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The potential of rumen fluid waste from slaughterhouses as an environmentally friendly source of enzyme additives for ruminant feedstuffs

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ABSTRACT

Rumen fluid disposal from slaughterhouses represents a major environmental challenge due to the presence of high levels of ammonia and phosphorus. The loading of these limiting nutrients into the soil and aquatic systems triggers eutrophication. Fortunately, the fluid is also rich in fibrolytic and other enzymes that could, alternatively, be used to enhance feed utilization in animals. Therefore, the aim of this study was to evaluate rumen fluid as a potential source of exogenous feed enzymes using a variety of test substrates. Hydrolytic enzyme activities of carboxymethyl cellulase, α-amylase and microcrystalline cellulase (avicellase) were measured in the rumen fluid to determine its enzymatic capabilities. Centrifuged and sonicated rumen fluid was used to pre-treat milled corn grain, barley grain, soybean meal, common vetch grain, bitter vetch grain, chickling vetch grain, alfalfa hay and common vetch straw substrates at 0, 1, 2, 3, or 4 mL per 100 g dry matter. Rumen fluid-treated substrates were subjected to a water solubility test and *in vitro* ruminal fermentation. The activities of carboxymethyl cellulase, avicellase and amylase were observed to be 377.8, 333.4 and 282 U/mL, respectively. Water solubility of dry matter in bitter vetch grain, chickling vetch grain, common vetch grain and soybean meal increased linearly with level of rumen fluid treatment. The highest solubility was observed in substrates treated with 4 mL rumen fluid per 100 g dry matter ($P < 0.05$). With the exception of common vetch grain, other feeds had the highest ($P < 0.05$) biogas production at 24 and 48 h when pre-treated with 3 and 4 mL rumen fluid per 100 g dry matter. Pre-treatment of feeds with rumen fluid significantly ($P < 0.05$) improved total fermentable fraction of corn grain, bitter vetch grain, chickling vetch grain, alfalfa hay and common vetch straw. However, digestible organic matter and metabolizable energy of common vetch grain were not influenced by rumen fluid pre-treatment. These results showed that rumen fluid has the potential to be used as an environmentally friendly source of exogenous feed enzymes that enhance feed utilization in ruminants.

1. Introduction

Rumen fluid contains high levels of ammonia and phosphorus thus its disposal from slaughterhouses causes environmental pollution. These rumen fluid nutrients cause eutrophication when discarded into the soil and waterways. It is, therefore, important to find viable alternative uses of rumen fluid from slaughterhouses in order to prevent environmental pollution.

Rumen fluid contains microbial enzymes such as xylanase, galactosidase, cellulase, hemicellulase and α-amylase that breakdown complex carbohydrates (Church, 1979). There is potential to exploit the enzymatic activity of rumen fluid as an alternative to conventional feed enzymes. Such an approach would contribute to sustainable, environmentally friendly animal production practices. Conventional feed enzymes are commonly added to animal diets to

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improve feed digestibility, rumen health, and production efficiency at a huge cost. Indeed, Alsersy et al. (2015) and Vallejo et al. (2016) reported an improvement in ruminal degradation of fibrous feeds pre-treated with conventional exogenous fibrolytic enzymes. Fibrous feeds contain high concentrations of hemicellulose and cellulose that limit their digestibility in ruminants and non-ruminant herbivores. Pre-treatment with exogenous fibrolytic enzymes enhances the digestibility of fibrous feeds by breaking the complex structure of carbohydrates (Vallejo et al., 2016) thereby reducing the quantities of feed nutrients excreted into the environment (Kholif et al., 2017).

The low digestibility of high fiber diets restricts their use as a major source of nutrients for high-producing ruminants (Salem et al., 2013; Vallejo et al., 2016) such as dairy and feedlot cattle. Therefore, it is vital to identify and evaluate cost-effective and environmentally friendly strategies that may be employed to improve degradation of such feeds in order to improve their value for high-producing ruminants. Several efforts made in this regard include the use of feed additives, exogenous fiber-degrading enzymes, direct fed microbials, and ionophores (Nsereko et al., 2000; Elghandour et al., 2016).

The rumen fluid is a rich and underutilized source of novel enzymes with a tremendous potential for industrial applications (Yue et al., 2013). The use of rumen fluid as a source of enzymes will allow for bioconversion and valorization of agricultural residues, organic fractions of municipal solid wastes as well as aquatic plants. This study was, therefore, designed to explore the potential of rumen fluid, often discarded from slaughterhouses, as a source of exogenous feed enzymes using corn grain, barley grain, soybean meal, common vetch grain, bitter vetch grain, chickling vetch grain, alfalfa hay and common vetch straw as feed substrates. *In vitro* ruminal fermentation parameters (biogas production and digestibility) of rumen fluid-treated feed substrates were determined.

2. Materials and methods

2.1. Rumen contents collection and preparation of enzymatic extracts

Rumen contents (fluid and particulate matter) were collected before feeding from different locations of the rumens of two fistulated Moghani sheep using a vacuum pump. The rumen contents were then transported to the laboratory in a pre-warmed container. Rumen contents were blended in a laboratory blender under constant purging with CO_2 to maintain anaerobic conditions before being filtered through four layers of cheesecloth. Thereafter, the rumen fluid was centrifuged at 1000 rpm for 10 min at 4 °C and the supernatant was sonicated by ultrasound (DT255H, Germany) in 4 cycles of 30 s (35 kHz) at 0 °C (Pan et al., 2003).

2.2. Enzymatic activity measurements

Hydrolytic enzyme activities of carboxymethyl cellulase (CMCase), α-amylase, and avicellase were measured in the ruminal preparations as described by Agarwal et al. (2000). To estimate the activity of CMCase, the reaction mixture containing 1 mL of 0.1 M phosphate buffer (pH 6.8), 0.5 mL of CMCase (1%), and 0.5 mL of enzyme solution was incubated for 60 min. For α-amylase, the reaction mixture containing l mL of buffer solution, 0.25 mL of soluble starch (1%) and 0.25 mL of enzyme solution was incubated for 30 min. The reaction was then terminated by adding 3 mL of dinitrosalicylic acid solution. To estimate the activity of avicellase, 1% substrate (avicel or microcrystalline cellulose) was suspended in the buffer and incubated at 10°C for 48 h (Tanaka et al., 1988). Assay mixture containing l mL of substrate suspension and l mL of enzyme solution was incubated for 60 min at 39 °C under constant gentle shaking. Reducing sugar was measured in all the samples using dinitrosalicylic acid technique with glucose as the standard. The enzymatic activity was obtained as μmole of reducing sugars produced per minute per mL and converted to U/mL under assay conditions (Agarwal et al., 2000).

2.3. Chemical analysis and rumen fluid treatment of feed samples

Soybean meal, common vetch grain, bitter vetch grain, chickling vetch grain, alfalfa hay, common vetch straw, corn grain and barley grain samples were milled to pass through a 1 mm screen for chemical analyses. The feed samples were analyzed for dry matter (method number 930.15), crude protein (CP, Kjeldahl $N \times 6.25$, method number 984.13), ether extract (EE, method number 920.39), and ash (method number 924.05) in accordance with AOAC (1997) procedures. The samples used for rumen fluid treatment were milled to pass through a 2 mm screen. The milled samples were then sprayed with different amounts of the centrifuged and sonicated rumen fluid preparation (1, 2, 3 and 4 mL per 100 g dry matter (DM)) and mixed thoroughly.

2.4. Dry matter solubility test

The aim of this test was to quantify DM solubility in water prior to ruminal incubation of feed samples pre-treated with rumen fluid as described by Colombatto et al. (2003). Untreated feed substrates were included as controls. For DM solubility test, triplicate amounts (1 g DM) of each feed sample were weighed into Erlenmeyer flasks and stored at 20 °C for 3 h. Subsequently, 100 mL distilled water was added to each flask and the mixtures were stored for 17 h at 20–22C. The residues were filtered using one layer polyester cheesecloth (52 ± 5 μm pore size, Gol Pooneh Safahan, Isfahan, Iran) after which DM loss was determined by difference.

2.5. In vitro ruminal fermentation

The fermentation of rumen fluid-treated feed samples was carried out in graduated glass syringes (100 mL capacity) according to a procedure recommended by Menke and Steingass (1988). The rumen fluid used for incubation was obtained from three ruminally fistulated Maghani rams (body mass = 65.2 ± 0.8 kg) fed a total mixed ration containing alfalfa hay and concentrates (1:1, wt/wt) prior to morning feeding. The rams were offered the ration in two similar quantities (up to 5% refusal) at 8 a.m. and 4 p.m. daily. The same amount of rumen fluid was collected from the three rams, mixed, stirred and strained through four layers of cheesecloth into a pre-warmed container and transported to the laboratory. Rumen fluid handling was done under constant purging with CO_2.

Oven-dried feed samples were weighed (200 ± 1 mg) in triplicate and transferred to 100 mL glass syringes fitted with plungers and incubated in a continuous rotary shaker. Ten mL of rumen fluid and 20 mL of McDougall's buffer solution were then added to the syringes. Three blanks (containing rumen fluid and buffer solution but without feed substrate) were included in each incubation run. Incubation was done at 39 °C and gas production was measured at 2, 6, 24, and 48 h post incubation. The average volume of gas produced from blanks was subtracted from the volume of gas produced per sample.

Metabolizable energy (ME) and digestible organic matter (DOM) of feed samples were estimated using equations proposed by Menke and Steingass (1988) as follows:

Table 1
Some enzymatic activities (U/mL)[a] in rumen fluid preparation[b].

Amylase	Avicelase	CMCase
377.8 ± 12.2	333.4 ± 9.9	282 ± 3.6

[a] 1 U/mL = 1 μmol glucose released per min.
[b] The rumen fluid was centrifuged at 1000 rpm for 10 min at 4 °C and the supernatant was sonicated by ultrasound in 4 cycles of 30s (35 kHz) at 0 °C.

$$ME(MJ/kgDM) = 1.06 + 0.1570GP + 0.0084CP + 0.0220EE - 0.0081CA \quad (1)$$

$$DOM(\%DM) = 9 + 0.99GP + 0.0595CP + 0.018CA \quad (2)$$

where CP is crude protein (%DM), GP is 24 h net gas production (mL/g DM), EE is crude fat (%DM) and CA is ash (%DM) of feeds.

2.6. Calculations

Cumulative gas production data were fitted to the model below using Fitcurve software (Chen, 1995):

$$Y = a + b\left(1 - e^{-ct}\right) \quad (3)$$

where *a* is the gas production from the immediately soluble fraction (mL); *b* is the gas production from the insoluble fraction (mL); *c* is the rate constant (%/h) of gas production from the insoluble fraction; *t* is the incubation time (h); and Y is the volume (mL) of gas produced at time t.

2.7. Statistical analysis

Dry matter solubility and *in vitro* ruminal fermentation data were analyzed based on a 4 × 8 factorial treatment design within a completely randomized experimental design. Main effects were rumen fluid pre-treatment (4 levels) and feed substrates (8 different feed samples). The analysis was done using the GLM procedure of SAS (2003) according to the following linear statistical model:

$$Y_{ijk} = \mu + RF_i + FS_j + (RF \times FS)_{ij} + e_{ijk} \quad (4)$$

where Y_{ijk} is the replicate observation (DM solubility or ruminal fermentation parameters) k (where k = 1, 2, 3) for feed substrate j and rumen fluid pre-treatment level i; μ is overall mean; RF_i is the influence of rumen fluid pre-treatment level i (where i = 1, 2, 3, 4); FS_j is the effect of feed substrate j (where j = 1, 2, …,8); $(RF \times FS)_{ij}$ is the interaction effect of rumen fluid pre-treatment level i and feed substrate j (where ij = 1; 1, 1; 2, 1; 3, 1; 4, …, 4; 8) and e_{ijk} is the random error associated with observation Y_{ijk}, assumed to be normally and independently distributed. Duncan test was used to separate means (SAS, 2003) and significant differences were declared at $P < 0.05$.

3. Results

3.1. Enzymatic activities of the rumen fluid

The cellulolytic activity of rumen fluid preparation was determined by its ability to hydrolyze carboxymethyl cellulose (CMC) and Avicel. The CMCase and avicellase activities were 377.8 and 333.4 U per mL rumen fluid, respectively. Amylase activity in rumen fluid was found to be 282 U per mL (Table 1).

3.2. Dry matter solubility

The level of rumen fluid treatment had a significant effect ($P < 0.05$) on the DM solubility of soybean meal, chickling vetch, and bitter vetch grains. However, no significant effect was observed on dry matter (DM) solubilities of common vetch grain, alfalfa hay, common vetch straw, corn grain and barley grain. Rumen fluid treatment increased ($P < 0.05$) the DM solubility of barley grain, soybean meal, bitter vetch grain, common vetch grain and chuckling vetch grain linearly. Untreated feed samples had the lowest DM solubility while those treated with 4 mL rumen fluid had the highest DM solubility (Table 2).

3.3. In vitro ruminal fermentation

Rumen fluid pre-treatment increased ($P < 0.05$) gas production of feed substrates linearly at 6, 24 and 48 h. Gas production after 6 h of incubation was highest ($P < 0.01$) when rumen fluid pre-treatment was applied at 4 mL/100 g DM of all feeds, except for alfalfa hay and common vetch grain. Differences were observed between barley, corn, soybean meal, and common vetch straw when rumen fluid pre-treatment was applied at a rate of 3 mL/100 g DM (Table 3).

The rapidly fermentable fraction (*a*) increased ($P < 0.05$) with rumen fluid pre-treatment in corn grain, bitter vetch grain, chickling vetch grain and common vetch straw. Pre-treatment with rumen fluid preparation had no significant effect on the potential fermentable fraction ($a + b$) in all feed substrates except for bitter vetch grain. The gas production rate of corn grain, soybean meal and chickling vetch grain increased with increasing levels of rumen

Table 2
Effect of rumen fluid preparation[a] on dry matter solubility of feeds (% of DM).

Item	Rumen fluid (RF) preparation[b]						P-value	
	RF0	RF1	RF2	RF3	RF4	RF Levels	Linear	Quadratic
Corn grain	45.1 ± 1.38	46.8 ± 1.46	47.1 ± 3.37	49.1 ± 4.15	50.6 ± 5.37	0.410	0.660	0.860
Barley grain	61.5 ± 1.72	65.1 ± 0.69	65.8 ± 0.63	66.1 ± 0.15	66.2 ± 1.61	0.690	0.001	0.013
Soybean meal	39.7 ± 0.98^{b}	41.0 ± 0.73^{b}	41.4 ± 0.72^{b}	43.4 ± 2.22^{b}	53.3 ± 5.36^{a}	0.006	0.001	0.009
Bitter vetch grain	63.5 ± 0.24^{c}	64.6 ± 0.49^{b}	65.7 ± 0.43^{a}	65.9 ± 0.37^{a}	66.2 ± 0.61^{a}	0.001	0.001	0.030
Common vetch grain	58.8 ± 1.33	59.7 ± 0.12	59.8 ± 0.73	60.1 ± 0.45	60.5 ± 0.14	0.131	0.016	0.655
Chickling vetch grain	62.6 ± 0.88^{b}	62.9 ± 0.22^{b}	63.9 ± 0.67^{ab}	64.4 ± 1.02^{a}	65.2 ± 0.51^{a}	0.008	0.001	0.752
Alfalfa hay	23.4 ± 0.99	23.7 ± 0.88	25.0 ± 1.06	25.2 ± 2.76	25.1 ± 1.12	0.365	0.059	0.860
Common vetch straw	23.9 ± 1.17	24.1 ± 1.90	24.3 ± 1.17	24.6 ± 0.92	25.6 ± 1.72	0.673	0.185	0.610

[a, b, c] Least square means in a row with differing letters differ significantly ($P < 0.05$).
[a] The rumen fluid was centrifuged at 1000 rpm for 10 min at 4°C and the supernatant was sonicated by ultrasound in 4 cycles of 30s (35 kHz) at 0 °C.
[b] Levels of rumen fluid preparation were: 0 (RF0), 1 (RF1), 2 (RF3), 3 (RF3) and 4 (RF4) mL per 100 g DM of feed substrate.

Table 3
Effect of pre-treating test feeds with rumen fluid preparation[a] on *in vitro* ruminal gas production at 6, 24 and 48 h (mL/g dry matter).

Item	Rumen fluid (RF) preparation[b]					*P-value*		
	RF0	RF1	RF2	RF3	RF4	RF Levels	Linear	Quadratic
Barley grain								
6	34.0 ± 0.81[b]	33.3 ± 0.47[b]	34.3 ± 0.47[a b]	35.0 ± 0.82[a]	35.2 ± 0.85[a]	0.014	0.003	0.329
24	62.5 ± 0.41[c]	62.0 ± 0.81[c]	63.9 ± 0.19[b]	66.0 ± 0.82[a]	66.1 ± 0.29[a]	0.001	0.001	0.189
48	63.2 ± 0.57[c]	65.0 ± 0.01[b]	65.1 ± 0.09[b]	65.7 ± 0.52[a]	65.2 ± 0.28[a b]	0.001	0.001	0.001
Corn grain								
6	28.1 ± 1.09[b]	28.5 ± 0.73[b]	29.2 ± 1.03[b]	33.8 ± 0.54[a]	33.9 ± 2.19[a]	0.001	0.001	0.174
24	61.2 ± 0.57[c]	61.8 ± 0.65[c]	63.7 ± 0.47[b]	65.3 ± 0.53[a]	65.4 ± 1.01[a]	0.001	0.001	0.369
48	63.1 ± 0.31[c]	63.7 ± 0.51[c]	64.6 ± 0.31[b]	65.6 ± 0.41[a]	65.9 ± 0.54[a]	0.001	0.001	0.638
Soybean meal								
6	22.2 ± 0.76[c]	23.5 ± 1.08[b c]	23.9 ± 1.39[b]	25.0 ± 0.73[b]	27.6 ± 0.88[a]	0.001	0.001	0.096
24	49.7 ± 1.17[c]	50.5 ± 0.41[b c]	51.9 ± 0.53[b]	53.1 ± 0.95[a b]	54.6 ± 1.54[a]	0.001	0.001	0.514
48	54.2 ± 0.87[b]	55.3 ± 0.54[a b]	54.9 ± 0.67[a b]	56.2 ± 1.32[a]	56.1 ± 0.62[a]	0.026	0.003	0.717
Bitter vetch grain								
6	25.2 ± 0.28[b]	24.8 ± 1.29[b]	25.0 ± 0.82[b]	26.2 ± 0.21[a b]	27.3 ± 1.89[a]	0.029	0.005	0.072
24	47.8 ± 1.03[b]	47.7 ± 2.62[b]	49.0 ± 0.82[a b]	50.7 ± 1.03[a]	50.4 ± 1.23[a]	0.031	0.004	0.981
48	66.2 ± 0.67[a b]	64.2 ± 0.44[b]	65.3 ± 0.69[b]	68.0 ± 0.15[a]	67.7 ± 0.46[a]	0.011	0.002	0.102
Common vetch grain								
6	14.1 ± 0.51	14.0 ± 0.82	13.9 ± 0.98	14.1 ± 0.34	15.2 ± 0.24	0.076	0.042	0.043
24	50.0 ± 0.82	50.2 ± 2.09	51.7 ± 2.87	52.7 ± 2.60	53.3 ± 2.62	0.229	0.026	0.892
48	53.7 ± 0.94	53.0 ± 0.82	54.3 ± 3.29	55.0 ± 2.45	55.5 ± 0.41	0.415	0.088	0.671
Chickling vetch grain								
6	10.0 ± 0.82[b]	10.7 ± 0.94[b]	10.3 ± 1.24[b]	10.2 ± 0.85[b]	13.4 ± 0.42[a]	0.001	0.001	0.005
24	48.9 ± 1.35[cd]	51.0 ± 0.82[b]	51.7 ± 0.47[b]	51.3 ± 2.05[b]	55.0 ± 0.82[a]	0.001	0.001	0.369
48	53.9 ± 1.35[b]	55.3 ± 1.89[b]	55.0 ± 0.82[b]	55.9 ± 0.54[a b]	57.7 ± 1.25[a]	0.010	0.001	0.431
Alfalfa hay								
6	13.7 ± 0.48[a]	11.3 ± 1.25[b]	12.9 ± 0.19[a]	13.7 ± 0.47[a]	13.7 ± 0.47[a]	0.001	0.044	0.007
24	30.6 ± 0.45[c]	31.0 ± 0.82[c]	33.4 ± 0.43[b]	35.8 ± 0.51[a]	35.9 ± 0.74[a]	0.001	0.001	0.688
48	36.5 ± 1.07[c]	36.3 ± 1.25[c]	39.4 ± 0.58[b]	42.6 ± 1.31[a]	43.9 ± 0.96[a]	0.001	0.001	0.152
Common vetch straw								
6	12.9 ± 0.67[b]	13.6 ± 0.57[a b]	14.3 ± 0.77[a]	14.5 ± 0.75[a]	14.3 ± 0.47[a]	0.019	0.003	0.110
24	25.8 ± 0.62[b]	26.5 ± 1.22[b]	26.7 ± 0.47[b]	29.0 ± 0.82[a]	28.7 ± 0.94[a]	0.001	0.001	0.919
48	32.9 ± 1.26[b c]	33.5 ± 0.41[b]	33.3 ± 1.25[b]	34.7 ± 0.47[a b]	35.0 ± 0.82[a]	0.022	0.002	0.589

[a] The rumen fluid was centrifuged at 1000 rpm for 10 min at 4 °C and the supernatant was sonicated by ultrasound in 4 cycles of 30s (35 kHz) at 0 °C.
[b] Levels of rumen fluid preparation were: 0 (RF0), 1 (RF1), 2 (RF3), 3 (RF3) and 4 (RF4) mL per 100 g DM of feed substrate.
[c] Least square means in a row with differing letters differ significantly ($P < 0.05$).

fluid pre-treatment while the opposite was true for common vetch straw (Table 4).

Rumen fluid pre-treatment did not influence ($P > 0.05$) metabolizable energy (ME) and digestible organic matter (DOM) of common vetch grain. However, in other feed substrates, there was a linear increase ($P < 0.05$) in ME and DOM with level of rumen fluid pre-treatment. The highest DOM and ME contents were obtained when substrates were pre-treated at a rate of 4 mL rumen fluid per 100 g DM (Table 5).

4. Discussion

This study investigated the potential of rumen fluid as a source of enzyme feed additives using an *in vitro* ruminal fermentation technique. Enzymatic activity results shown in Table 2 confirm the presence of active enzymes in the rumen fluid preparation. The increase in dry matter solubility of tested feeds also confirms the presence of active enzymes in the prepared ruminal additive. Rumen fluid pre-treatment enhanced the solubility of DM due to the presence of microbial enzymes that pre-digested cell walls and other components of feed samples. Other researchers (Colombatto et al., 2003; Elghandour et al., 2013) also reported an increase in soluble DM of enzyme-treated feeds. Yue et al. (2013) also proposed that rumen fluid could be used as a source of enzymes.

Various classes of enzymes are required to breakdown the complex structural components of plant cell walls to their simpler molecules to enable the release of soluble cell contents (Morgavi et al., 2012). The rumen is an anaerobic microbial ecosystem, made up of bacteria, archaea, fungi, and protozoa that produce a vast array of enzymes. These enzymes synergistically digest complex molecules to their simple components. Consequently, the observed higher DM solubility of substrates pre-treated with rumen fluid may be due to the activity of these rumen fluid enzymes produced by rumen microbes.

The *in vitro* ruminal gas production technique was employed in this study to determine the effects of rumen fluid pre-treatment on fermentation kinetics of test feed substrates. Changes in gas production kinetics of enzyme-treated feeds have been reported previously. Elghandour et al. (2013) stated that increasing doses of an enzyme preparation from a ruminal bacterium, *Ruminococcus flavefaciens*, increased gas production from four fibrous feeds at all incubation times. Similarly, rate of gas production in two of the four fibrous feeds increased in response to higher doses of the enzyme preparation. Colombatto et al. (2003) observed higher *in vitro* ruminal organic matter digestibility for enzyme-treated feeds using an *in vitro* gas production method. This effect is attributed to the increase in degradation rate achieved via a combined effect of direct enzyme hydrolysis and synergistic action between the endogenous (ruminal) and exogenous enzymes. Wallace et al. (2001) examined the effect of two enzymatic preparations on the fermentation of corn and grass silages using an *in vitro* ruminal gas production method and reported that the rate of gas production increased at concentrations much higher than the recommended application rates. They also observed the highest correlation between increased gas production and enzyme activities against micro-granular cellulose (Wallace et al., 2001). In this study, estimated metabolizable energy and digestible organic matter were influenced by the rumen fluid enzymatic preparation. This is

Table 4
Effect of pre-treating test feeds with rumen fluid preparation[a] on *in vitro* ruminal gas production parameters (*a* (mL/g DM), *b* (mL/g DM), *a*+*b* (mL/g DM), *c* (%/h)) of feeds.

Item	Rumen fluid (RF) preparation[b]					*P-value*		
	RF0	RF1	RF2	RF3	RF4	RF Levels	Linear	Quadratic
Barley grain								
a	−1.4 ± 1.63	−1.7 ± 2.76	−1.5 ± 0.70	−1.7 ± 1.07	−1.3 ± 1.74	0.764	0.689	0.858
b	65.8 ± 1.92	66.0 ± 2.72	67.7 ± 0.55	68.5 ± 0.55	68.0 ± 2.19	0.162	0.027	0.485
a + *b*	67.2 ± 3.47	67.7 ± 3.51	69.1 ± 1.25	70.2 ± 1.57	69.4 ± 3.67	0.593	0.155	0.601
c	14.6 ± 0.11[a]	13.4 ± 0.04[b]	14.1 ± 0.04[a]	14.4 ± 0.17[a]	14.6 ± 0.12[a]	0.033	0.001	0.201
Corn grain								
a	5.9 ± 0.88[b]	6.0 ± 0.29[b]	6.9 ± 0.57[a b]	6.7 ± 0.88[a b]	7.6 ± 0.43[a]	0.015	0.002	0.622
b	59.0 ± 0.49	59.7 ± 0.57	59.3 ± 0.26	60.0 ± 0.94	59.4 ± 0.32	0.201	0.218	0.197
a + *b*	64.9 ± 0.48[c]	65.6 ± 0.33[b]	66.2 ± 0.32[b]	66.7 ± 0.49[a b]	67.0 ± 0.57[a]	0.001	0.001	0.341
c	8.5 ± 0.29[b]	8.5 ± 0.30[b]	8.7 ± 0.47[b]	9.9 ± 0.26[a]	9.6 ± 0.21[a]	0.001	0.001	0.542
Soybean meal								
a	2.1 ± 1.38	3.0 ± 0.66	2.5 ± 0.74	3.4 ± 0.77	3.0 ± 1.67	0.551	0.243	0.579
b	53.9 ± 2.03	53.9 ± 0.49	54.4 ± 0.99	54.1 ± 0.49	54.6 ± 1.45	0.906	0.426	0.945
a + *b*	56.0 ± 1.42	56.9 ± 0.74	57.0 ± 0.30	57.5 ± 0.31	57.6 ± 0.26	0.060	0.006	0.475
c	7.1 ± 0.10[b]	7.1 ± 0.12[b]	7.5 ± 0.06[b]	7.5 ± 0.05[b]	8.4 ± 0.13[a]	0.001	0.001	0.009
Bitter vetch grain								
a	7.5 ± 0.90[a b]	8.6 ± 1.13[a b]	6.2 ± 0.63[b c]	7.3 ± 0.96[a b]	9.3 ± 2.15[a]	0.026	0.308	0.035
b	59.2 ± 0.86[b]	57.5 ± 0.24[c]	60.9 ± 0.81[a]	61.8 ± 1.59[a]	59.3 ± 1.37[b]	0.001	0.024	0.057
a + *b*	66.8 ± 0.16[b]	66.1 ± 1.33[b]	67.1 ± 1.23[b]	69.0 ± 0.69[a]	68.5 ± 0.86[a]	0.003	0.001	0.503
c	5.9 ± 0.15	5.9 ± 0.08	6.18 ± 0.22	6.1 ± 0.15	6.1 ± 0.49	0.397	0.156	0.540
Common vetch grain								
a	−2.7 ± 0.72	−3.7 ± 0.74	−3.4 ± 076	−3.5 ± 1.07	−2.3 ± 1.76	0.344	0.519	0.063
b	59.7 ± 0.43	60.3 ± 0.47	61.2 ± 1.56	62.2 ± 2.25	62.2 ± 1.26	0.068	0.006	0.618
a + *b*	62.4 ± 0.86	64.0 ± 0.79	64.7 ± 2.30	65.7 ± 3.27	64.4 ± 2.99	0.383	0.129	0.229
c	6.0 ± 0.17	6.2 ± 0.12	6.4 ± 0.21	6.4 ± 0.49	6.4 ± 0.69	0.606	0.169	0.437
Chickling vetch grain								
a	−7.8 ± 0.59[b]	−7.9 ± 0.44[b]	−9.7 ± 1.67[a]	−8.4 ± 1.29[a b]	−6.1 ± 0.64[c]	0.003	0.089	0.001
b	66.8 ± 0.87[d]	68.2 ± 0.51[c]	69.6 ± 1.40[b]	72.2 ± 0.33[a]	72.0 ± 0.90[a]	0.001	0.001	0.277
a + *b*	74.6 ± 1.46[c]	76.1 ± 0.40[b c]	79.3 ± 3.02[a]	80.6 ± 0.99[a]	78.1 ± 1.49[a b]	0.001	0.001	0.007
c	6.2 ± 0.19[b]	6.6 ± 0.02[b]	7.1 ± 0.35[a]	6.2 ± 0.41[b]	6.4 ± 0.28[b]	0.003	0.922	0.004
Alfalfa hay								
a	5.0 ± 0.32	4.1 ± 1.39	5.5 ± 0.48	5.4 ± 0.25	5.1 ± 0.40	0.078	0.162	0.771
b	35.7 ± 0.38[d]	37.0 ± 0.88[c]	38.8 ± 0.22[b]	41.8 ± 0.29[a]	42.6 ± 0.60[a]	0.001	0.001	0.882
a + *b*	40.7 ± 0.31[c]	41.1 ± 1.16[c]	44.3 ± 0.58[b]	47.3 ± 0.26[a]	47.7 ± 0.27[a]	0.001	0.001	0.839
c	4.6 ± 0.21	4.7 ± 0.62	4.4 ± 0.16	4.5 ± 0.24	4.5 ± 0.11	0.652	0.602	0.427
Common vetch straw								
a	4.2 ± 0.14[b c]	2.8 ± 2.04[c]	5.9 ± 0.55[a]	4.7 ± 0.38[a b]	5.2 ± 0.12[a b]	0.006	0.027	0.852
b	34.5 ± 0.13[c]	35.0 ± 0.28[c]	36.3 ± 0.76[b]	40.3 ± 0.32[a]	40.0 ± 0.68[a]	0.001	0.001	0.204
a + *b*	38.8 ± 0.20[c]	37.8 ± 1.76[c]	42.2 ± 0.42[b]	44.9 ± 0.16[a]	45.2 ± 0.79[a]	0.001	0.001	0.599
c	4.1 ± 0.19[a b]	4.3 ± 0.51[a]	3.6 ± 0.26[c]	3.7 ± 0.20[b c]	3.6 ± 0.21[c]	0.013	0.004	0.640

[a] The rumen fluid was centrifuged at 1000 rpm for 10 min at 4 °C and the supernatant was sonicated by ultrasound in 4 cycles of 30s (35 kHz) at 0 °C.
[b] Levels of rumen fluid preparation were: 0 (RF0), 1 (RF1), 2 (RF3), 3 (RF3) and 4 (RF4) mL per 100 g DM of feed substrate. '*a*' is gas produced by the soluble fraction, '*b*' is gas produced by the insoluble fraction, (*a* + *b*) is the potential gas production and 'c' is the gas production rate constant for the insoluble fraction (%/h).
[c] Least square means in a row with differing letters differ significantly ($P < 0.05$).

consistent with previous findings (Colombatto et al., 2003; Elghandour et al., 2013). Cheng et al. (1991) suggested that exogenous enzymes could improve the microbial attachment to plant cell wall components. They could also improve the hydrolytic capacity of the rumen microbes because of possible synergistic effects with rumen microbial enzymes (Morgavi et al., 2012), which further stimulates the growth of rumen bacteria (Nsereko et al., 2000). Martin and Nisbet (1992) proposed that exogenous feed enzymes could be a source of growth factors that stimulate rumen bacterial growth and thus enhance the digestive capacity of the rumen. Exogenous enzyme additives have been reported to improve the nutritive values of feeds while reducing enteric methane and carbon dioxide emissions (Hernandez et al., 2017; Kholif et al., 2017).

Like most human activities, intensive ruminant production contributes to environmental pollution due to improper waste disposal as well as enteric methane emissions. In this study, pre-treatment of test feeds with the rumen fluid enzymatic preparation enhanced *in vitro* ruminal fermentation and resulted in higher estimated digestibility and metabolizable energy content of feeds. This positive effect means that using rumen fluid as a feed additive can reduce the amount of nutrients excreted into the environment by animals as well as reducing dietary energy losses. Enhancing feed utilization in ruminants has the added benefit of reducing enteric methane emissions thereby protecting the environment. Indeed, Salem et al. (2013, 2015) reported that the inclusion of exogenous fibrolytic enzymes improves feed and nutrient utilization thereby reducing excretion of nutrients to the environment. Excessive excretion of nutrients due to inefficient digestibility and high CH_4 emissions are major constraints in achieving sustainable ruminant production (Hristov et al., 2015). Application of rumen fluid as an enzyme source for ruminant feeds is an environmentally friendly avenue through which rumen fluid from slaughterhouses can be managed. Thus, rumen fluid can be a new and inexpensive source of exogenous enzymes for the animal feed industry.

5. Conclusions

This study explored the potential of rumen fluid pre-treatment to improve ruminal fermentation parameters and digestibility of a variety of feed substrates. Pre-treatment with rumen fluid improved the simulated fermentation of corn grain, bitter vetch grain, chickling vetch grain, alfalfa hay and common vetch straw. These findings suggest that, when applied at a rate of 4 mL/100 g DM, rumen fluid has the potential to enhance nutritive value of

Table 5
Effect of pre-treating test feeds with rumen fluid preparation[a] on organic matter digestibility (DOM, %) and metabolizable energy (ME, MJ/kg) of feeds.

Item	Rumen fluid (RF) preparation[b]					*P-value*		
	RF0	RF1	RF2	RF3	RF4	RF Levels	Linear	Quadratic
Barley grain								
DOM	71.7 ± 0.40[c]	71.2 ± 0.81[c]	73.0 ± 0.19[b]	75.1 ± 0.80[a]	75.2 ± 0.29[a]	0.001	0.001	0.189
ME	11.0 ± 0.06[c]	10.9 ± 0.13[c]	11.2 ± 0.03[b]	11.6 ± 0.13[a]	11.6 ± 0.05[a]	0.001	0.001	0.181
Corn grain								
DOM	70.2 ± 0.57[c]	70.8 ± 0.64[c]	72.6 ± 0.46[b]	74.2 ± 0.52[a]	74.4 ± 0.99[a]	0.001	0.001	0.369
ME	10.7 ± 0.09[c]	10.9 ± 0.10[c]	11.2 ± 0.07[b]	11.4 ± 0.08[a]	11.4 ± 0.16[a]	0.001	0.001	0.365
Soybean meal								
DOM	61.2 ± 1.16[c]	62.0 ± 0.41[b c]	63.3 ± 0.53[b]	64.6 ± 0.95[a b]	66.0 ± 1.53[a]	0.001	0.001	0.514
ME	9.3 ± 0.18[c]	9.5 ± 0.07[b c]	9.7 ± 0.08[b]	9.8 ± 0.15[a b]	10.1 ± 0.24[a]	0.001	0.001	0.513
Bitter vetch grain								
DOM	58.0 ± 1.02[b]	57.8 ± 2.59[b]	59.1 ± 0.81[a b]	60.9 ± 1.03[a]	60.5 ± 1.22[a]	0.031	0.004	0.981
ME	8.8 ± 0.16[b]	8.8 ± 0.41[b]	9.0 ± 0.13[a]	9.3 ± 0.16[a]	9.2 ± 0.19[a]	0.022	0.002	0.847
Common vetch grain								
DOM	60.1 ± 0.81[c]	60.3 ± 2.07[c]	61.8 ± 2.84[b]	62.7 ± 2.57[a]	63.4 ± 2.59[a]	0.229	0.026	0.892
ME	9.1 ± 0.13[a b]	9.2 ± 0.33[a b]	9.4 ± 0.45[a b]	9.6 ± 0.41[a]	9.7 ± 0.41[a]	0.186	0.019	0.997
Chickling vetch grain								
DOM	59.0 ± 1.34[c]	61.1 ± 0.81[b]	61.8 ± 0.47[b]	61.5 ± 2.03[b]	65.1 ± 0.80[a]	0.001	0.001	0.369
ME	8.9 ± 0.21[c]	9.3 ± 0.13[b]	9.4 ± 0.07[b]	9.4 ± 0.32[b]	10.0 ± 0.13[a]	0.001	0.001	0.521
Alfalfa hay								
DOM	45.2 ± 0.39[c]	45.5 ± 0.73[c]	47.7 ± 0.38[b]	49.8 ± 0.45[a]	49.9 ± 0.66[a]	0.001	0.001	0.689
ME	6.7 ± 0.06[c]	6.8 ± 0.11[c]	7.1 ± 0.06[b]	7.4 ± 0.07[a]	7.4 ± 0.10[a]	0.001	0.001	0.681
Common vetch straw								
DOM	44.6 ± 0.28[b]	45.2 ± 0.68[b]	45.4 ± 0.51[b]	47.5 ± 0.34[a]	47.2 ± 0.68[a]	0.001	0.001	0.858
ME	6.5 ± 0.10[b]	6.6 ± 0.17[b]	6.6 ± 0.07[b]	6.9 ± 0.14[a]	6.9 ± 0.14[a]	0.001	0.001	0.919

[a] The rumen fluid was centrifuged at 1000 rpm for 10 min at 4 °C and supernatant was sonicated by ultrasound in4 cycles of 30 s (35 kHz) at 0 °C.
[b] Levels of rumen fluid preparation were: 0 (RF0), 1 (RF1), 2 (RF3), 3 (RF3) and 4 (RF4) mL per 100 g DM of feed substrate.
[c] Least square means in a row with differing letters differ significantly ($P < 0.05$).

ruminant feeds. Its use for this purpose may reduce the environmental threat posed by the disposal of rumen fluid from slaughterhouses while simultaneously reducing enteric methane emissions from ruminant production.

Conflicts of interest

The authors declare no conflict of interest.

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