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Effect of Exogenous Enzymes on Nutrient Digestibility and Ruminal Fermentation of Holstein Cows

Research Article

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ABSTRACT

This study aimed to evaluate the effect of different exogenous enzymes or their combination on nutrient digestibility and ruminal fermentation of Holstein cows. Five ruminally cannulated adult Holstein cows were distributed in a 5 × 5 Latin square design and received the same basal diet consisting of 30% of corn silage and 70% of concentrate but with inclusion of different exogenous enzymes comprising five treatments (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 enzyme pool: basal diet with 30 g enzyme mixture (7.5 g of amylase, 15 g of xylanase and 7.5 g of cellulase + protease)/cow/day). Accordingly, the experiment was conducted in five periods of 23 days each. Therefore, data were collected to evaluate nutrient digestibility and ruminal fermentation parameters. The different exogenous enzymes or their combination (enzyme pool) did not affect ($P>0.05$) nutrient digestibility, ruminal pH, protozoa population, ammonia nitrogen and methane concentration and production. The enzyme combination and cellulase + protease increased ($P<0.05$) the concentration and production of acetate and propionate as well as total short-chain fatty acids, but did not significantly affect the relative energy loss of methane concering the other rumen fermentation products (acetate, propionate, and butyrate). Therefore, the dietary supplementation of different exogenous enzymes or their combination in Holstein cows did not improve feed value, but the enzyme combination has shown a tendency to reduce the relative energy loss of methane.

KEY WORDS digestibility, enzymes, methane, relative energy loss, short-chain fatty acids.

INTRODUCTION

The increase in greenhouse gases (GHG) in the atmosphere has been identified as one of the leading causes of climate change as it increases the potential for global warming.

Methane (CH_4) is considered the second largest contributor to a global warming, with global warming potential 25 times greater than carbon dioxide (CO_2) and lifetime in the atmosphere of 9 to 15 years, and an annual growth rate of 7% (IPCC, 2007).

Agricultural soils and livestock production (mainly the enteric fermentation of domesticated ruminants such as cattle, buffalos, sheep, and goats) are some of the sectors responsible for the emission of gases (CO_2, CH_4, and N_2O) that cause the increase of greenhouse effect in the atmosphere (Carega and Dantas, 2017).

According to IPCC (2014), global GHG emissions from agricultural production in the 2000-2010 were estimated at 5 to 5.8 gigatonnes of carbon dioxide equivalent ($GtCO_2eq$) per year. The US Environmental Protection Agency (EPA, 2012) stated that the global CH_4 emissions from enteric fermentation increased by 7% from 1990 to 2005 (from 1764 to 1894 megatonnes of carbon dioxide equivalent ($MtCO_2eq$)) and, over this period, the global livestock populations have increased. From 2005 to 2030, CH_4 emissions from enteric fermentation are projected to increase about 18-22%, from 1894 to 2320 $MtCO_2eq$ (EPA, 2012). Even though, according to Lynch (2019), there are still insufficient data available to comprehensively address essential questions regarding the climate impacts of agricultural production. Global GHG emissions from the industrial and waste/wastewater sector grew from 10.4 $GtCO_2eq$ in 1990 to 13.0 $GtCO_2eq$ in 2005 and then to 15.4 $GtCO_2eq$ in 2010 (IPCC, 2014), showing that the industry-related GHG emissions have continued to increase and are higher than GHG emissions from livestock sector. Therefore, the environmental impact caused by the industrial sector may be higher than that of the livestock sector.

In addition to environmental problems, CH_4 production from enteric fermentation of ruminants leads to feed gross energy losses ranging from 2 to 15% (Wanapat *et al*. 2015), depending on the quantity, quality, and type of feed consumed (EPA, 2012). Therefore, considering the importance of ruminant production, it is essential to establish economically viable ways to reduce CH_4 production (Popova *et al*. 2013) which may include increasing livestock productivity, improving nutritional management, manipulation of rumen fermentation, changes in diet composition, dietary addition of CH_4 production inhibitors or defaunation (Shibata and Terada, 2010).

In over three decades, the increasing trend on feed additives, such as exogenous enzymes (ExE), gained force owing to a ban on antibiotic growth promoters (Sujani and Seresinhe, 2015). The ExE used in ruminants' diets can be characterized into main categories as fibrolytic, amylolytic, and proteolytic based on a specific substrate on which their enzyme activity can perform (Sujani and Seresinhe, 2015). The ultimate function of ExE is to supply maximum nutrients from digestible, potentially digestible, and indigestible fractions of cell walls (Mocherla *et al*. 2017) as they enhance dry matter (DM) and nutrient digestibility, degradability and disappearance rate (Mocherla *et al*. 2017; Tirado-González *et al*. 2017; Devant *et al*. 2020). The application of exogenous fibrolytic enzymes (EFE) enhances rumen fermentation of roughages, although the magnitude of the effects may depend on the fibrous substrate fermented and the amount of enzyme added (Elghandour *et al*. 2016; Abid *et al*. 2019), and can lead to a more outstanding total short-chain fatty acid (SCFA) production in the rumen (Silva *et al*. 2016).

Exogenous enzymes, such as amylase supplementation, have shown positive effects on DM intake, rumen, total tract starch digestibility, feed efficiency, milk, and meat production (Meschiatti *et al*. 2019; Toseti *et al*. 2020; Amaro *et al*. 2021). According to Eun and Beauchemin (2005), the use of exogenous proteolytic enzymes has been ignored because it is assumed that they would cause excessive protein degradation in the rumen, resulting in inefficient nitrogen use, but in their study, adding exogenous proteolytic enzymes to the diet increased total tract digestibility of DM, organic matter (OM), crude protein (CP) as well as fiber, although not affecting short-chain fatty acid profile. Nevertheless, protease enzyme products may be more effective in high-concentrate diets (Vera *et al*. 2012).

Therefore, the inclusion of ExE in ruminant feeding can be crucial in reducing enteric CH_4 production, considered as a GHG, as it is widely known that the more productive the animal, the less is CH_4 emission (Mocherla *et al*. 2017). Thus, the effect of ExE on reducing CH_4 production contributes to enhancing feed energy efficiency. Then, in tropical areas where most of the feed is from fibrous resources, the addition of ExE to diet is crucial since the production of CH_4 is maximum with low-yielding animals (Mocherla *et al*. 2017).

Knowing the effects of ExE, this study was performed with the hypothesis that adding different ExE in ruminant diets or their combination would improve feed value by enhancing nutrient digestibility and rumen fermentation and, accordingly, reduce the relative energy loss (REL) of CH_4 concering the other rumen fermentation products. Therefore, the objective was to evaluate the effect of supplementing different ExE (Amylase, Xylanase, Cellulase + protease, and a mixture of these) on nutrient digestibility and ruminal fermentation of Holstein cows.

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 Animal Science and Food Engineering of Sao Paulo University (USP-Brazil) under the protocol number CEUA 9296281113.

Treatments, experimental design, and feeding management

Five adult Holstein cows, non-pregnant and non-lactating, carrying rumen cannula and having a mean body weight of 923 kg (±86), were kept in a roofed shed in the individual pen with free access to sand bedding. The experiment was conducted in a 5 × 5 Latin square design, using the animal within each period as the experimental unit. The animals were allocated to one of the 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, SP, Brazil); (3) Xylanase: basal diet with 15 g of xylanase/cow/day (Fibrozyme©, ALLTECH, SP, Brazil); (4) Cellulase + protease: basal diet with 7.5 g cellulase + protease/cow/day (Allzyme VegPro PO©, ALLTECH, SP, Brazil); 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 the form of total mixed ration in a ratio of 30% of corn silage and 70% of the concentrate as a normal diet the animals received as high producing dairy cows. 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 23 days each, but the last two days of each period, the cows were kept on pasture. The first 15 days were for diet adaptation and the next six days for data collection. Therefore, evaluations were recorded at the following times: the apparent total tract digestibility of dry matter (DM), crude protein (CP), non-fiber carbohydrates (NFC), and neutral detergent fiber (NDF) 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 feces collection); rumen pH, rumen fermentation products (CH_4, short-chain fatty acids (SCFA), and NH_3-N) and rumen protozoa on day 21.

Evaluation of apparent total-tract digestibility

The digestibilities of DM, CP, NFC, and NDF were determined 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 feces collection.

The apparent digestibility was calculated based on the Cr_2O_3 content of the diet and feces according to Conceição *et al.* (2007), using equations 1 and 2. Between days 16 and 20, samples of feed were collected to determine feed DM content.

Table 1 Ingredients and chemical composition of basal diet

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

[1] Vitamin and mineral mixture, quantity per kg of product: vitamin A: 200000 IU; vitamin D_3: 50000 IU; vitamin E: 1500 IU; 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 and Zinc: 3200 mg.
[2] Determined through chemical analysis.
[3] Estimated by the Spartan Dairy Ration Evaluator/Balancer software, version 3.0.3.

DMD= 100 – 100 × Cr203 (%) in diet / Cr203 (%) in feces (1)

ND= 100 – 100 × % Cr203 d / % Cr203 f × % Nf / % Nd (2)

Where:

DMD: DM digestibility.

ND: nutrient digestibility.

% Cr_2O_3d: chromium oxide content in the diet.

% Cr_2O_3f: chromium oxide content in feces.

% Nd: nutrient content in the diet.

% Nf: nutrient content in feces.

The DM content of feed and feces was determined by drying using a forced air oven at 65 ˚C for 72 h according to AOAC (1995). All analyses were corrected for the analytical DM content determined at 105 ˚C for 16 h. The ash was obtained by calcination in a muffle furnace at 550 ˚C for 4 h.

The CP was obtained by the total N content (N×6.25) using the micro-Kjeldahl technique (method 920.87; AOAC, 1990). The ether extracts (EE) was obtained using ANKOM XT15 Extractor® equipment (method Am 5-04; AOCS, 2005). The NDF and acid detergent fiber (ADF) were obtained by the method of Van Soest *et al.* (1991). The dietary NDF was obtained by thermostable α-amylase. The NFC content was obtained by subtracting the sum of CP, EE, ash, and NDF (expressed in percentage of DM) from 100.

Rumen pH measurment

The pH measurement was continuously performed using a data logger (Model T7-1 LRCpH, Dascor®, CA, USA). The system consisted of a pH probe enclosed in a protective shield that allowed the rumen liquid to percolate freely but prevented the electrode from contacting the rumen epithelium. Weights were attached to each probe to ensure that it remained in the ventral sac of the rumen. The probes were programmed so that the electrodes measured and recorded the rumen pH every 10 min over the measurement period. The probes were inserted into the rumen of cows to measure the rumen pH (at every 10 min) for 24 h. The pH data were recorded as minimum, mean, and maximum pH. The area under the curve and duration of time in which pH was below 6.2, 6.0, and 5.8 were also calculated. According to Penner *et al.* (2007), the pH 5.8 indicates the threshold of sub-acute rumen acidosis, and pH 6.0 and 6.2 indicate the thresholds of healthy rumen conditions (Penner and Beauchemin, 2010). The area under the curve was calculated by multiplying the absolute value of the deviations in pH by the time (min) spent below the threshold established for each measurement, divided by 60 and expressed as pH unit per hour according to Moya *et al.* (2011).

Evaluation of ruminal fermentation products

The ruminal fermentation products were evaluated using the *ex-situ* (micro-rumen) technique described by Rodrigues *et al.* (2012) and Perna Junior *et al.* (2017). This technique consists of placing rumen content in flasks (micro-rumen) and incubating in a thermostatic bath, simulating rumen conditions for 30 min (Figure 1).

Sampling from rumen content

Glass flasks of 50 mL capacity (Frascolex, São Paulo, Brazil) were firstly identified and weighed. Then, at zero (0), 3, 6, 9, and 12 h after morning feeding, the rumen content was separately collected in solid and liquid fractions. On this day, the cows were fed after the first collection (about 8:30 a.m.) and after the last collection (about 8:30 p.m.). Both rumen fractions (solid and liquid) were placed in flasks (about 10 g of the solid fraction and 20 mL of the liquid fraction). The flasks were then capped with rubber stoppers and sealed with aluminium sealing wax through specific pliers. Afterward, they were "washed" with CO_2 using two needles for gas inlet and outlet to ensure an anaerobic environment. Four flasks per cow were prepared for each sampling time, two of which were immediately placed in an autoclave to inactivate the fermentative process (under temperature and pressure) for 15 min. The other two flasks were immediately incubated for 30 min in a thermostatic bath at 39 ˚C. At the end of the incubation time the fermentative process was also inactivated in the autoclave. After the flasks cooled at room temperature, the volume of gas and the concentration of CH_4, SCFA, and ammonia in each flask were measured. The Figure 1 shows the diagram of the entire procedure.

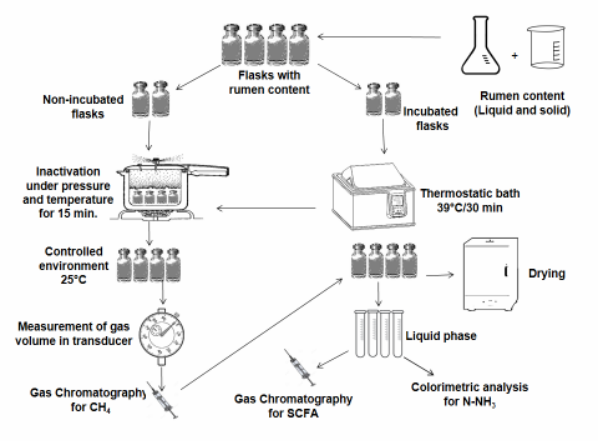

Figure 1 Diagrammatic representation of *ex-situ* ruminal fermentation technique

Gas volume and methane measurement

In a temperature-controlled environment (25 ˚C), the volume of gas produced in the incubated and non-incubated flasks was measured using a pressure transducer (Data logger Universal AG5000, Genesis SM®, Barueri, SP, Brazil) connected to a reader with a syringe and needle. The volume was measured by dragging the accumulated gas in the upper part of the flask using the syringe connected to the transducer until a zero-pressure reading. The volume displaced by the gas produced in the flask was recorded to determine the production of CH_4 gas. The total gas volume was obtained by the sum of that obtained in the syringe plus the headspace of the flask. After measuring by the transducer, the determination of CH_4 concentration was performed by gas chromatography, according to Kaminski *et al.* (2003), by injecting 0.5 mL of gas into a chromatograph (Trace 1300, Thermo Fisher Scientific®, Rodano, Milan, Italy).

Calculation of liquid volume, solid content and concentration of SCFA

The volume of liquid within the incubated and non-incubated flasks was calculated by the difference between the weight of the flask containing the sample after drying at a 105 ˚C and the weight of the flask containing the sample before drying. The solid content of the flask was obtained by the difference, in weight, between the flask containing the sample after drying and the empty flask (obtained before flasks were filled).

For SCFA (acetate, propionate, and butyrate) concentration, 4 mL of rumen fluid content of each flask were collected and centrifuged at 2000 × g for 20 min, and 2 mL of supernatant were added to a test tube containing 0.4 mL of formic acid, then sealed and frozen at -20 ˚C for further analysis, according to Erwin *et al.* (1961). The SCFA were measured through gas chromatography (Focus GC, Thermo Scientific®, Rodano, Milan, Italy) by using a glass column with 1.22 m length and 0.63 cm diameter packed with 80/120 Carbopack B-DA/4% (Supelco, Sigma-Aldrich, St. Louis, MO, USA).

Calculation of SCFA and methane yield

All calculations of SCFA and CH_4 yield were performed according to Rodrigues *et al.* (2012) and Perna Junior *et al.* (2017). Methane production was obtained multiplying the total volume of gas (mL) produced in each flask by the concentration of CH_4 in the gas phase (mmol/mL) obtained in an incubated flask, and then subtracting what was produced in a non-incubated flask (equation 3). The individual quantification of SCFA was obtained by multiplying the volume of liquid (mL) and the concentration of each SCFA (mmol/mL) obtained in the incubated flask, and then subtracting the production obtained in a non-incubated flask (equation 4). Subsequently, the CH_4 and SCFA production was expressed based on the solid content of the flasks (grams or kilograms). This content was obtained by the difference between the weight of the flask containing dry sample (105 ˚C) and the weight of the empty flask.

CH_4 Prod.= (CH_4Conc.×total gas vol.)T_{30} – (CH_4Conc.×total gas vol.)T_0 (3)

Where:

CH_4 Prod.: CH_4 production at the time between rumen content injection in the flask and inactivation.

CH_4 Conc.: CH_4 concentration (mmol/mL).

Total gas vol.: total volume of gas (obtained by the sum of the volume determined by the transducer and the headspace (mL)).

T_{30}: incubation time of 30 min.

T_0: incubation time of zero min.

SCFA Prod.= (SCFA Conc.×total Liq. vol.)T_{30} – (SCFA Conc.×total Liq. vol.)T_0 (4)

Where:

SCFA Prod.: SCFA production at the time between rumen content injection in the flask and inactivation.

SCFA Conc.: SCFA concentration (mmol/mL).

Total Liq. vol.: total volume of liquid in the flask (obtained by the weight difference before and after drying (mL)).

T_{30}: incubation time of 30 min.

T_0: incubation time of zero min.

Calculation of relative energy loss

After CH_4 and SCFA were quantified, each product was multiplied by the respective combustion heat to express the CH_4 production as a percentage of the energy from the fermentation produced. Therefore, the relative energy loss (REL) was considered as the ratio between the energy contained in CH_4 produced and the sum of the energy contained in all quantified fermentation products (CH_4 and SCFA), expressed as a percentage. Thus, theoretical chemical values of the combustion heat were used, assuming that acetate, propionate, butyrate, CH_4, and CO_2 present 3.49, 4.98, 5.96, 13.16, and 0.0 kcal/g or 209.40, 368.52, 524.48, 210.56, and 0.0 kcal/mol, respectively. The REL was calculated according to Rodrigues *et al.* (2012), (equation 5).

REL (%)= 100 × ($\varepsilon CH_4/\varepsilon CH_4+\varepsilon C_2+\varepsilon C_3+\varepsilon C_4$)

Where:

REL: relative energy loss of methane concering the other fermentation products.

εCH_4: methane energy (kcal/g or kcal/mol).

εC_2: acetate energy (kcal/g or kcal/mol).

εC_3: propionate energy (kcal/g or kcal/mol).

εC_4: butyrate energy (kcal/g or kcal/mol).

Determination of ammonia concentration and balance

To determine rumen ammonia (NH_3-N) concentration, 2.0 mL of centrifuged liquid of each flask were added to a test tube with 1 mL of 1 N of H_2SO_4 solution, and then analyzed through colorimeter, according to Kulasek (1972) and adapted by Foldager (1977). The balance was obtained by the difference of NH_3-N concentration between the 30 min incubated flasks with the non-incubated flasks. For a better interpretation, the balance data were estimated per hour (equation 6). By following this procedure, it was possible to evaluate whether the balance of ammonia production in the rumen was positive or negative.

NH_3-N balance (mg/dL.h)= [Conc. 30 min (mg/dL) – Conc. 0 min (mg/dL)] × 2 (6)

Where:
Conc. 30 min: NH_3-N concentration in incubated flasks.
Conc. 0 min: NH_3-N concentration in non-incubated flasks.

Protozoa counting

The rumen content for protozoa counting was collected simultaneously with that for rumen fermentation products at zero (0), 3, 6, 9, and 12 h after the morning meal. Equal portions of solid and liquid fractions were mixed and homogenized, then about 10 mL were inserted in flasks containing 20 mL of formaldehyde at 18.5%. Next, 1 mL of this content was stained for 4 h with two drops of 2% brilliant green. Afterward, 9 mL of glycerol at 30% were added. Then, the Neubauer Enhanced Bright-Line counting chamber (1 mL capacity) (Hausser Scientific Partnership®, Horsham, PA, USA) was filled and coupled to the optical microscope and 100 optical fields were counted according to Dehority (1993). Three genera of protozoa were identified: *Isotricha*, *Dasytricha*, *Entodinium*, and the subfamily *Diplodiniinae*.

Statistical analysis

The data were analyzed using Statistical Analysis System (SAS, 2013). First, they were evaluated to discrepant information (outliers) and normality of residues by the 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 dry matter intake (DMI), nutrient digestibility, and ruminal pH, the model included the treatment effect as a fixed effect and the animal and period effects as random effects. 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.

The data for CH_4 and SCFA production, NH_3-N concentration and balance and rumen protozoa counting were analyzed using the mixed model procedure (PROC MIXED) and to the model was added the factor "measures repeated over time", referring to the different sampling hours.

The analysis by the time was performed only when the interactions between time and treatment were significant. For the analyses, among the 15 different covariance structures were tested, and that which best fit the statistical model was chosen based on the lowest value of the Corrected Akaike Information Criterion (AICC) according to Wang and Goonewardene (2004). The comparison of means among treatments was performed using Pdiff test at a 5% significance level.

RESULTS AND DISCUSSION

The different ExE did not affect nutrient digestibility (P>0.05). On average, the digestibilities of DM, CP, NDF, NFC, EE and organic matter (OM) were 68%, 69%, 46%, 79%, 77, 70%, respectively, with total digestible nutrients (TDN) equal to 70% (Table 2). There was no effect (P>0.05) of enzymes on rumen pH profile, regardless of treatment. The cows had minimum, mean and maximum pH of 5.27, 6.0, and 6.78, respectively (Table 3). The time (min) in which the pH remained below 5.8, 6.0 and 6.2 and the pH area below 5.8, 6.0 and 6.2 were also not influenced by the treatments (P>0.05).

There was no effect of the different ExE on total and differential count of rumen protozoa (P>0.05) (Table 4). The genus *Entodinium* represented 97.41% of the total protozoa in the rumen and was present in higher concentrations in all treatments, representing an average concentration of 443.88 × 10^3/mL.

The different ExE did not affect the concentration or production of rumen NH_3-N (Table 5); the mean rumen nitrogen in the flasks before incubation (T_0) was 14.83 mg/dL and after incubation (T_{30}) was 16.63 mg/dL.

There was no effect (P>0.05) of the different ExE on CH_4 production (g/kg/day) or concentration (mmol/L) as well as on the relative energy loss (REL) of CH_4 concering the other rumen fermentation products (Table 6). The average CH_4 production was 28.25 g/kg/day and for REL was 20.70%.

However, the addition of enzymes significantly affected the production of propionate, acetate and total SCFA when compared to the control diet.

The enzyme pool treatment showed higher production of acetate (P<0.05) when compared to control, amylase and xylanase treatments, but did not differ (P>0.05) from the diet that included C + P; accordingly, increased feed gross energy (GE) (kcal/kg/day) released in the form of acetate.

Table 2 Dry matter intake and nutrient digestibility of basal diet in cows fed different exogenous 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
Digestibility							
DM	65.27	67.72	67.65	68.53	68.84	0.606	NS
CP	65.58	69.44	67.92	74.02	70.04	1.294	NS
EE	72.34	77.62	79.71	75.95	81.85	1.359	NS
NDF	39.24	43.02	47.17	51.41	50.59	1.746	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
TDN	67.15	69.60	69.64	70.45	71.45	0.583	NS

DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; NFC: non-fiber carbohydrates; OM: organic matter and TDN: total digestible nutrients
C + P: cellulase + protease and Pool: amylase + xylanase + (C+P).
SEM: standard error of the means.
NS: non significant.

Table 3 The ruminal pH of Holstein cows fed different exogenous enzymes

Variable	Treatments					SEM	P-value
	Control	Amylase	Xylanase	C + P	Pool		
Rumen pH (day)							
Minimum	5.25	5.41	5.13	5.38	5.19	0.06	NS
Mean	5.98	6.16	5.95	6.01	5.89	0.06	NS
Maximum	6.68	6.95	6.71	6.87	6.69	0.06	NS
pH time (min/day)							
Below 5.8	572	388	562	496	635	67.44	NS
Below 6.0	742	556	722	708	875	67.54	NS
Below 6.2	926	742	860	942	1132	72.34	NS
pH area (h.pH/day)							
Below 5.8	2.47	1.88	4.47	2.14	3.02	0.61	NS
Below 6.0	4.65	3.44	6.61	4.21	5.54	0.80	NS
Below 6.2	7.43	5.61	9.24	6.91	8.88	0.99	NS

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

Table 4 Total and differential count of protozoa in cows fed different exogenous enzymes

Variables	Treatments					SEM	P-value		
	Control	Amylase	Xylanase	C + P	Pool		Treat.	Time	Interaction[1]
Protozoa ($\times 10^3$/mL)									
Dasytricha	2.88	4.00	3.22	3.65	2.88	0.228	NS	NS	NS
Diplodiniinae	6.67	7.15	5.62	8.06	8.21	0.423	NS	NS	NS
Entodinium	399.5	460.5	429.5	451.7	478.2	14.24	NS	NS	NS
Isotricha	0.960	1.344	1.488	1.056	1.488	0.173	NS	NS	NS
Total	410.0	473.0	439.8	464.4	490.8	14.59	NS	NS	NS
Protozoa (%)									
Dasytricha	0.774	0.898	0.849	0.870	0.622	0.061	NS	NS	NS
Diplodiniinae	1.570	1.563	1.337	1.639	1.565	0.074	NS	NS	NS
Entodinium	97.44	97.29	97.49	97.28	97.54	0.110	NS	NS	NS
Isotricha	0.205	0.249	0.313	0.202	0.266	0.031	NS	NS	NS

[1] Interaction between treatment and time.
C + P: cellulase + protease and Pool: amylase + xylanase + (C+P).
SEM: standard error of the means.
NS: non significant.

Table 5 Concentration and balance of ruminal ammonia nitrogenin of cows fed different exogenous enzymes

Variables	Treatments					SEM	P-value		
	Control	Amylase	Xylanase	C + P	Pool		Treat.	Time	Interaction[1]
NH_3-N concentration									
0 min (mg/dL)	14.16	14.76	15.20	14.75	15.30	0.483	NS	NS	NS
30 min (mg/dL)	16.11	16.62	17.13	16.42	16.86	0.525	NS	NS	NS
NH_3-N balance									
mg/dL.h	3.89	3.72	3.85	3.34	3.12	0.291	NS	NS	NS

[1] Interaction between treatment and time.
NH_3-N balance= (30 min–0 min) × 2.
C + P: cellulase + protease and Pool: amylase + xylanase + (C+P).
SEM: standard error of the means.
NS: non significant.

Table 6 Methane and short-chain fatty acid (SCFA) production as well as REL of cows fed different exogenous enzymes

Variables	Treatments					SEM	P-value		
	Control	Amylase	Xylanase	C + P	Pool		Treat.	Time	Interaction
Acetic acid									
0 min (mmol/L)	72.71	66.95	67.39	69.28	69.32	0.702	NS[3]	NS	NS
30 min (mmol/L)	77.30	71.92	71.42	74.20	76.02	0.747	NS	NS	NS
Difference (mmol/L)	4.58^{bc}	4.96^{bc}	4.03^{c}	5.52^{ab}	6.70^{a}	0.250	0.0033	NS	NS
Production (g/kg.day)	185.6^{bc}	210.3^{bc}	167.9^{c}	239.4^{ab}	282.3^{a}	10.695	0.0146	NS	NS
Gross energy (GE, kcal/kg.day)	647.8^{bc}	733.9^{bc}	586.0^{c}	835.6^{ab}	985.3^{a}	37.32	0.0146	NS	NS
Propionic acid									
0 min (mmol/L)	20.70	18.64	19.44	18.36	18.73	0.428	NS	NS	NS
30 min (mmol/L)	22.29	20.57	21.12	20.38	21.06	0.451	NS	NS	NS
Difference (mmol/L)	01.59	01.94	01.67	02.02	02.32	0.081	NS	NS	NS
Production (g/kg.day)	79.71^{c}	101.8^{abc}	87.61^{bc}	106.9^{ab}	121.5^{a}	4.334	0.0107	NS	NS
GE (kcal/kg.day)	396.90	507.30	436.30	532.60	605.00	21.585	NS	NS	NS
Butyric acid									
0 min (mmol/L)	11.62	10.24	10.14	10.82	10.39	0.271	NS	NS	NS
30 min (mmol/L)	13.03	11.66	11.29	12.21	12.00	0.298	NS	NS	NS
Difference (mmol/L)	1.47	1.43	1.13	1.39	1.62	0.056	NS	NS	NS
Production (g/kg.day)	87.6	89.2	69.7	87.3	100.4	3.386	NS	NS	NS
GE (kcal/kg.day)	521.9	531.3	415.9	520.3	598.5	20.184	NS	NS	NS
Total SCFA									
0 min (mmol/L)	105.03	95.83	96.99	98.46	98.44	1.298	NS	NS	NS
30 min (mmol/L)	112.63	104.16	103.84	107.39	109.08	1.379	NS	NS	NS
Difference (mmol/L)	7.65^{bc}	8.33^{bc}	6.84^{c}	8.94^{ab}	10.64^{a}	0.350	0.0011	NS	NS
Production (g/kg.day)	352.9^{bc}	401.3^{bc}	325.3^{c}	433.7^{ab}	504.2^{a}	16.45	0.0020	NS	NS
GE (kcal/kg.day)	1566.8^{b}	1772.6^{ab}	1438.3^{c}	1888.6^{ab}	2188.8^{a}	69.525	0.0020	NS	NS
Methane									
0 min (mmol/flask)	0.022	0.022	0.022	0.023	0.023	0.0004	NS	0.0059	NS
30 min (mmol/flask)	0.093	0.085	0.082	0.089	0.096	0.0015	NS	0.0030	NS
Difference (mmol/flask)	0.070	0.064	0.060	0.066	0.074	0.0014	NS	0.0110	NS
Production (g/kg.day)	28.49	27.72	25.65	28.59	30.82	0.5819	NS	0.0028	NS
GE (kcal/kg.day)	374.8	364.8	351.3	376.2	405.5	7.1230	NS	0.0028	NS
REL[1] (%)	21.80	20.48	22.78	19.57	18.88	0.6628	NS	NS	NS

[1] REL: relative energy loss of methane concering the other rumen fermentation products.
C + P: cellulase + protease and Pool: amylase + xylanase + (C+P).
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.
NS: non significant.

For the production of propionate, the pool treatment presented higher production (P<0.05) when compared to control and xylanase treatments (but did not differ (P>0.05) from the diets that included C + P and amylase), though no effect was observed on GE released in the form of this SCFA. The different treatments did not affect (P>0.05) the production of butyrate. The concentration and production of total SCFA were higher in enzyme pool treatment (P<0.05) when compared to control, xylanase and amylase treatments but did not differ when compared to C + P diet.

Exogenous enzymes, acting together with enzymes produced by rumen microorganisms, potentiate the degradation of DM and nutrients such as structural carbohydrates (Beauchemin *et al.* 2003; Mocherla *et al.* 2017; Elsiddig, 2019). Some studies have shown that addition of EFE in ruminant diet promotes 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 the digestive enzymes in diet of steers on DM, CP and NDF digestibility. The total tract digestibility of DM, NDF and CP was also unaffected in Ran *et al.* (2019) study. Giraldo *et al.* (2008) found no effect on diet digestibility when EFE 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). Moharrery (2014) conducted an *in vitro* study adding fibrolytic enzymes directly to the ruminal fluid and observed no significant increase on DM or cellulose degradation. 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).

Eun and Beauchemin (2005) evaluated the effects of a proteolytic feed enzyme on feed intake, digestion, rumen fermentation, and milk production and observed an increased total-tract digestibility of DM, CP, and NDF. Rojo *et al.* (2015) evaluated the influence of cellulase addition to dairy goat diets on digestion and fermentation and observed a greater digestibility of DM 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 digestibility of DM and starch, but protease did not have additional benefits on nutrient digestibility. Song *et al.* (2018), evaluating the effects of EFE 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.

Rumen pH is a potential parameter which affects rumen digestibility of nutrients. Adequate rumen pH maintenance is a characteristic generally determined by the type of diet. The pH 5.8 indicates the threshold for cases of sub-acute rumen acidosis (Penner *et al.* 2007) and the pH 6.0 and 6.2 are thresholds indicative of healthy conditions favouring a better cellulolytic activity (Penner and Beauchemin, 2010). Although there were no differences among treatments on rumen pH, the mean pH observed in the present study was about 6, indicating great conditions to enhance fiber digestibility which was about 46.29% for NDF, but with a tendency to increase in all diets which included enzymes (Table 2). Similarly, no treatment effect was found on rumen pH in the study conducted by Silva *et al.* (2016) when included xylanase in diets for dairy cows.

The hypothesis when this study was carried out was that the use of ExE such as xylanase and cellulase would improve feed digestibility but such impact was not observed. Beauchemin *et al.* (2003) reported potential increases in voluntary feed intake due to improvements in rumen fiber digestion, increasing feed passage rate through digestive tract by EFE supplementation. Nonetheless, different studies have shown inconsistent effects of ExE on rumen DM and nutrient degradation. The review by Mocherla *et al.* (2017) about the effects of ExE on rumen digestion found that the function of ExE varies with various factors, which is the reason why various contradicting results were reported. According to Tirado-González *et al.* (2017), the response of ExE may depend upon the mixture of 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).

Ammonia production in rumen generally exceeds the use capacity by rumen microorganisms, resulting in accumulation and subsequent absorption and conversion to urea by the liver (Rodrigues, 2016). The efficiency of microbial protein synthesis is one of the most critical factors to reduce the concentration of ammonia in the rumen and it can be improved by diets with high total digestible nutrients to supply the energy required for bacterial activity (Seo *et al.* 2010). The use of different ExE in the present study did not impact the production or concentration of rumen NH_3-N. Peters *et al.* (2010) found no effect of EFE on rumen NH_3-N in dairy cows; equally, Elghandour *et al.* (2016) observed no effect of EFE in *in vitro* rumen fermentation study on the production and concentration of rumen NH_3-N. Although evaluating the effects of increasing doses of xylanase in dairy cows, Silva *et al.* (2016) observed a negative quadratic effect on rumen NH_3-N concentration. Salem *et*

al. (2013), using a combination of different ExE in cross-bred steers, observed increased concentrations of rumen NH_3-N.

The concentration of *Entodinium* protozoa found in this study (97.41%) corroborates with Dehority (2003) and Perna Junior *et al*. (2017) and it may be characteristic of diet (70% of concentrate) as, according to Brown *et al.* (2006), in high concentrate diets this genus can be represented in more than 90%. Avellaneda *et al*. (2009) found no effect of EFE on rumen protozoa counts in Suffolk lambs. Although there was no effect of treatments on rumen protozoa count in the present study, it is widely known that the reduction of protozoa in the rumen may contribute to the reduction of enteric CH_4 production as methanogens (*archaea*) can be found closely associated with ciliate protozoa, adhering to their cell surface or in intracellular medium (Finlay *et al*. 1994; Patra and Saxena, 2011).

The application of ExE is pointed to impact rumen fermentation depending on the substrate fermented and the amount of enzyme added (Elghandour *et al*. 2016; Abid *et al*. 2019; Meschiatti *et al.* 2019; Toseti *et al.* 2020; Amaro *et al.* 2021) and can lead to a greater total SCFA production in rumen (Silva *et al*. 2016). This study was carried out hypothesising that adding different ExE in ruminant diets would ameliorate feed value by enhancing nutrient digestibility and ruminal fermentation by increasing total SCFA production and, accordingly, reducing the REL of CH_4 concering the other rumen fermentation products (acetate, propionate and butyrate). Comparing the different ExE used in the present study, the combination of enzymes (enzyme pool) and cellulase + protease were the only treatments that showed a significant effect on increasing the production of acetate and propionate with a consequent increase in the total SCFA production. However, amylase and xylanase appeared to have no effect. Methane production (g/kg/day) or concentration (mmol/L) was not influenced by the use of different ExE or their combination; nonetheless, the enzyme pool had the highest total SCFA production followed by the diet containing cellulase + protease, but even though the reduction in REL was not significant (but there was a tendency for enzyme combination), showing that the feed value did not improve by the use of different ExE. Among all treatments, the enzyme combination was more robust in increasing the concentration and production of acetate and total SCFA and the consequent increase in the gross energy released in the form of these SCFA, showing the most significant effect of all treatments. In their study, Silva *et al.* (2016), observed a linear increasing effect on acetate, propionate, butyrate and total SCFA production but there was no effect on the concentration. In ExE combination study of Salem *et al*. (2013), an increase in total SCFA production was also observed. Using different levels of EFE for lactating Holstein cow diets, Chung *et al*. (2012) observed a linear increased enteric CH_4 production, but using a multi-enzyme in an *in vitro* study, Faramarzi-Garmroodi *et al*. (2016) found no effect on CH_4 production.

The hypothesis for carrying out this study was based on the fact that many studies, such as Carulla *et al*. (2005), Animut *et al*. (2008), Tiemann *et al*. (2008) and Jayanegara *et al*. (2012), have shown that factors that induce reduced enteric CH_4 production are generally associated with reduced fiber digestion, as fiber digestion is directly proportional to the production of enteric CH_4. This fact prompted the thought that the use of ExE (such as EFE) may not reduce CH_4 production, but because digestive enzymes increase nutrient use by increasing nutrient digestibility, they lead to greater SCFA production and, in this case, the relative production of CH_4 may be reduced, i.e., the REL of CH_4 concering the other rumen fermentation products may be lower.

Different studies have shown inconsistent results on effects of ExE on nutrient degradation and ruminal fermentation. Some studies, including this study, show lack or little effect of ExE, others show different effects (Golder *et al*. 2019); however, some factors which contribute to the type or magnitude of response of these enzymes are pointed out. Many studies, such as Bowman *et al*. (2002), Beauchemin *et al*. (2003), Elghandour *et al*. (2016), Mocherla *et al*. (2017), Tirado-González *et al.* (2017) and Abid *et al.* (2019), have concluded that the response of ExE may depend on the mixture of enzymes, the diet composition, enzyme products, dosage, and the method of enzyme application. 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.

CONCLUSION

The utilization of different exogenous enzymes or their combination in cows' feeding did not affect on nutrient digestibility, rumen pH, rumen protozoa counts, or rumen ammonia and methane production. Nonetheless, the enzyme combination and cellulase + protease increased total SCFA production but the relative energy loss of methane concering the other rumen fermentation products did not change. Therefore, the supplementation of different exogenous enzymes or their combination in Holstein cow diets did not significantly improve feed value, but the enzyme combination has shown a tendency to reduce the relative

energy loss of methane. Further studies are recommended specially those which give much emphasis on the method of enzyme application and optimum dosage.

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REFERENCES

Abid K., Jabri J., Beckers Y., Yaich H., Malek A., Rekhis J. and Kamoun M. (2019). Effects of exogenous fibrolytic enzymes on the ruminal fermentation of agro-industrial by-products. *South African J. Anim. Sci.* **49,** 612-618.

Amaro F.X., Kim D., Agarussi M.C.N., Silva V.P., Fernandes T., Arriola K.G., Jiang Y., Cervantes A.P., Adesogan A.T., Ferraretto L.F., Yu S., Li W. and Vyas D. (2021). Effects of exogenous α-amylases, glucoamylases, and proteases on ruminal *in vitro* dry matter and starch digestibility, gas production, and volatile fatty acids of mature dent corn grain. *Transl. Anim. Sci*. **5,** 1-16.

Animut G., Puchala R., Goetsch A.L., Patra A.K., Sahlu T., Varel V.H. and Wells J. (2008). Methane emission by goats consuming diets with different levels of condensed tannins from Lespedeza. *Anim. Feed Sci. Technol.* **144,** 212-227.

AOAC. (1995). Official Methods of Analysis. 12th Ed. Association of Official Analytical Chemists, Arlington, Washington, DC., USA.

AOAC. (1990). Official Methods of Analysis. Vol. I. 15th Ed. Association of Official Analytical Chemists, Arlington, VA, USA.

AOCS. (2005). Official methods and recommended practices of the AOCS. 7th Ed. The American Oil Chemists' Society, Boulder, IL., USA.

Avellaneda J.H., Pinos-Rodríguez J.M., González S.S., Bárcena R., Hernández A., Cobos M., Hernández D. and Montañeze O. (2009). Effects of exogenous fibrolytic enzymes on ruminal fermentation and digestion of Guinea grass hay. *Anim. Feed Sci. Technol.* **149,** 70-77.

Beauchemin K.A., Colombatto D., Morgavi D.P. and Yang W.Z. (2003). Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *J. Anim. Sci*. **81,** 37-47.

Bowman G.R., Beauchemin K.A. and Shelford J.A. (2002). The proportion of the diet to which fibrolytic enzymes are added affects nutrient digestion by lactating dairy cows. *J. Dairy Sci*. **85,** 3420-3429.

Brown M., Ponce C. and Pulikanti R. (2006). Adaptation of beef cattle to high-concentrate diets: Performance and ruminal metabolism. *J. Anim. Sci*. **84,** 25-33.

Carega M.F.C.S. and Dantas A. (2017). Metano ruminal e o uso de taninos condensados como estratégia de mitigação. *Nucl. Anim.* **9,** 51-64.

Carulla J.E., Kreuzer M., Machmuller A. and Hess H.D. (2005). Supplementation of *Acacia mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. *Australian J. Agric. Res.* **56,** 961-970.

Chung Y.H., Zhou M., Holtshausen L., Alexander T.W., McAllister T.A., Guan L.L., Oba M. and Beauchemin K.A. (2012). A fibrolytic enzyme additive for lactating Holstein cow diets: Ruminal fermentation, rumen microbial populations, and enteric methane emissions. *J. Dairy Sci*. **95,** 1419-1427.

Conceição D., Jacques R., Bento F., Simonetti A., Selbach P. and Camargo F. (2007). Redução de cromo hexavalente por bactérias isoladas de solos contaminados com cromo. *Ciência Rural.* **37,** 1661-1667.

Dehority B.A. (1993). Laboratory Manual for Classification and Morphology of Rumen Ciliate Protozoa. CRC Press, Inc., Boca Raton, Florida.

Dehority B.A. (2003). Rumen Microbiology. Nottingham University Press, Nottingham, United Kingdom.

Devant M., Yu S., Genís S., Larsen T. and Wenting L. (2020). Effects of exogenous glucoamylase enzymes alone or in combination with a neutral protease on apparent total tract digestibility and feces D-lactate in crossbred angus bulls fed a ration rich in rolled corn. *Animals*. **10,** 1-8.

Elghandour M.M.Y., Kholif A.E., Hernández J., Mariezcurrena M.D., López S., Camacho L.M., Márquez O. and Salem A.Z.M. (2016). Influence of the addition of exogenous xylanase with or without pre-incubation on the *in vitro* ruminal fermentation of three fibrous feeds. *Czech J. Anim. Sci.* **61,** 262-272.

Elsiddig M.A. (2019). A review on effects of exogenous fibrolytic enzymes in ruminant nutrition. *Merit Res. J. Agric. Sci. Soil Sci.* **7,** 170-176.

Encinas C.M.A., Villalobos G.V., Viveros J.D., Flores G.C., Almora E.A. and Rangel F.C. (2018). Animal performance and nutrient digestibility of feedlot steers fed a diet supplemented with a mixture of direct-fed microbials and digestive enzymes. *R. Bras. Zootec.* **47,** 1-7.

Environmental Protection Agency (EPA). (2012). Global Anthropogenic Non-CO_2 Greenhouse Gas Emissions: 1990-2030. Washington, DC., USA.

Erwin E.S., Marco G.J. and Emery E.M. (1961). Volatile fatty acids analyses of blood and rumen fluid by gas chromatography. *J. Dairy Sci*. **44,** 1768-1771.

Eun J.S. and Beauchemin K.A. (2005). Effects of a proteolytic feed enzyme on intake, digestion, ruminal fermentation, and milk production. *J. Dairy Sci.* **88,** 2140-2153.

Faramarzi-Garmroodi A., Danesh Mesgaran M. and Vakili A.R. (2016). *In vitro* estimation of microbial nitrogen production and ruminal fermentation responses to levels and applying duration of a commercial enzyme mixture. *Iranian J. Appl. Anim. Sci*. **6**, 87-93.

Finlay B.J., Esteban G., Clarke K.J., Williams A.G., Embley T.M.

and Hirt R.P. (1994). Some rumen ciliates have endosymbiotic methanogens. *FEMS Microbiol. Lett*. **117,** 157-162.

Foldager J. (1977). Protein requirement and non protein nitrogen for high producing cow in early lactation. Ph D Thesis. Michigan State Univ., East Lasing, Michigan.

Giraldo L.A., Tejido M.L., Ranilla M.J., Ramos S. and Carro M.D. (2008). Influence of direct-fed fibrolytic enzymes on diet digestibility and ruminal activity in sheep fed a grass hay-based diet. *J. Anim. Sci*. **86,** 1617-1623.

Golder H.M., Rossow H.A. and Lean I.J. (2019). Effects of in-feed enzymes on milk production and components, reproduction, and health in dairy cows. *J. Dairy Sci.* **102,** 8011-8026.

IPCC. (2007). Climate Change 2007: The Physical Science Basis. In Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom.

IPCC. (2014). Climate change 2014: Mitigation of Climate Change. Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom.

Jayanegara A., Leiber F. and Kreuzer M. (2012). Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from *in vivo* and *in vitro* experiments. *J. Anim. Physiol. Anim. Nutr*. **96,** 365-375.

Kaminski M., Kartanowics R., Jastrzebski D. and Kaminski M.M. (2003). Determination of carbon monoxide, methane and carbon dioxide in refinery hydrogen gases and air by gas chromatography. *J. Chromatogr. A*. **989,** 277-283.

Kulasek G.A. (1972). A micromethod for determination of urea in plasma, whole blood and blood cells using urease and phenol reagent. *Polish Arch. Weter*. **15,** 801-810.

Lynch J. (2019). Availability of disaggregated greenhouse gas emissions from beef cattle production: A systematic review. *Environ. Impact Assess. Rev*. **76,** 69-78.

Meschiatti M.A.P., Gouvêa V.N., Pellarin L.A., Batalha C.D.A., Biehl M.V., Acedo T.S., Dórea J.R.R., Tamassia L.F.M., Owens F.N. and Santos F.A.P. (2019). Feeding the combination of essential oils and exogenous α-amylase increases performance and carcass production of finishing beef cattle. *J. Anim. Sci.* **97,** 456-471.

Mocherla V.A.N., Suryanarayana S. and Kavitha P. (2017). Role of exogenous fibrolytic enzymes in ruminant digestion-A review. *Int. J. Curr. Microbiol. Appl. Sci.* **11,** 1400-1408.

Moharrery A. (2014). Influence of fibrolytic enzymes on the *in vitro* hydrolysis and fermentation of different types of roughages treatment. *Iranian J. Appl. Anim. Sci*. **4,** 515-520.

Moya D., Mazzenga A., Holtshausen L., Cozzi G., González L.A., Calsamiglia S., Gibb D.G., Mcallister T.A., Beauchemin K.A. and Schwartzkopf-Genswein K. (2011). Feeding behavior and ruminal acidosis in beef cattle offered a total mixed ration or dietary components separately. *J. Anim. Sci*. **89,** 520-530.

Neumann M., Leão G.F.M., Vigne G.L.D., Dos Santos C.L., Venancio B.J. and Dochwat A. (2018). Xylanase - complex efficacy in high-energy diet for bulls finished in feedlot. *Act. Sci. Anim. Sci.* **40,** 1-7.

Patra A.K. and Saxena J. (2011). Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. *J. Food Sci. Agric.* **91,** 24-37.

Penner G.B. and Beauchemin K.A. (2010). Variation among cows in their susceptibility to acidosis: Challenge or opportunity. *Adv. Dairy Sci. Technol*. **22,** 173-187.

Penner G.B., Beauchemin K.A. and Mutsvangwa T. (2007). Severity of ruminal acidosis in primiparous Holstein cows during the periparturient period. *J. Dairy Sci*. **90,** 365-375.

Perna Junior F., Cassiano E.C.O., Martins M.F., Romero L.A., Zapata D.C.V., Pinedo L.A., Marino C.T. and Rodrigues P.H.M. (2017). Effect of tannins-rich extract from Acacia mearnsii or monensin as feed additives on ruminal fermentation efficiency in cattle. *Livest. Sci.* **203,** 21-29.

Peters A., Lebzien P., Meyer U., Borchert U., Bulang M. and Flachowsky G. (2010). Effect of exogenous fibrolytic enzymes on ruminal fermentation and nutrient digestion in dairy cows. *Arch. Anim. Nutr*. **64,** 221-237.

Popova M., Morgavi D.P. and Martin C. (2013). Methanogens and methanogenesis in the rumens and ceca of lambs fed two different high-grain-content diets. *Appl. Environ. Microbiol.* **79,** 1777-1786.

Ran T., Saleem A.M., Shen Y., Ribeiro Jr G.O., Beauchemin K.A., Tsang A., Yang W. and McAllister T.A. (2019). Effects of a recombinant fibrolytic enzyme on fiber digestion, ruminal fermentation, nitrogen balance, and total tract digestibility of heifers fed a high forage diet. *J. Anim. Sci*. **97,** 3578-3587.

Rodrigues P.H.M., Pinedo L.A., Solorzano L.A.R., Perna Junior F., Martins M.F., Castro A.L., Godoy G.L.A. and Marino C.T. (2012). Descrição da metodologia *ex-situ* de estudo da fermentação ruminal (micro-rúmen) com vista à mensuração da produção de metano. Pp. 55-59 in Annu. Meet. Basileira Soc. Zootech., Brasilia DF.

Rodrigues P.H.M. (2016). Control and manipulation of ruminal fermentation. Pp. 157-188 in Ruminology. D.D. Millen, M.D.B. Arrigoni and R.D.L. Pacheco, Eds. Springer International Publishing, Switzerland.

Rojo R., Kholif A.E., Salem A.Z.M., Elghandour M.M.Y., Odongo N.E., Montes de Oca R., Rivero N. and Alonso M.U. (2015). Influence of cellulase addition to dairy goat diets on digestion and fermentation, milk production and fatty acid content. *J. Agric. Sci.* **153,** 1514-1523.

Salem A.Z.M., Gado H.M., Colombatto D. and Elghandour M.M.Y. (2013). Effects of exogenous enzymes on nutrient digestibility, ruminal fermentation and growth performance in beef steers. *Livest. Sci.* **154,** 69-73.

SAS Institute. (2013). SAS®/STAT Software, Release 9.3. SAS Institute, Inc., Cary, NC. USA.

Seo J.K., Yang J., Kim H.J., Upadhaya S.D., Cho W.M. and Ha J.K. (2010). Effects of synchronization of carbohydrate and protein supply on ruminal fermentation, nitrogen metabolism and microbial protein synthesis in Holstein steers. *Asian-Australasian J. Anim. Sci*. **23,** 1455-1461.

Shibata M. and Terada F. (2010). Factors affecting methane production and mitigation in ruminants. *J. Anim. Sci.* **81,** 2-10.

Silva T.H., Takiya C.S., Vendramini T.H.A., Ferreira de Jesus E.,

Zanferari F. and Rennó F.P. (2016). Effects of dietary fibrolytic enzymes on chewing time, ruminal fermentation, and performance of mid-lactating dairy cows. *Anim. Feed Sci. Technol*. **221,** 35-43.

Song S.D., Chen G.J., Guo C.H., Rao K.Q., Gao Y.H., Peng Z.L., Zhang Z.F., Bai X., Wang Y., Wanga B.X., Chen Z.H., Fu X.S. and Zhu W.L. (2018). Effects of exogenous fibrolytic enzyme supplementation to diets with different NFC/NDF ratios on the growth performance, nutrient digestibility and ruminal fermentation in Chinese domesticated black goats. *Anim. Feed Sci. Technol.* **236,** 170-177.

Sujani S. and Seresinhe R.T. (2015). Exogenous Enzymes in Ruminant Nutrition: A Review. *Asian J. Anim. Sci.* **3,** 85-99.

Tiemann T.T., Lascano C.E., Wettstein H.R., Mayer A.C., Kreuzer M. and Hess H.D. (2008). Effect of the tropical tannin-rich shrub legumes *Calliandra calothyrsus* and *Flemingia macrophylla* on methane emission and nitrogen and energy balance in growing lambs. *Animal.* **2,** 790-799.

Tirado-González D.N., Miranda-Romero L.A., Ruíz-Flores A., Medina-Cuéllar S.E., Ramírez-Valverde R. and Tirado-Estrada G. (2017). Meta-analysis: Effects of exogenous fibrolytic enzymes in ruminant diets. *J. Appl. Anim. Res.* **46,** 771-783.

Toseti L.B., Goulart R.S., Gouvêa V.N., Acedo T.S., Vasconcellos G.S., Pires A.V., Leme P.R., Netto A.S. and Silva S.L. (2020). Effects of a blend of essential oils and exogenous α-amylase in diets containing different roughage sources for finishing beef cattle. *Anim. Feed Sci. Technol.* **269,** 114643-114649.

Van Soest P.J., Robertson J.B. and Lewis B.A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysscharides in relation to animal nutrition. *J. Dairy Sci.* **74,** 3583-3597.

Vera J.M., Smith A.H., ZoBell D.R., Young A.J. and Eun J.S. (2012). Effects of an exogenous proteolytic enzyme on growth performance of beef steers and *in vitro* ruminal fermentation in continuous cultures. *Prof. Anim. Sci*. **28,** 452-463.

Wanapat M., Cherdthong A., Phesatcha K. and Kang S. (2015). Dietary sources and their effects on animal production and environmental sustainability. *Anim. Nutr.* **1,** 196-203.

Wang Z. and Goonewardene L.A. (2004). The use of MIXED models in the analysis of animal experiments with repeated measures data. *Canadian J. Anim. Sci.* **84,** 1-11.

Yang Y., Ferreira G., Corl B.A. and Campbell B.T. (2019). Production performance, nutrient digestibility, and milk fatty acid profile of lactating dairy cows fed corn silage- or sorghum silage-based diets with and without xylanase supplementation. *J. Dairy Sci.* **102,** 2266-2274.

Zilio E.M.C., Del Valle T.A., Ghizzi L.G., Takiya C.S., Dias M.S.S., Nunes A.T., Silva G.G. and Rennó F.P. (2019). Effects of exogenous fibrolytic and amylolytic enzymes on ruminal fermentation and performance of mid-lactation dairy cows. *J. Dairy Sci.* **102,** 4179-4189.