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Research Article
Effect of Exogenous Fibrolytic Enzymes on Ruminal Fermentation and Gas Production by RUSITEC, *in vitro* Abomasum and Ileum Digestibility

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Abstract

Background and Objective: Numerous *in vitro* studies have showed that exogenous fibrolytic enzyme can enhance fiber degradation of roughage. The aim of this study was to evaluate the effect of an Exogenous Fibrolytic Enzymes (EFE) product applied to a total mixed ration on ruminal fermentation, gas production and nutrient digestion *in vitro* whole digestive tract. **Materials and Methods:** The total mixed ration contained 55% concentrates, 22.5% corn silage and 22.5% alfalfa hay on a dry matter basis. The EFE multi enzyme feed additive powder produced from *Ruminococcus flavefaciens* was used in this study. Four levels of the EFE (0.0, 0.4, 0.8 and 1.2 g) were used E0, E2, E4 and E6, respectively, these levels of EFE were added directly into 20 g of diet in nylon bags and incubated in the RUSITEC for 48 h *in vitro* fermentation. Statistical analyses of continuous data were performed by use of SAS with repeated measures or Tukey's test. **Results:** Results of the rumen experiment showed no significant difference ($p>0.05$) in pH-values, dry matter, organic matter digestibility, crude protein digestibility, gas production, CH_4 and total volatile fatty acids production among all treatments in RUSITEC by using different EFE levels. The digestibility of neutral detergent fibre, acid detergent fiber and NH_3-N concentrations were increased ($p<0.05$) with increasing EFE levels. The *in vitro* abomasum and *in vitro* ileum digestibility experiment, the dry matter, organic matter digestibility and total volatile fatty acids were unchanged ($p>0.05$). NH_3-N concentrations were also increased ($p<0.05$) with increasing EFE levels. Moreover, in the *in vitro* abomasum experiment, the neutral detergent fibre digestibility and acid detergent fiber digestibility was increased ($p<0.05$) with increasing EFE levels. **Conclusion:** The results indicate that using high doses of EFE (VTR®) containing *Ruminococcus flavefaciens* in TMR (45:55) could improve the neutral detergent fibre digestibility and acid detergent fiber digestibility in rumen and small intestine.

Key words: Forages, exogenous fibrolytic enzymes, fibre degradation, feed intake

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The efficiency by which ruminants obtain energy from structural plant polysaccharides and, in turn, produce high quality meat and milk protein is increasingly important if the demands of an expanding human population are to be met[1]. Improving utilization of local forages would enhance production efficiency and reduce costs. Various strategies have been attempted to improve forage quality for ruminant livestock including treatment with physical agents such as heat, steam and pressure; with chemicals such as acids, alkalis and NH_3; with biological agents such as white rot fungi; via natural selection, breeding or molecular engineering and enzyme technology[2]. However, none of these methods is widely used for improving forage quality and ruminant animal performance.

Many studies have confirmed that the addition of exogenous fibrolytic enzymes to feeds can improve degradability of Dry Matter (DM) and Neutral Detergent Fibre (NDF) *in vitro*[2-4] and enhance digestion of forage and milk production in dairy cows[5]. For low quality forage, Bhasker *et al.*[6] showed that the application of exogenous fibrolytic enzymes improved *in vitro* Gas Production (GP) and degradation of cereal straws. Kholif *et al.*[7] reported a 28% improvement in Acid Detergent Fibre (ADF) digestibility when exogenous enzymes containing xylanase activity were added to a high-concentrate diet. Rajamma *et al.*[8] reported no effects when the same enzyme product was added to a high-grain barley-based feedlot finishing diet containing 17% forage on a dry matter basis. Although positive, as well as no effects were reported in the literature, results from research on the effects of exogenous fibrolytic enzymes on ruminant's diets are variable and not conclusive[9].

Although the reasons for this discrepancy are unknown, it could be due to differences in enzyme activity, application rate and composition, type of diet fed to the animals, physiological stage of the animal, time of enzyme delivery, ruminal activity and enzyme stability, enzyme feed specificity and the portion of the diet to which enzymes are applied[10]. The hypothesis was that the dietary application of selected EFE could act synergistically to benefit rumen fermentation that enhance abomasum and ileal digestibility. The objectives of this study were to assess the effects of three doses of EFE (VTR®) on the *in vitro* ruminal fermentation, methane emissions and total tract digestibility of a TMR with 45:55 forages:concentrate ratio (F:C).

MATERIALS AND METHODS

This study was approved by the Animal Care and Use Committee of the College of Animal Science and Technology of the Northwest Agriculture and Forestry University Shaanxi province, Yangling, People's Republic of China. The experiment was conducted during the autumn 2016.

Apparatus and animals: This study was carried out by using the rumen simulation technique (RUSITEC) (Sanshin Co. Ltd., Tokyo, Japan). According to the method described by Kajikawa *et al.*[11], the inoculum used in the fermenters was obtained from three rumen cannulated goats (40 kg mean body weight). Rumen content was collected through the ruminal fistula before morning feeding and strained through four layers of cheese cloth to separate the liquid and solid fractions. On the first day, each fermenter was filled with 350 mL of warmed buffer, 350 mL of rumen fluid, one bag containing 70 g of wet solid rumen digesta and one bag containing 20 g DM of dietary substrate. The buffer was modified according to McDougall[12]. For the preparation of McDougall's buffer 9.8 g of $NaHCO_3$, 9.25 g of $Na_2HPO_4 \cdot 12H_2O$, 0.57 g of KCl, 0.47 g of NaCl, 0.13 g of $MgCl_2 \cdot 6H_2O$ and 0.0455 g of $CaCl_2$ were dissolved in sufficient volume of distilled water to get 1 L solution. Chemicals were procured from (Jinhuada Chemical. Sci-tech Co., Ltd, Guangdong and Aoboxing Bio-tech CO., LED. Beijing, China). The bag containing the solid rumen digesta was removed after 24 h and a bag containing of dietary substrate was added. Thereafter, one bag was replaced daily in the morning so that each bag remained in the fermenter for 48 h.

Experimental diets, procedure and sampling: In this study, the single factor experimental design was used. The substrate consisted of 45% roughage and 55% concentrate (DM basis). The roughage contained alfalfa hay and corn silage (22.5:22.5%). The ingredients and the chemical composition of the diet are shown in Table 1. Before formulating the diet, roughage and concentrate were ground through 4 and 2 mm sieves, respectively. Both feed components were weighed independently and were carefully mixed before applying the experimental treatments. In the first experimental trial, all fermentation vessels were supplied with 11 g DM of concentrate and 9 g DM of roughage (4.5 g DM of corn silage: 4.5 g DM of alfalfa) hay, which was placed into nylon bags.

In 4 treatments, the doses of enzymes inclusion were on (% DM); 0, 0.4, 0.8 and 1.2 g in replication (E0, E2, E4 and E6,

Table 1: Ingredient Composition of The Concentrate Supplement (DM basis)

Items	Percentage
Corn	50.00
Soybean meal	9.73
Wheat bran	18.00
Cottonseed meal	11.00
Corn germ	7.00
Calcium carbonate	1.00
Calcium hydrogen phosphate	1.12
Salt (NaCl)	1.00
Mineral-vitamin premix*	0.35
Sodium carbonate	0.80
Chemical composition of total mixed ration (DM%)#	
DM	89.70
OM	93.48
CP	16.50
NDF	58.92
ADF	32.95

*Vitamin-mineral mix contained kg^{-1}: 450 mg of nicotinic acid, 600 mg of Mn, 950 mg of Zn, 430 mg of Fe, 650 mg of Cu, 30 mg of Se, 45 mg of I, 20 mg of Co, 800 mg of vitamin E, 45 000 IU of vitamin D and 120 000 IU of vitamin A, #Total mixed ration contained (55% concentrates, 22.5% corn silage and 22.5% alfalfa hay), DM: Dry matter, OM: Organic matter, CP: Crude protein, NDF: Neutral detergent fiber, ADF: Acid detergent fibre

respectively). It was directly added into nylon bag with two replicates per treatment. The enzymes (fermentation extracts of *Ruminococcus flavefaciens*) powder preparations were procured from (Guangdong VTR (Veterinary Technology Research) Bio-tech Co., Ltd, Zhuhai, Guangdong, China). In the preliminary experiment, the enzyme mixture was assayed for several enzymatic activities and was found to contain (units g^{-1} of enzyme preparation) 17500 units of β-glucanase, 2000 units of xylanase and 3500 units of cellulase activity.

Experimental treatments were randomly assigned to one of the eight fermenters. The experiment was conducted over two independents 15 days incubation periods, with 8 days for adaptation and 7 days for sample collection. Apparent ruminal fibre digestibility, total VFA, N_3H-N and Gas production were determined on days 8, 9 and 10 following the procedure of Zhao *et al*.[13].

On day 11, 4 mL of each fermenter fluid was collected at 0, 3, 6, 9 and 12 h after replacing the feedbag and the pH was measured immediately. On day 15, 20 mL of fermenter fluid as a liquid fraction were collected for the *in vitro* abomasum digestibility.

***In vitro* abomasum and ileum digestibility:** The three-step procedure of Boisen and Fernandez[14] was modified to 2-step thus using incubation of feed sample with pepsin for 60 min, followed by incubation with small intestinal fluid for 18 h, the *in vitro* abomasum digestibility of DM of the undigested RUSITEC residue was determined. Firstly, the RUSITEC undigested residue was weighed (2.40±0.05 g) into 100 mL bottles in three replicates for each treatment. The *in vitro* ileum digestibility of DM of the *in vitro* abomasum undigested residue was determined, using second step. Firstly, the abomasum-undigested residue was weighed (0.45±0.05 g) into 100 mL bottles in triplicate and 10 mL of abomasum fluid and 30 mL of artificial saliva[12] were added. Each experiment used 48 bottles. Insolubilized and precipitated materials were collected after filtration and then dried. The residues were analyzed for OM, NDF and ADF. The fluid was collected and frozen at -80°C for determination of VFA and the NH_3-N concentration. For Small Intestinal Fluid (SIF) collection, a sheep was slaughtered and immediately total small intestinal digesta (duodenum to ileum end) was collected according to method described by Liang[15].

Analytical procedures: Substrates and substrate residues after 48 h of incubation were dried at 65°C and ground through a 1 mm screen for chemical analysis. The DM, ash and N were determined according to AOAC[16]. The NDF and ADF content Van Soest *et al*.[17] of both feed and fermentation residues were determined using a fiber analyzer unit without use of an alpha amylase but with sodium sulphite. Total and individual VFA were calculated according to Jiao *et al*.[18]. NH_3-N in the samples was analyzed according to method described by Russi *et al*.[19]. The concentration of methane was analyzed by GC (Model 663-30; Hitachi High-Technologies Corporation, Tokyo, Japan).

Statistical analysis: Differences of *in vitro* measurements were analyzed by the PROC MIXED procedure (SAS Inc., Cary, NC, USA) version (6.2.9)[20] general linear model option of SPSS 17.0 (SPSS Inc., Chicago, IL, USA) procedure considering dose rate of administration as fixed effects in a completely randomized design. Differences between treatment means were separated by Tukey's test at 0.05 p level.

RESULTS

***In vitro* rumen digestibility:** In the present study, results showed that exogenous fibrolytic enzymes treatments did not effect on pH before replacing feed or average of the pH over 12 h after replacing feed ($p = 0.13$) (Table 2). The values of pH ranged from (6.2-7.2). No significant difference was observed in the apparent digestibility of DM, OM and CP among the treatments, while, the apparent digestibility of NDF and ADF were significantly ($p = 0.02$, $p<0.01$) upregulated in dose of EFE (Table 2).

Table 2: Effect of dietary Exogenous Fibrolytic Enzyme (EFE) addition on pH and apparent nutrient digestibility of the diet in the RUSITEC fermenters, *in vitro* abomasum and *in vitro* ileum digestibility (n = 4)

	Experimental treatments					
Items	E0	E2	E4	E6	SEM*	p-value
Rusitec fermenters						
pH before feeding	6.75	6.75	6.75	6.80	0.02	0.33
pH# (0-12 h)	6.78	6.80	7.00	6.82	0.07	0.13
Rumen apparent digestibility (DM%)						
DM	64.31^{ab}	66.96^{ab}	62.91^{b}	68.75^{a}	0.85	0.04
OM	67.17^{b}	69.51^{ab}	66.18^{b}	71.62^{a}	0.74	0.02
NDF	38.91^{b}	41.58^{b}	42.01^{b}	49.19^{a}	1.19	>0.01
ADF	9.57^{b}	11.52^{b}	11.80^{b}	17.12^{a}	0.82	>0.01
CP	67.23	69.60	65.56	60.65	0.75	0.05
***In vitro* abomasum digestibility (DM%)**						
DM	12.77	14.76	17.10	19.23	0.94	0.06
OM	12.27	14.55	17.01	17.77	1.10	0.29
NDF	17.70^{b}	18.92^{b}	18.35^{b}	23.11^{a}	0.65	>0.01
ADF	8.93^{b}	8.96^{b}	10.91^{ab}	12.31^{a}	0.47	0.01
***In vitro* ileum digestibility (DM%)**						
DM	55.31	51.83	54.88	51.71	0.76	0.18
OM	56.64	53.29	55.73	53.39	0.78	0.34
NDF	41.41	38.35	37.76	36.41	0.84	0.19
ADF	24.48^{ab}	23.75^{ab}	18.41^{b}	27.75^{a}	1.25	0.05

$^{a-c}$Within a row, means without a common superscript letter differ, $p \leq 0.05$ (Tukey's test), #Mean pH of values determined at 0, 3, 6,9 and 12 h after feeding, *SEM: Standard error of means, DM: Dry matter, OM: Organic matter, CP: Crude protein, NDF: Neutral detergent fiber, ADF: Acid detergent fibre

Table 3: Effect of dietary Exogenous Fibrolytic Enzyme (EFE) addition on molar proportions of Volatile Fatty Acids (VFA) and Ammonia-N in the RUSITEC fermenters (n = 4)

	Experimental treatments					
Items	E0	E2	E4	E6	SEM*	p-value
Total VFA (mmol L^{-1})	61.49	64.66	62.62	65.57	5.59	0.95
Individual VFAs (mol/100 mol)						
Acetate (A)	59.61	57.89	56.95	56.30	1.29	0.32
Propionate (P)	19.93	18.34	17.01	16.55	0.62	0.26
Butyrate	9.83	9.49	9.38	08.83	0.75	0.82
Ratio A:P	2.98	3.10	3.07	3.21	0.17	0.72
Methane/VFA (mol mol^{-1})	0.18	0.21	0.20	0.18	0.03	0.79
Ammonia-N (mg L^{-1})	22.77^{c}	25.60^{bc}	29.26^{b}	37.63^{a}	0.89	>0.01

$^{a-c}$Within a row, means without a common superscript letter differ, $p \leq 0.05$ (Tukey's test), *SEM: Standard error of means

As shown in Table 3, the NH_3-N was increased ($p<0.01$) with the addition of EFE when compared with the control. Moreover, dietary addition of EFE did not affect ($p>0.05$) the total and individual of volatile fatty acids in the RUSITEC.

The results of GP in fibrolytic enzyme treated group are presented in (Table 4). The GP production was found unchanged ($p>0.05$) in all treatment groups compared with control, indicating that dietary addition of EFE has no effect on CH_4 production ($p = 0.66$).

***In vitro* abomasum digestibility:** The nutrient digestibility coefficients of experimental ratios were presented in (Table 2). The NDF digestibility and ADF digestibility in the treatment groups were comparatively higher that of control treatment, while no significant difference was observed between treatment groups and control treatment for OM digestibility.

As shown in Table 5, there were comparatively no significant differences in total and composition of volatile fatty acids ($p>0.05$) of in treatment groups and control. Moreover, NH_3-N concentrations were significantly increased ($p = 0.05$) with increasing EFE level.

***In vitro* ileum digestibility:** The digestibility dry matter, organic matter and neutral detergent fiber were remained unaffected in all treatment groups ($p>0.05$) compared to control (Table 2). The NH_3-N concentration and acid detergent

Table 4: Effect of dietary Exogenous Fibrolytic Enzyme (EFE) addition on daily production and composition of Gas in the RUSITEC fermenters (n = 4)

	Experimental treatments					
Items	E0	E2	E4	E6	SEM*	p-value
GP# (mol d^{-1})	1442.30	1507.10	1644.10	1636.30	225.11	0.89
Hydrogen (%)	0.45	0.36	0.51	0.76	0.35	0.87
Methane (%)	11.10	11.12	11.29	9.72	0.98	0.66
Carbon dioxide (%)	41.22	40.68	40.71	43.84	5.91	0.51

[a-c]Within a row, means without a common superscript letter differ, $p \leq 0.05$ (Tukey's test), *SEM: Standard error of means, #GP: Gas production

Table 5: Effect of dietary EFE addition on molar proportions of Volatile Fatty Acid (VFA) in vitro abomasum digestibility (n = 4)

	Experimental treatments					
Items	E0	E2	E4	E6	SEM*	p-value
Total VFA (mmol L^{-1})	48.26	46.17	49.64	50.03	3.05	0.81
Individual (mol/100 mol)						
Acetate (A)	55.43	58.17	53.70	54.07	2.18	0.49
Propionate (P)	17.10	17.34	16.71	16.14	0.63	0.58
Butyrate	12.96	12.95	11.22	10.85	0.48	0.22
Ratio A:P	3.31	3.36	3.26	3.20	0.17	0.91
Ammonia-N (mg L^{-1})	10.97^{b}	12.82^{ab}	14.52^{ab}	16.42^{a}	1.21	0.05

[a-c]Within a row, means without a common superscript letter differ, $p \leq 0.05$ (Tukey's test), *SEM: Standard error of means

Table 6: Effect of dietary Exogenous Fibrolytic Enzyme (EFE) addition on molar proportions of Volatile Fatty Acids (VFA) *in vitro* ileum digestibility (n = 4)

	Experimental treatments					
Items	E0	E2	E4	E6	SEM*	p-value
Total VFA (mmol L^{-1})	23.74	22.95	24.70	23.55	0.46	0.13
Individual (mol/100 mol)						
Acetate (A)	72.22	73.27	73.11	73.72	0.92	0.71
Propionate (P)	11.44	11.30	11.89	11.96	0.38	0.36
Butyrate	9.04	8.77	8.15	8.19	0.51	0.56
Ratio A:P	6.31	6.69	6.14	6.27	0.21	0.33
Ammonia-N (mg L^{-1})	16.32^{c}	20.01^{b}	21.18^{ab}	22.09^{a}	0.36	>0.01

[a-c]Within a row, means without a common superscript letter differ, $p \leq 0.05$ (Tukey's test), *SEM, standard error of means

fibre were significantly ($p<0.05$) increased with increasing EFE level, while the addition of EFE did not affect the total VFA compared to control treatment (Table 6).

DISCUSSION

***In vitro* rumen digestibility:** The pH plays a vital function in conserving normal homeostasis of gastro intestinal tract. Optimal pH is necessary for normal digestion of nutrient. In current study, the pH and apparent digestibility of the diet was assessed by *in vitro* RUSITEC fermenter. The results showed that EFE treatments did not effect on pH before replacing feed or mean of the pH over 12 h after replacing feed. The values of pH ranged from (6.2-7.2). The exogenous fibrolytic enzymes were added with the speculation that the enzymes could enhance substrate degradation and enhance the utilization of the fiber portion of the diet.

Rumen pH value was not altered after addition with EFE when compared with the control. This might imply that the number of lactic acid utilizing bacteria has increased in the rumen, resulting in low concentration of lactic acid and high pH. This result was contrary to those Torres *et al.*[21] and Lu *et al.*[22]. Moreover, the inclusion of exogenous fibrolytic enzymes has improved the DM digestibility and OM digestibility when compared with the control. Which is in agreement with, Colombatto *et al.*[23] treated alfalfa stem with six levels (0, 0.51, 1.02, 2.55, 5.1 and 25.5 µL g^{-1} of alfalfa stem) of EFE and reported a linear increase in the *in vitro* DM and OM digestibility with increasing enzyme levels. Beauchemin *et al.*[24] suggested that the addition of EFE improved digestion and the colonization of ruminal microorganism of cell wall which promoted the utilization of cellulose by microorganism and increased the DM digestibility. On the contrary, in other studies it was proved the conclusions drawn by Lu *et al.*[22]. Vicini *et al.*[25] similarly reported no effect on DM digestibility and OM digestibility of high forage and low concentrate diet (60:40 ratio) treated with EFE.

Differences in DM and NDF rumen digestibility of a total mixed ration were observed between control and enzyme addition. Degradation of DM in the rumen was dependent on level of enzyme added. Exogenous fibrolytic enzymes have the ability to increase the initial rate but not the extent of DM digestion when used in ruminant diets. In this study, nutrient digestibility was similar among all treatments. When compared to control, it was observed that exogenous cellulase and xylanase supplementation had positive effects on apparent total tract digestibility of nutrients in rumen. Similarly, Gaafar *et al.*[26] reported that dietary inclusions of fibrolytic enzymes can increase nutrient digestibility for ruminants at a certain extent. On the contrary, Bhasker *et al.*[6] have similarly affirmed that supplementing exogenous fibrolytic enzymes did not alter the nutrient utilization in sheep fed on a maize Stover-based total mixed ration. Similarly, Lewis *et al.*[27] have demonstrated that the digestibility of NDF and ADF were not improved by feeding fibrolytic enzymes in dairy cows.

Mendoza *et al.*[28] suggested that the response to exogenous enzymes depends on the quality of feed, particularly the proportion of the NDF that is potentially digestible in the rumen These authors hypothesized that the addition of exogenous enzymes will improve the digestibility of forages that have high proportion of potentially digestible fraction. This hypothesis may explain well the observed positive effect of the enzyme product on the degradability of DM, NDF and ADF evaluated in this study. Furthermore, the differences might be related to the dosage of commercial enzymes used. As for N balance, it was noted that retained N and also the availability of retained N and digested N were increased at the low dose of EFE supplementation. Similarly, Chandra *et al.*[29] reported that dietary inclusion of cellulase and xylanase mixture at a low dose could improve nutrient digestibility in Murrah buffaloes receiving a total mixed ration containing wheat straw (45%), green maize (15%) and concentrate (40%).

Total VFA are the end-product of ruminal bacteria fermentation and represent the major provider of energy[30]. In current study, the total and individual volatile fatty acids concentration was not altered in response to EFE in the diets. The lack of response to addition of EFE has been noted for rumen fermentation[31,22]. Current results are also in agreement with Yang *et al.*[32] who noted no effects of EFE on ruminal fermentation. In versus, some experiments reported that EFE addition could increase the total volatile fatty acids concentration[6]. However, in the present study, the unchanged individual VFAs for using EFE was not expected, as the degradation was enhanced with increasing EFE. The lack of complete consistency between degradation and fermentation end products production might be due to the type of substrates, EFE sources and the efficiency of enzymes (i.e., ruminal enzyme and EFE) on microbial growth, which affect forage fibre utilization by Lu *et al.*[22].

The total VFA concentration, individual VFA molar proportion and ratio of acetate to propionate were not altered in response to EFE in the diets. The lack of response to addition of EFE has been reported for ruminal fermentation patterns of ruminants[21]. Results were likewise in agreement with Yang *et al.*[24] who reported no effects of fibrolytic enzymes on ruminal fermentation.

The NH_3-N concentration production in the effluent relies on the range of crud protein degradation and N uptake by rumen microorganism. NH_3-N concentration is fundamental for microbial protein synthesis as the favored and main N source of ruminal bacteria[33]. In current study, observed that the concentration of NH_3-N was significantly increased compared to the control group. Which might have that enhanced bacterial utilization of on nitrogen the results supported the findings of earlier study. Similar effect was reported by Rajamma *et al.*[8] reported elevated NH_3-N concentrations after addition of EFE in buffalo bulls. Arriola *et al.*[34] reported similar results after the addition of EFE in the ration of lactating dairy cattle. In contradiction, Gaafar *et al.*[26] who added EFE to mixed ration of lactating buffaloes.

Production of Gas (GP) was not significantly affected by the addition of EFE. This study results are corroborated with findings of Colombatto *et al.*[23] who noted no improvement in gas production for corn silage treated with EFE. Beauchemin *et al.*[24] stated that the lack of response to enzyme supplementation might be due to insufficient supply of enzymes. Some studies reported that the fibrolytic enzyme did not affect GP[35]. This seems to be dependent on many factors such as source, type and dose of enzyme, type of diets fed to the animals and enzyme application methods and method of administration[36].

Variability and lack of response from the EFE might have been influenced by several factors, including diet type and level of the enzyme activities provided, temperature and pH of the rumen environment for fibre digestion. Regarding the varying proportion of the diets, the readily fermentable portion of the diet may have altered the pH and reduced the enzymes for fibre digestion. Therefore, the effect of the EFE *in vitro* (RUSITEC) depend on the source, diet type and composition and the specific enzyme activities contained within the enzyme preparations.

The forage in diet was fermented by the microorganism in rumen, released H^+ and CO_2 provided the source to produce methane by methanogens[37]. Production of CH_4 was not affected after dietary addition of EFE in the rumen[23]. Moreover, McGinn *et al.*[38] reported no effect of EFE on NDF, ADF and CH_4 production in steers fed barley silage based diet. In the current study, the dietary addition of fibrolytic enzyme showed no significant effects on CH_4 production after 24 h of incubation. However, high doses of EFE enhanced the production of CH_4 when fed to lactating dairy cow[31]. The production of CH_4 was reported to be influenced by several factors, including composition of diet, ruminal pH and microorganism populations[39]. These contradictory results might be due to different fibrolytic enzymes used or different experimental conditions.

***In vitro* abomasum digestibility:** There are few reports on the effects of the addition of EFE in abomasum digestibility of nutrients. In the present study, there was no significant difference in the digestibility values of OM due to the increasing levels of EFE. On contrast, Lewis *et al.*[32] reported no effect of EFE on in situ digestibility of OM during the initial phase of digestion.

Moreover, the NH_3-N concentration was improved with increasing fibrolytic enzyme supplementation. According to Beauchemin *et al.*[40] EFE treatments were argued to increase the neutral detergent fiber. Therefore, the added EFE increased the digestion of NDF of substrate leads to release of more energy and ultimately improving microbial protein synthesis[41]. Avellaneda *et al.*[42] noted a significant improve ($p<0.05$) in NH_3-N concentration in sheep's fed on guinea grass addition with EFE. Changes in NH_3-N concentration were observed when EFE were added to feed suggesting that EFE may have affected microbial growth and activity.

***In vitro* ileum digestibility:** In the present study, ilial digestibility of DM and OM did not effected significantly. These results supported by Avellaneda *et al.*[42] who noted the lineal application of EFE thru a ruminal cannula to sheep's fed guinea grass hay did not affect total tract digestion. Our results were similarly agreed with Reddish and Kung Jr.[43] reported no effect of enzyme mixture on *in vitro* digestion of TMR even when added in high doses and also observed unaltered nutrient digestion in lambs fed diets treated with enzyme mixture. In the present study found that the EE levels had significant increase the digestibility of ADF. Krause *et al.*[44] reported 28% increase in ADF digestibility with increasing the EFE levels compared with the control treatment. Higher digestibility might be due to synergistic effect of enzyme on ruminal microflora.

Desirable effects of supplementation of fiber degrading enzymes to in diets leads to improved VFA production, which represents an increase in available energy. However, in the present study, non-significant differences were found in the total and individual VFA concentration in ileum digesta among the treatments. Nonetheless, our findings are corroborated with the results of Chung *et al.*[31] who noted a numeric but not significant increase in ileal concentration of total VFA after xylanase supplementation to wheat-based diets.

CONCLUSIONS

The addition of EFE did not affect DM digestibility, OM digestibility, CP digestibility, total VFA, GP and CH_4 production, while increasing the digestibility of, NDF, ADF and NH_3-N concentration and with increasing EFE treatments in the RUSITEC. Nevertheless, in the *in vitro* abomasum and *in vitro* ileum digestibility experiments, OM, DM digestibility and total VFA were not significantly affected with increasing EFE levels. However, the NH_3-N concentration and ADF digestibility was significantly increased with increasing EFE when compared to control. The results indicated that using low doses of EFE (VTR®) containing *Ruminococcus flavefaciens* in TMR (45:55) could improve the digestibility of DM, OM, NDF, ADF and in rumen. However, further research is needed to validate this method using an *in vivo* model.

SIGNIFICANCE STATEMENTS

This study discovers the effect of exogenous fibrolytic enzymes on ruminal fermentation that can be beneficial for forage utilization in rumen and small intestine. This study will help the other researchers to uncover the critical areas of improvements the nutrient digestibility-that many researchers were not able to explore. Thus, a new theory on additional Fibrolytic enzymes and possibly other related combinations, could be of significant importance.

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