text{Nutrient (g)} = (\text{added or eliminated/biodigested nutrient (\%)} \times \text{DM (g)}) / 100 \quad (8)$$

The nutrient removal, in percentage, was calculated by using the added and recovered nutrient content and expressed in g/kg of DM according to the following equation:

$$\text{Removed nutrient (\%)} = (\text{added nutrient (g)} - \text{ercovered nutrient (g)}/\text{added nutrient (g)}) / 100 \quad (9)$$

Laboratory analysis

The samples of substrates before and after anaerobic digestion were collected and dried in an oven with ventilation and constant air renewal at 65 ˚C for 72 hours, according to AOAC (1995). Then, they were milled with wily-type knives in 1 mm sieves and stored in properly sealed vials. The DM was determined at 105 ˚C for 16 hours (method 930.15; AOAC, 1995). The mineral matter (MM) was obtained by calcination in a muffle oven at 550 ˚C for 5 hours (AOAC, 1990). The TS (TS=100-humidity) and VS (VS=TS-MM) contents of the substrates were determined with adaptations to the methodology described in APHA (2012). The total nitrogen (N) content was determined by the micro-Kjeldahl technique (method 920.87; AOAC, 1990). The Neutral detergent fibre (NDF) was determined by the method described by Van Soest *et al*. (1991). The hydrogen ion potential (pH) was measured by portable pH meter (Hanna Instruments®, HI 8424, Italy).

Statistical analysis

The data were analysed by using Statistical Analysis System (SAS, 2013). First, they were evaluated in relation to the presence of discrepant information (outliers) and normality of residues by Shapiro-Wilk test. When the normality premises were not met, the data were transformed. The data were then submitted to analysis of variance and a significance level of 5% was adopted.

For the DMI, digestibility, feeding behaviour and disappearance rate, the model included the treatment effect as a fixed effect and the animal and period effects as random factors. The statistical model was used according to the equation below:

$$y_{ijk} = \mu + T_i + P_j + A_k + e_{ijk}$$

Where:

Y_{ijk}: observation concerning treatment (i) + period (j) + animal (k).
μ: overall mean.
T_i: effect of treatment (fixed effect).
P_j: effect of period (random effect).
A_k: animal effect (random effect).
e_{ijk}: random error associated with each observation.

For anaerobic digestion test, biogas production was obtained in each biodigester by biogas measurement for about 6 months (165 days). The frequency of biogas measurement was performed following gas meter capacity and the speed of gas production. For this reason, production and time for filling were considered as variables over time, not allowing to perform statistical analysis in repeated measurement. In this way, gas production over time was used to run Gompertz model using non-linear procedures (PROC NLIN) in SAS software. The data obtained in Gompertz model, as well as other data, were all analysed by using SAS. Before the data were analysed, they were also evaluated in relation to the presence of discrepant information (outliers) and normality of residues by Shapiro-Wilk test. When the normality premise was not met, the data were transformed. They were next submitted to analysis of variance, using mixed model procedure (PROC MIXED). The model included treatment effect as a fixed effect. The statistical model used was described according to equation below:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where:
Y_{ij}: response variable.
μ: overall mean.
T_i: effect of treatment (fixed effect).
e_{ij}: random error associated with each observation.

RESULTS AND DISCUSSION

There were no differences among treatments (P>0.05) on feeding behaviour (Table 3). The average number of daily meals (number of eating events) was 9.76, the total daily time spent eating was 158.8 minutes and 59.13 minutes to eat 1 kg of NDF. For rumination, an average of 13.96 events per day were observed, with a total daily time in this parameter of 301.60 minutes and 115.54 minutes to ruminate 1 kg of NDF.

The average number of events (NE) of mastication per day was 23.72, the total daily masticating time was 460.40 minutes and the time to masticate 1 kg of NDF was 173.48 minutes. The addition of ExE in diet did not affect DMI of cows (P>0.05).

The average daily DMI per cow was 14.41 kg, corresponding to 1.54% of body weight (BW) and 85.25 g/kg of metabolic weight ($BW^{0.75}$) (Table 4). The different ExE did not affect nutrient digestibility (P>0.05). On average, the digestibility was 67.60% for DM, 69.40% for CP, 46.29% for NDF, 42.33% for ADF, 77.49% for EE, 78.76% for NFC, 69.77% for OM and 67.93% for gross energy (GE), with total digestible nutrients (TDN) equal to 69.66%. Accordingly, no differences were found in nutrient excretion (Table 5). The average daily excretion was 4.70 kg for DM, 1.74 kg for NDF, 1.74 kg for NFC and 4.18 kg for OM. Correspondingly, the addition of ExE in the diet of cows showed no differences (P>0.05) on the rumen dynamic variables concerning rumen content volume and rumen DM disappearance rate (Table 6).

The theoretical non-significant biogas production (in general) was observed about 150 days after biodigesters were filled. Therefore, the biodigestion process was interrupted on day 165. The TS and VS of substrates (after biodigestion) from the different treatments did not show differences amongst them (P>0.05). However, biodigestion process provided an average of 29.28% TS reduction and 35.09% VS reduction. Likewise, the differences were neither observed on TS or VS removal efficiency nor on pH values (Table 7).

There was no effect (P>0.05) of treatments on the variables of biogas production (Table 8), i.e. ExE did neither influence total gas production nor the concentration and production of CH_4 from faeces of cows fed diets containing these enzymes. The potential for CH_4 production was similar among treatments. On average, 0.029 litre of CH_4 was produced per gram of faeces added, 0.236 litre of CH_4 per gram of VS added, and 0.714 litre of CH_4 per gram of VS removed.

Feed intake is fundamentally important in nutrition by establishing the amount of nutrients for health and production. Rumen fill is one of different factors pointed to affect DMI. Therefore, feeds of low digestibility (high NDF content) place constraints on DMI because of slow clearance from rumen (NRC, 2016). The use of ExE, such as cellulase and xylanase, is supposed to improve DMI in ruminants (Rojo *et al.* 2015; Silva *et al.* 2016; Mocherla *et al.* 2017; Tirado-González *et al.* 2017; Golder *et al.* 2019; Meschiatti *et al.* 2019; Devant *et al.* 2020). In the present study, the different ExE (amylase, xylanase and cellulose + protease or their combination) showed to have no effect on DMI. In the same way, many other studies found no effect of ExE on DMI.

Mohamed *et al.* (2013) and Shadmanesh (2014), both evaluating the effect of dietary suppplement with fibrolytic enzymes on productive performance of early lactating dairy cows, found no effect on DMI.

Table 3 Feeding behaviour of Holstein cows fed different enzymes

Variables	Treatments					SEM	P-value
	Control	Amylase	Xylanase	C + P	Pool		
Eating							
Number of events	10.20	10.00	9.000	8.800	10.80	0.396	NS
Total time eating (min)	172.0	143.0	161.0	150.0	168.0	5.614	NS
Neutral detergent fibre (NDF, min/kg)	57.82	57.82	62.60	59.33	58.06	1.842	NS
Ruminating							
Number of events	13.00	14.60	13.80	15.00	13.40	0.674	NS
Total time ruminating (min)	297.0	317.0	288.0	307.0	299.0	13.51	NS
NDF (min/kg)	119.9	115.7	118.5	113.4	110.2	4.821	NS
Masticating							
Number of events	23.20	24.60	22.80	23.80	24.20	0.855	NS
Total time masticating (min)	469.0	460.0	449.0	457.0	467.0	16.48	NS
NDF (min/kg)	169.2	176.0	181.1	172.8	168.3	6.060	NS

C + P: cellulase + protease and Pool: amylase + xylanase + (C + P).
SEM: standard error of the means.
NS: non significant.

Table 4 Dry matter intake (DMI) of Holstein cows fed different enzymes

Variables	Treatments					SEM	P-value
	Control	Amylase	Xylanase	C + P	Pool		
Dry matter intake							
kg/cow/day	14.66	14.27	14.01	14.35	14.77	0.409	NS
% body weight (BW)	1.56	1.53	1.50	1.55	1.58	0.039	NS
g/kg $BW^{0.75}$	86.38	84.36	82.75	85.52	87.26	2.120	NS

C + P: cellulase + protease and Pool: amylase + xylanase + (C + P).
SEM: standard error of the means.
NS: non significant.

Table 5 Total apparent digestibility of dry matter and its fractions of Holstein cows fed different enzymes

Variables	Treatments					SEM	P-value
	Control	Amylase	Xylanase	C + P	Pool		
Digestibility (%)							
DM	65.27	67.72	67.65	68.53	68.84	0.606	NS[4]
CP	65.58	69.44	67.92	74.02	70.04	1.294	NS
NDF	39.24	43.02	47.17	51.41	50.59	1.746	NS
ADF	35.05	41.98	42.23	44.00	48.39	1.853	NS
EE	72.34	77.62	79.71	75.95	81.85	1.359	NS
NFC	78.67	79.94	78.41	77.25	79.54	0.713	NS
OM	67.37	69.72	69.65	70.63	71.48	0.576	NS
GE	65.13	67.74	68.21	68.66	69.90	0.602	NS
TDN	67.15	69.60	69.64	70.45	71.45	0.583	NS
Excretion (kg/d)							
DM	5.06	4.67	4.57	4.60	4.59	0.139	NS
NDF	1.97	1.85	1.65	1.58	1.65	0.064	NS
NFC	1.76	1.64	1.74	1.87	1.70	0.074	NS
OM	4.53	4.18	4.09	4.09	4.02	0.127	NS

DM: dry matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fibre; ADF: acid detergent fibre; NFC: non-fibre carbohydrates; OM: organic matter; GE: gross energy; C + P: cellulase + protease and Pool: amylase + xylanase + (C + P).
SEM: standard error of the means.
NS: non significant.

Encinas *et al*. (2018), evaluating the performance and nutrient digestibility of feedlot steers fed a diet supplemented with digestive enzymes, found no effect on DMI. Ran *et al.* (2019) evaluated effects of a recombinant fibrolytic enzyme on fibre digestion, rumen fermentation, nitrogen balance, and total tract digestibility of heifers fed a high forage diet but did not observe any effect on feed intake. Evaluating the effects of supplementing xylanase in dairy cows, Yang *et al*. (2019) found no effect on DMI. Zilio *et al*. (2019) also found no differences on DMI when evaluated effects of exogenous fibrolytic and amylolytic enzymes in dairy cows. The meta-analysis, performed by Tirado-González *et al*. (2017), which used 586 records extracted from 74 journal articles evaluating effects of exogenous fibrolytic enzymes in ruminant diets revealed that DMI of dairy cows was not affected, but there was an improvement of DMI of beef cattle. Inconsistent results were also found in a literature review work carried out by Sujan and Seresinhe (2015). According to Bowman *et al*. (2002), the effects of fibrolytic enzymes (and other kind of ExE) on DMI may depend on enzyme products and the method of applying of enzymes. In the present study, the lack of effect of different treatments on DMI is considered to be a reflection of a fact also observed in the study, which is the lack of effect on feeding behaviour. Therefore, if ExE did not incite significant changes on feeding behaviour, most probably DMI would also be unchanged.

Exogenous enzymes, acting together with enzymes produced by rumen microorganisms, potentiate the degradation of DM and nutrients such as structural carbohydrates and increase the rate of fibre degradation, increasing digestive efficiency of feed (Beauchemin *et al.* 2003; Mocherla *et al.* 2017; Elsiddig, 2019). Some studies have shown that the addition of fibrolytic enzymes in ruminant diet promotes increased cellulase and xylanase activity (Neumann *et al.* 2018; Golder *at al.* 2019), whereas proteases increase proteolytic activity in rumen (Eun and Beauchemin, 2005). This effect was not observed in the present study as digestibility of NDF and CP, as well as total DM was similar for all treatments. Encinas *et al*. (2018) also did not observe any effect of addition of digestive enzymes in diet of steers on DM, CP and NDF digestibility. The total tract digestibility of DM, OM, NDF, and ADF were also unaffected in Ran *et al.* (2019) study. Giraldo *et al.* (2008) reported having found no effect on diet digestibility when exogenous fibrolytic enzyme preparation was administered (12 g/animal/day) directly into the rumen of sheep. Xylanase supplementation did not affect nutrient digestibility in the study of Yang *et al*. (2019). The jointly or separately supplementation of xylanase and amylase enzymes had no impact on total tract digestion of nutrients in dairy cows in studies performed by Zilio *et al*. (2019) and Silva *et al*. (2016).

Different from the results of the present study, as well as other studies mentioned, Eun and Beauchemin (2005), evaluating effects of a proteolytic feed enzyme on intake, digestion, rumen fermentation, and milk production, observed an increased total tract digestibility of DM, OM, CP, ADF and NDF. Rojo *et al*. (2015), evaluating the influence of cellulase addition to dairy goat diets on digestion and fermentation, observed a greater digestibility of DM, OM and NDF. Devant *et al.* (2020) evaluated effects of exogenous glucoamylase alone or in combination with a neutral protease on apparent total tract digestibility and observed an increased apparent total tract digestibility of DM and starch, but protease did not have additional benefits on nutrient digestibility. Song *et al*. (2018), evaluating the effects of fibrolytic enzymes observed a significant increased digestibility of NDF in Chinese domesticated black goats. In meta-analysis of Tirado-González *et al*. (2017), cellulase and xylanase enhanced *in vivo* DM digestibility in low-forage (forage:concentrate<50%) grass-based diets. In forage:concentrate ≥ 50% legume-based diets, cellulase and xylanase enhanced *in situ* DM and NDF disappearance rate, but there were no effects in forage:concentrate ≥ 50% grass-based diets. In the same study, in forage:concentrate ≥ 50% diets, *in vitro* DM degradability was improved mainly by cellulase, but fibre degradability was improved when cellulase and xylanase were used jointly in sheep rumen liquid for *in vitro* evaluations.

In the present study, DM and nutrient degradability was not evaluated, but no effect of the different treatments on rumen dynamics (regarding disappearance rate) was observed, however, it is thought that the greater the DM degradability, the greater the disappearance rate. Therefore, as there was no effect of enzyme addition on feeding behaviour and DMI accompanied by a lack of effect on digestibility, the lack of effect on degradability and disappearance rate is not surprising.

The hypothesis when this study was carried out was that the use of ExE such as xylanase and cellulase would improve feed digestibility and, accordingly, increase DMI, but such was not observed. Beauchemin *et al*. (2003) reported potential increases in feed voluntary intake due to improvements on rumen fibre digestion, increasing feed passage rate through digestive tract by fibrolytic enzyme supplementation. Nonetheless, different studies have shown inconsistent results on effects of ExE on rumen DM and nutrients degradation. The review by Mocherla *et al.* (2017) on the effects of ExE on rumen digestion found that the function of ExE varies with various factors, that is the reason why various contradicting results were reported.

Table 6 Rumen mass and rumen disappearance rate of Holstein cows fed different enzymes

Variables	Treatments					SEM	P-value
	Control	Amylase	Xylanase	C + P	Pool		
Rumen DM (%)	12.22	11.98	12.88	12.44	13.16	0.22	NS
Liquid mass (kg)	53.56	52.33	51.11	53.45	51.04	1.92	NS
Liquid mass (% BW)	5.64	5.57	5.45	5.70	5.45	0.13	NS
Solid mass (kg)	7.48	7.14	7.56	7.38	7.83	0.28	NS
Solid mass (% BW)	0.78	0.76	0.80	0.79	0.83	0.02	NS
Total rumen mass (kg)	61.04	59.48	58.67	60.83	58.87	2.16	NS
Total rumen mass (% BW)	6.42	6.33	6.26	6.50	6.28	0.14	NS
Solid turnover (%/d)	206.1	203.2	187.5	196.6	194.2	4.65	NS
Solid mass kt (%/h)	8.58	8.47	7.81	8.19	8.09	0.19	NS
Solid mass kt (kg/h)	0.620	0.602	0.592	0.604	0.620	0.02	NS

DM: dry matter; BW: body weight; kt: disappearance rate; C + P: cellulase + protease and Pool: amylase + xylanase + (C + P).
SEM: standard error of the means.
NS: non significant.

Table 7 Biodigestion and removal efficiency of nutrients from anaerobic batch-type biodigesters supplied with the waste of Holstein cows fed different enzymes

Variables	Treatments					SEM	P-value
	Control	Amylase	Xylanase	C + P	Pool		
Added nutrients (%)							
Total solids	4.27	4.35	4.15	4.13	4.33	0.0700	NS
Volatile solids	3.59	3.97	3.71	3.61	3.94	0.0816	NS
Recovered nutrients (%)							
Total solids	3.22	3.47	3.12	3.10	3.34	0.0801	NS
Volatile solids	2.60	2.27	2.50	2.43	2.65	0.0842	NS
Removal efficiency (%)							
Total solids	28.51	33.09	30.23	27.40	26.02	2.4424	NS
Volatile solids	32.11	35.98	37.31	34.81	35.73	2.2338	NS
pH after biodigestion	7.07	7.02	7.00	7.00	7.11	0.0208	NS

C + P: cellulase + protease and Pool: amylase + xylanase + (C + P).
SEM: standard error of the means.
NS: non significant.

Table 8 Gas production (total biogas and CH_4) in batch-type biodigesters with the waste of Holstein cows fed different enzymes

Variables	Treatments					SEM	P-value
	Control	Amylase	Xylanase	C + P	Pool		
Total biogas (L)	15.86	14.86	15.75	15.34	16.08	1.0828	NS
CH_4 (L)	10.88	10.22	11.73	10.67	11.57	0.7010	NS
CH_4 (%)	68.63	68.84	74.48	69.53	71.98	0.8753	NS
CH_4/faeces (L/g)	0.030	0.026	0.030	0.030	0.030	0.0095	NS
CH_4/added volatile solids (L/g)	0.243	0.243	0.225	0.240	0.230	0.0094	NS
CH_4/removed volatile solids (L/g)	0.743	0.603	0.655	0.726	0.640	0.0615	NS

C + P: cellulase + protease and Pool: amylase + xylanase + (C + P).
SEM: standard error of the means.
NS: non significant.

According to Tirado-González *et al.* (2017), the response of ExE may depend upon the mixture of digestive enzymes, as well as the diet composition, but it may also depend on enzyme products, dosage and the method of enzyme application (Bowman *et al.* 2002; Beauchemin *et al.* 2003; Mocherla *et al.* 2017). According to Beauchemin *et al.* (2003), ruminant feed enzyme additives, primarily xylanases and cellulases, are concentrated extracts which result from bacterial or fungal fermentation with specific enzymatic activities, therefore, the variation of the response can also be attributed to activities and characteristics of enzymes supplied, as well as to experimental conditions in which energy is not the limiting nutrient.

Although there was no effect of the different treatments on substrate pH before (Table 2) or after (Table 7) anaerobic digestion, the substrate pH before anaerobic digestion ranged between 6.28 and 6.57, but after anaerobic digestion ranged between 7.00 and 7.11. This shows a pH increase during biodigestion process, indicating that the different treatments (including control) created better pH conditions

for CH_4 production as Rabiu *et al.* (2014), Mshandete *et al.* (2006) and Gunaseelan (1995) stated that the pH of a normal and healthy anaerobic digestion system for CH_4 production is generally in the range of 7.00 to 8.50.

Removed VS are used to measure the performance of biodigestion process in addition to being a direct indicator of metabolic activity of microbiological community. Davidsson *et al.* (2008) reported that the reduction of VS in cattle manure by anaerobic digestion process ranges from 30% to 45%, while Dohányos and Zábranská (2001) stipulated the range of 25% to 50%. In the present study, the VS removal efficiency renged from 32.11% (control treatment) to 37.31% (xylanase treatment) and averaging 35% (Table 7), indicating a good process performance. Orrico Junior *et al.* (2010), evaluating the efficiency of anaerobic digestion process in batch-type digesters supplied with cattle manure fed diet 1 (60% roughage:40% concentrate) and diet 2 (40% roughage:60% concentrate), observed a variation on VS removal, where the increase in forage proportion led to a lower VS removal efficiency and a respective lower anaerobic digestion efficiency. This indicates that VS removal efficiency might depend on diet composition since cattle manage to use a large part of concentrate and part of roughage, the undegradable fraction present in roughage ends up excreted in faeces and this fraction has low utilisation in biodigester.

According to Mocherla *et al.* (2017), ExE cause the breakdown of fibre cell walls, degrade proteins and reduce effects of anti-nutritive factors, making nutrients more available both for the animal and for anaerobic bacteria present in biodigesters. This causes greater availability of nutrients and consequently increases biogas production. Nevertheless, this effect was not observed in the present study, where enzymes did not interfere in the results of biogas and CH_4 production. The lack of differences among treatments, in addition to other factors, may be related to the composition of substrates, where enzymes did not promote changes in the composition of manure that made up the substrates and then, they remained similar with the control treatment and did not provide changes in biogas production.

In the present study, despite having forage in the diet, CH_4 concentration among treatments averaged 70.60%. Using the same type of digesters (batch-type digesters), Orrico *et al.* (2007) obtained 66.55% of CH_4, while Silva (1998) described that the composition of biogas may vary from 60 to 70% of CH_4 in batch-type digesters. Thus, biodigestion process proved to be efficient since Soussana *et al.* (2010) stated that the greater the proportion of CH_4, the more efficient the biogas for energy production. Gopalan *et al.* (2013) reported an average CH_4 yield of 0.17 litre per gram of added VS (L/g VS), with a variation of 0.07-0.28 L/g VS, these data are in accordance with this study, in which there was a CH_4 yield of 0.24 L/g VS. Nonetheless, the average values found by Møller *et al.* (2004) and Perna Junior (2018) are a little higher, as they found 0.40 L/g VS and 0.34 L/g VS, respectively. This shows that CH_4 production from added VS may depend on study conditions.

The management of cattle manure has become increasingly challenging because its production continues to rise while the regulations on manure management have become increasingly stringent (Baek *et al.* 2020). Cow manure represents a surplus manure waste in agricultural food sectors and requires proper disposal. Anaerobic digestion has raised global interest owing to apparent environmental benefits which include simultaneous waste diminishment and renewable energy generation (Li *et al.* 2021).

According to some studies above cited, the manipulation of rumen fermentation by using ExE to increase digestibility and consequently feed efficiency in ruminants is a widely studied topic. Therefore, for the present study, assays of anaerobic digestion of manure from ruminants fed ExE were not found. The anaerobic digestion assay in the present study was carried out with the hypothesis that the use of ExE in cows' feeding would increase nutrient digestibility and reduce faecal excretion of OM and, consequently, reduce greenhouse gas production potential from faeces. Nonetheless, the hypothesis was not confirmed since the ExE did not appear to have any effect on all evaluated parameters, which may have been caused by some factors mentioned above, as some studies (Bowman *et al.* 2002; Beauchemin *et al.* 2003; Mocherla *et al.* 2017; Tirado-González *et al.* 2017) indicate that the response of ExE may depend on the mixture of the enzymes, diet composition, enzyme products, method of application, activities and characteristics of the enzymes, dosage, as well as experimental conditions.

Although ExE did not have a significant effect on all parameters evaluated in the present study, the enzyme combination and cellulase + protease showed a tendency to increase digestibility of CP, NDF and ADF (Table 5), showing that they might have tended to improve the efficiency of utilisation of these nutrients.

CONCLUSION

The utilisation of exogenous enzymes in cows' feeding did not have any impact on feeding behaviour, dry matter intake, nutrient digestibility, rumen disappearance rate or on methane production from faeces. Therefore, enzymes did not increase the efficiency of nutrient utilisation and, accordingly, did not alter the potential of methane production from faeces by means of anaerobic digestion. Among the threatments, the enzyme combination and cellulase + prote-

ase may have slightly improved the efficiency of CP, NDF and ADF use by the animals.

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