ORIGINAL ARTICLE

Effect of exogenous xylanase on rumen *in vitro* gas production and degradability of wheat straw

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ABSTRACT

The objective of this study was to determine effects of xylanase on *in vitro* gas production (GP) and *in sacco* degradability of wheat straw. Rumen fluid was obtained from three Mongolian native goats fitted with permanent rumen cannulas. The trial consisted of five doses (0, 0.5, 1.0, 1.5, 2.0 μL/g of substrate) of a commercial xylanase (Dyadic® xylanase PLUS, Dyadic International, Inc., Jupiter, FL, USA). For the *in sacco* degradability, different levels of xylanase enzyme were added directly onto 2 g of wheat straw in nylon bags and incubated in the rumen for 3, 6, 12, 24 and 48 h to estimate degradability of wheat straw. Total GP increased ($P < 0.001$) at all times of incubation at intermediate levels of xylanase. Methane production had a similar pattern at 3 and 12 h of incubation; increased linearly at 24 h of incubation, and was unaffected at 6 and 48 h of incubation. Rumen NH_3-N concentration increased linearly at 3 h and the highest values were observed with intermediate enzyme levels. All ruminal volatile fatty acids increased linearly with intermediate levels of the fibrolytic enzyme. The *in sacco* rate of dry matter degradation decreased linearly ($P = 0.020$) with increasing enzymes. Intermediate levels of xylanase improved rumen kinetic fermentation and degradability. The outcome of this research indicated that the application of xylanase enzyme could improve *in vitro* GP fermentation of wheat straw.

Key words: degradability, gas production, wheat straw, xylanase.

INTRODUCTION

In Mongolia, the low and seasonal yield of native pastures causes a feed gap between the annual pasture provision and the requirements of grazing ruminants. Therefore, there is a growing need to improve the feeding value of locally available resources such as wheat straw for supplementary feeding. Wheat straw constitutes an important source of roughage for ruminants. It typically contain more than 70% neutral detergent fiber (NDF) on a dry matter (DM) basis, with less than 40% total digestive tract digestibility of the NDF, even under ideal feeding conditions (NRC 2001).

Increasing efficiency of feed utilization has many advantages, including reducing the amount of undigested nutrients, reduction of nitrogenous compound excretion (Kuelling *et al.* 2003) and reduction of methane emissions (Johnson & Johnson 1995) which can be achieved by manipulating animal diets using different feed additives. Biologically active enzymes, as animal feed additives, are now produced at low cost due to recent advances in fermentation technology and biotechnology.

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Xylanases are the major enzyme group that can break β1–4 linkages joining sugar molecules of xylans in plant cell wall components (Dawson & Tricarico 1999; Beauchemin *et al.* 2003). Several studies with exogenous fibrolytic enzymes (EFE) have reported increased microbial activities in the rumen which resulted in enhancement of animal performance (Gado *et al.* 2009; Khattab *et al.* 2011; Salem *et al.* 2013). However, despite the importance of enzyme formulation, the relationship between enzymatic activities and improvements in forage utilization is not well established (Eun *et al.* 2007).

However, results with EFE addition in ruminant systems have been highly variable and inconsistent, with some studies showing substantial improvements of feed digestibility and animal performance (Salem *et al.* 2013), while others have reported negative effects or none at all (Baloyi 2008). Some of the reasons postulated for the inconsistent results is that the effects of EFE are influenced by factors such as enzyme source, the type and dose of enzyme, composition of diet, enzyme application method and even the level of animal productivity (Beauchemin *et al.* 2003; Carro *et al.* 2007).

Although many researchers have investigated the effect of using EFE to improve the feeding value of hay or straw using *in vitro* techniques in many parts of the world, for example Carro *et al.* (2007) in Spain, Eun *et al.* (2007) in Canada, Khattab *et al.* (2011) in Egypt and Valdes *et al.* (2015) in Mexico, this is the first study of this kind in Mongolia. The aim of this experiment was to investigate effects of direct addition of xylanase on *in vitro* gas production (GP) and *in sacco* DM degradability of wheat straw.

MATERIALS AND METHODS

Samples of wheat straw (*Triticum aestivum* var. 106) were collected from fields of the Institute of Plant and Agriculture in Darkhan Uul province of Mongolia. Average summer temperature of the study site is 25 to 30°C during summer, winter temperatures of −10 to −30°C and over 260 sunny days per year. Straw samples were ground in a Wiley mill to pass a 1.5 mm sieve and stored in plastic bags for subsequent chemical analysis, *in vitro* incubation with enzyme and *in sacco* degradability. These trials were conducted at the Feed Evaluation Laboratory of Mongolian State University of Agriculture and Research Institute of Animal Husbandry, Mongolia.

In vitro incubation

Rumen fluid was collected from three Mongolian native goats (35 ± 4.4 kg of body weight) fitted with permanent rumen cannulas. Goats were fed a ration (8.1 MJ net energy for maintenance and 114 g crude protein) of 1 kg miscellaneous grass hay and 0.2 kg wheat bran in two equal portions at 08.00 and at 17.00 hours) formulated to meet the nutrient requirements of growing lambs (NRC 1985) for 2 weeks. Ruminal contents were obtained immediately before the morning feeding, mixed and strained through four layers of cheese-cloth into a flask with an O_2-free headspace. Ten mL of particle-free ruminal fluid was added to each bottle and 40 mL of the buffer solution (Goering & Van Soest 1970), with no trypticase was immediately added in a proportion 1:4 (v/v).

The buffer solution for the *in vitro* GP was based on the *in vitro* rumen digestibility buffer solution and was prepared as described by Goering and Van Soest (1970). Activities of EFE xylanase (Dyadic® xylanase PLUS; Dyadic International, Inc., Jupiter, FL, USA) was measured using the assay of Bailey *et al.* (1992) where the enzyme activities were cellulase 30 000 to 36 000 units/g and beta-glucanase from 7500 to 10 000 units/g. Activities of EFE xylanase were determined at pH 5.5 and 37°C.

Samples of wheat straw were dried at 55°C for 48 h and ground through a Wiley mill as described above. Enzyme solution was diluted with water (1:3 ratio v/v), to allow enzyme-substrate interaction (Beauchemin *et al.* 2003). Samples of 0.5 g straw were weighed into 120 mL serum bottles and the xylanase enzyme was added directly with the substrate in the bottles at the rate of 0, 0.5, 1.0, 1.5 and 2.0 μL/g of substrate DM. Bottles were kept at room temperature (21-23°C) for 16 h before adding buffered ruminal fluid in order to create a stable enzyme-feed complex. Substrate in non-treated bottles (blanks) was treated with the corresponding amount of distilled water.

A total of 135 bottles with or without substrate (i.e. three bottles of each triplicate sample in three runs in different weeks with each of the five doses of xylanase), plus three bottles as blanks (i.e., rumen fluid only), were incubated for 0, 3, 6, 9, 12, 24 and 48 h. Forty mL of Goering and Van Soest (1970) buffer solution was added to each bottle at 39°C under continuous flushing with CO_2. Bottles were withdrawn from the shaker-incubator after the scheduled inoculation time and total GP was measured in all bottles using a pressure transducer and a calibrated syringe. A gas sample of 5 mL from each bottle were taken and injected to Bio^{anyt} system GC-2010 (Shimadzu GC-2010 GC-gas chromatograph; Shimadzu, Tokyo, Japan) to measure CH_4 concentrations.

At the end of each incubation period, bottles were uncapped and pH was immediately measured. After 10 min of centrifugation at 4°C at $4000 \times g$, 4 mL of bottle contents were sampled for determination of total and individual volatile fatty acids (VFA) and ammonia N (NH_3-N) concentrations. Finally, the content of the bottles was transferred to previously weighed filter crucibles and solid residue was washed in a Foss Fiber Analyzer and the crucibles were dried at 60°C for 48 h to estimate apparent DM degradability. Residues were analyzed for NDF and acid detergent fiber (ADF) to estimate fiber degradability.

Procedures for analysis of VFA, CH_4 and NH_3-N were described by Carro *et al.* (1999).

In sacco degradability

Straw samples were ground through a 2 mm screen. About 2 g (DM basis) of wheat straw was placed into pre-weighed nylon bags (12 cm × 5 cm, 45 μm pore size) and four levels of enzyme 0.5, 1.0, 1.5, 2.0 μL/g DM were added directly onto substrates in the bags. For control (0 μL/g) no enzyme were added.

Nylon bags with straw samples were incubated with enzyme substrate for 16 h at room temperature and then incubated in the rumen of goats for 3, 6, 12, 24 and 48 h. The 0 h time bags were treated in the same manner as the other bags upon removal from the rumen. Bags retrieved at the indicated times were washed thoroughly in cold tap water

Table 1 Effect of enzyme levels on ruminal pH, total gas production and methane emission of wheat straw at different times of incubation

Batch time (h)	Enzyme levels, µL/g					SEM	P	
	E0	E0.5	E1.0	E1.5	E2.0		Linear	Quadratic
pH								
3	6.84	6.73	6.74	6.71	6.73	0.017	< 0.001	< 0.001
6	6.90	6.78	6.82	6.77	6.80	0.021	< 0.001	< 0.001
12	6.81	6.77	6.74	6.72	6.72	0.029	0.016	0.781
24	6.84	6.77	6.73	6.70	6.73	0.035	0.006	0.688
48	6.74	6.70	6.70	6.63	6.67	0.031	0.185	0.556
Total gas production, mL/g dry matter								
3	8.63	27.03	28.39	26.09	21.31	0.313	< 0.001	< 0.001
6	12.16	32.66	33.33	33.00	23.00	0.774	< 0.001	< 0.001
12	17.83	39.16	38.33	36.66	33.83	0.288	< 0.001	< 0.001
24	25.50	48.00	45.00	45.83	43.50	1.901	< 0.001	< 0.001
48	44.50	73.30	69.30	67.00	63.43	1.110	< 0.001	< 0.001
Methane, mL/g dry matter								
3	0.00	4.06	4.18	4.82	4.16	0.073	< 0.001	< 0.001
6	4.64	5.33	5.16	5.43	5.46	0.442	0.221	0.198
12	6.54	6.89	6.84	8.11	8.31	0.944	0.143	< 0.001
24	7.77	8.67	9.47	9.52	9.71	0.307	< 0.001	0.771
48	10.89	9.79	9.18	10.93	10.91	0.772	0.057	0.817

until the rinse was clear. The bags were dried at 60°C for 72 h and weighed to determine DM loss at different times of *in sacco* incubation. All dried samples were kept at −20°C until subsequent analyses.

One gram of samples was used for NDF analyses which were determined using an ANKOM200 Fibre Analyzer unit (ANKOM Technology Corporation, Macedon, NY, USA). The NDF was assayed without use of an alpha amylase but with sodium sulfite in the NDF and expressed without residual ash. Samples of straw were also analyzed for DM (#934.01), ash (#942.05), N (#954.01) and EE (#920.39) according to AOAC (1997).

Calculations and statistical analyses

Disappearance of DM, ADF and NDF at each incubation time was calculated from the portion remaining after incubation in the rumen. Disappearance rates were fitted to the equation of Ørskov and McDonald (1979) as:

$$Y_{(t)} = a + b\left(1 - e^{-ct}\right)$$

where: $Y_{(t)}$ is the proportion of the incubated material degraded at time t (hours of incubation); 'a' is the water soluble and instantly degradable fraction; 'b' is the potentially degradable fraction; 'c' is the fractional rate of degradation of fraction b (/h). The effective degradability of DM in each species was then estimated (Ørskov & McDonald 1979) by the equation: effective degradability (g/kg DM) = $a + bc/c + k$. In this equation, k refers to the fractional outflow rate of small particles from the rumen. A value of 0.05 fraction/h was used for k.

The experimental design was completely randomized where results were subjected to least squares analysis of variance using the MIXED procedure of SAS (2002). During *in vitro* incubation, data of each of the three runs within the same sample were averaged. Mean values of each individual sample within each treatment (i.e. three samples of each) were used as the experimental unit. Orthogonal polynomial contrasts were used to examine their responses (linear or quadratic) to increasing xylanase levels. Differences were considered significant at $P < 0.05$.

RESULTS

Wheat straw contained (g/kg on DM basis): crude protein, 40; ether extract, 6; NDF, 788; ADF, 473; and ash, 59.

After 48 h of incubation, the pH ranged from 6.63 to 6.74 and did not differ among treatments (Table 1). The pH decreased linearly with increasing levels of xylanase at 12 ($P = 0.016$) and 24 ($P = 0.006$) hours of incubation. The lowest pH values were at intermediate enzyme levels (i.e. 1.0 to 1.5 µL/g) at 3 and 6 h of incubation (linear and quadratic effects; $P < 0.001$). Total GP increased quadratically ($P < 0.001$) with increasing levels of xylanase. Methane production was increased (linear and quadratic effects; $P < 0.001$) at 3 and 12 h of incubation; it increased linearly at 24 h of incubation, and was unaffected at 6 and 48 h of incubation (Table 1).

Rumen NH_3-N concentration increased linearly at 3 h of incubation, was not affected at 6 h of incubation and had the highest values at intermediate enzyme levels at 12, 24 and 48 h of incubation (linear and quadratic effects; $P < 0.006$). All ruminal VFA concentrations increased linearly or had highest values with intermediate levels of xylanase (Table 2).

The *in sacco* rate of DM degradation decreased linearly ($P = 0.020$) with increasing enzyme levels, but the a, b and effective degradability values were not affected. *In sacco* degradation of NDF was not affected by xylanase levels (Table 3).

Table 2 Effects of enzyme levels on ruminal NH_3–N (mg/L) and VFA (mmol/L) concentrations of wheat straw at different times of incubation

Batch time (hours)	Enzyme levels, µL/g					SEM	P	
	E0	E0.5	E1.0	E1.5	E2.0		Linear	Quadratic
NH_3–N								
3	152.1	161.8	171.2	102.7	164.9	2.64	< 0.001	0.926
6	188.2	171.9	181.0	167.3	182.7	9.22	0.106	0.080
12	236.1	228.4	233.4	243.7	232.3	2.58	< 0.001	< 0.001
24	259.5	284.2	288.1	281.2	284.5	2.71	< 0.001	0.006
48	290.9	323.3	318.4	328.5	303.8	3.13	< 0.001	< 0.001
Total VFA								
3	23.8	33.4	34.3	33.4	31.8	1.28	< 0.001	< 0.001
6	25.4	37.0	39.2	43.0	40.3	1.72	< 0.001	0.003
12	32.6	43.1	47.7	46.1	43.9	3.78	0.005	0.352
24	40.2	52.7	54.3	50.8	45.6	2.80	< 0.001	0.297
48	48.2	62.0	63.6	63.9	61.6	2.00	< 0.001	0.019
Acetate								
3	11.5	15.3	15.6	15.3	14.5	0.58	< 0.001	0.002
6	12.1	17.3	17.7	19.4	18.5	1.01	< 0.001	0.008
12	15.8	21.4	24.3	23.5	22.5	2.06	0.003	0.335
24	21.9	27.5	28.2	26.5	24.0	1.43	0.001	0.374
48	27.4	33.1	33.8	33.2	33.5	0.74	< 0.001	0.037
Propionate								
3	6.28	9.79	10.03	9.62	9.20	0.391	< 0.001	< 0.001
6	6.47	9.47	10.94	12.14	11.03	0.593	< 0.001	0.099
12	nd	nd	nd	nd	nd			
24	nd	nd	nd	nd	nd			
48	nd	nd	nd	nd	nd			
Butyrate								
3	3.04	4.81	4.99	4.82	4.61	0.208	< 0.001	< 0.001
6	3.39	5.71	5.96	6.41	5.96	0.423	< 0.001	0.006
12	3.66	6.05	7.06	6.82	6.36	0.904	0.006	0.361
24	4.63	7.21	7.48	6.97	6.08	0.412	< 0.001	0.304
48	5.21	8.05	8.34	8.17	7.80	0.207	< 0.001	0.003
Valerate								
3	1.01	1.66	1.77	1.74	1.64	0.079	< 0.001	0.001
6	1.32	2.31	2.37	2.62	2.42	0.162	< 0.001	0.003
12	2.14	3.33	3.58	3.47	3.27	0.428	0.013	0.485
24	2.57	3.78	3.84	3.57	3.13	0.219	< 0.001	0.152
48	2.51	4.01	4.03	4.05	3.81	0.148	< 0.001	0.004

VFA, volatile fatty acids; nd, not determined.

DISCUSSION

Exogenous fibrolytic enzymes may improve the nutritive value of agricultural by-products due to enhanced attachment by rumen microorganisms (Nsereko *et al.* 2002), creation of stable enzyme feed complexes (Kung *et al.* 2000), and alteration of fiber structure, which stimulates microbial colonization (Giraldo *et al.* 2004). However, EFE can cause release of reducing sugar which can enhance ruminal microbial colonization (Alsersy *et al.* 2015). Results of the current study show that the rate of GP increased for wheat straw by the addition of xylanase. The amount of gas produced with xylanase at all incubation times may be related to internal plant cell wall structures of wheat straw. It has been reported that some commercial EFE increased total GP and rates of *in vitro* fermentation of feed (Wallace *et al.*

2001). Increased cell wall content was considered to suppress microbial activity through a reduction in the availability of rapidly fermented carbohydrates (Wilson & Hatfield 1997). However, it has been shown that EFE could potentially improve fiber degradation through a hydrolytic action prior to feeding or *in vitro* incubation (Giraldo *et al.* 2004; Elghandour *et al.* 2013). The increase in GP of xylanase-treated wheat straw at low and moderate levels, at all the incubation times, supports the hypothesis that a suitable enzyme dose improves fermentation efficiency, and/or that xylanase addition stimulated fermentation (Nsereko *et al.* 2002). Increased dose of xylanase may have prevented binding of enzymes to substrate receptors, which reduced proportional attachment by ruminal microorganisms to fiber (Treacher & Hunt 1996). Colombatto *et al.* (2003a) concluded that increasing the level of enzyme from 1× to

Table 3 Effect of enzyme levels on ruminal DM and NDF degradability (%) of wheat straw at different times of incubation in ruminally cannulated goats

| | | Enzyme levels, μL/g | | | | | SEM | *P* | |
		E0	E0.5	E1.0	E1.5	E2.0		Linear	Quadratic
In vitro	Dry matter								
	a	1.1	5.0	5.0	4.9	5.9	1.89	0.016	0.022
	b	41.1	69.6	52.2	30.2	93.1	4.00	0.025	0.037
	c (/h)	0.029	0.016	0.015	0.041	0.007	0.0981	0.520	0.652
	ED	16.2	22.2	17.2	18.5	17.6	2.97	0.236	0.326
	Neutral detergent fiber								
	a	0.3	2.2	2.1	2.6	2.0	0.07	0.001	0.025
	b	10.7	7.6	7.5	8.4	8.9	1.44	0.221	0.456
	c (/h)	0.06	0.08	0.10	0.08	0.10	0.329	0.015	0.060
	ED	6.3	7.0	7.0	7.8	7.8	1.94	0.143	0.154
In sacco	Dry matter								
	a	5.5	10.8	9.6	11.1	10.9	0.77	0.057	0.169
	b	26.8	35.2	23.5	28.0	27.0	2.19	0.354	0.593
	c (/h)	0.05	0.02	0.07	0.04	0.04	0.008	0.020	0.142
	ED	19.3	20.5	23.5	22.8	22.5	2.10	0.566	0.347
	Neutral detergent fiber								
	a	0.6	3.2	3.7	4.1	4.4	1.80	0.252	0.658
	b	31.5	43.0	26.1	49.2	61.6	4.11	0.525	0.647
	c (/h)	0.025	0.014	0.038	0.012	0.009	0.0111	0.648	0.235
	ED	11.1	12.6	14.9	13.6	13.6	2.16	0.761	0.125

a is the fraction that immediately disappears from the *in sacco* bag (intercept), *b* the insoluble fraction that is potentially degraded over time, *c* the fractional rate of degradation of fraction *b*, *c* is the rate of degradation (/h) and ED is the effective degradability.

5× increased the rate and extent of GP, but levels of 10× levels were ineffective.

Some studies reported that the fibrolytic enzyme did not affect GP (Giraldo *et al*. 2007a; Ruiz *et al*. 2013). 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 (Beauchemin *et al*. 2003; Carro *et al*. 2007), causing inconsistent results.

Methane emission was increased due to the addition of xylanase. Giraldo *et al*. (2007a) found that addition of xylanase at 15 and 30 IU/g of mixture of grass hay and concentrate at 60:40 increased methane emission. Increased methane emission with EFE in most studies was consistent with a greater production (Giraldo *et al*. 2007b). Acetate production is associated with the release of H_2, which can be used by methanogenic bacteria to form methane (Stewart *et al*. 1997). Only a few studies have investigated the effects of exogenous enzymes on methane production, and results are conflicting (Giraldo *et al*. 2007b).

The ruminal VFA concentrations increased with low level of xylanase which is consistent with Wang *et al*. (2002) and Giraldo *et al*. (2005) who showed that treatment of wheat straw with an enzyme preparation containing xylanase and β-gluconase activities increased VFA at 4 and 6 h of incubation. Increase of VFA may have been due to increased fiber digestion and altered ruminal fermentation (Nsereko *et al*. 2002), enhanced attachment and colonization to the plant cell wall material by rumen microorganisms

(Wang *et al*. 2001; Nsereko *et al*. 2002). Enzyme preparation can contain sugars that are fermented by ruminal microorganisms, thus increasing VFA production (Colombatto *et al*. 2003b).

Differences in DM and NDF rumen degradability of wheat straw 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 (Wang *et al*. 2002). Xylanase enzyme increased both potentially degradable fraction and degradation rate of NDF and ADF. The positive results of enzymes on *in vitro* degradation of substrate fiber are consistent with those reported by Yang *et al*. (2000) and Salem *et al*. (2015). The mode of action by which enzymes can affect digestion is still subject to speculation (Elwakeel *et al*. 2007). It has been suggested that improved degradation could be related to the fact that enzymes treatments increase bacterial colonization of feed particles (Yang *et al*. 2000), and reduce the release of sugar-digested portion of the feed-attracted bacteria to the site of digestion and enhance attachment of bacteria to undigested feed particles (Yang *et al*. 2000; Wang *et al*. 2001). The solubilized carbohydrates would also provide energy that would lead to rapid microbial growth, shortening the lag time for microbial colonization (Yang *et al*. 2000).

Dawson and Tricarico (1999) suggested that the most active period for enzyme effect is during the first

6 to 12 h of the digestive process, prior to bacterial colonization of feed substrates.

Greater effects with low or moderate amounts of xylanase than greater amounts of this enzyme agrees with the results reported by Beauchemin *et al.* (2000), in which greater amounts of enzyme preparations did not improve DM digestibility compared with reduced amounts of enzymes. Beauchemin *et al.* (2003) speculated that excessive enzymes applied to the feed may bind to sites used by rumen bacteria and make them unavailable, creating a barrier against microbial colonization.

Newbold (1997) noted that most enzymes function within a few hours of feeding before being degraded by the proteolytic activity of rumen microbes. In our study, applying xylanase to wheat straw increased, linearly, both the degradation rate and extent of DM and NDF in the rumen only at 3, 6 and 12 h after incubation. These results suggest that the treatment with xylanase stimulated the initial phases of feed degradation, but the effects were reduced as incubation time progressed. Lewis *et al.* (1999) added a xylanase-cellulase enzymes solution to total mixed ration of dairy cows based on alfalfa hay and corn silage, and reported an increase in milk production for the intermediate enzyme level compared with the low or high levels of enzyme addition. It is difficult to compare the dosages in our study with those used by Lewis *et al.* (1999) because a different enzyme product was used and the conditions under which the enzymatic activities were determined were not given. Similarly, in our study, the optimal dose for the degradability of wheat straw was intermediate dosage (1.0 to 1.5 µL/g) compared with the low or high levels of enzyme addition.

Conclusions

The effects of fibrolytic enzymes supplementation on *in vitro* GP and ruminal fermentation activities observed in the current study demonstrated that the addition of intermediate levels of xylanase (i.e. 1.0 to 1.5 µL/g), in general, improved GP, rumen NH_3-N concentration and volatile fatty acids in addition to enhancing *in sacco* degradability at early stages of fermentation.

Implications

Large amounts of straws may cause many pollution problems in some places. The addition of xylanase enzyme may improve its nutritive value as a ruminant feeds. Moreover, improved nutritive value of straws could aid in reducing endemic feed shortages for animal production in Mongolia and other developing countries.

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