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Effects of compound treatment of exogenous feed enzymes and microwave irradiation on *in vitro* ruminal fermentation and intestinal digestion of *guar* meal

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ABSTRACT

Effects of compound treatments of microwave irradiation (900 W) for 0, 2 and 4 min and exogenous feed enzymes at the application rate of 0, 250 and 500 g/ton on fermentation kinetics and digestibility of *guar* meal were evaluated by *in vitro* gas production and 3-step *in vitro* digestion techniques. Cumulative gas production was recorded at 2, 4, 6, 8, 12, 16, 24, 36, 48, 72 and 96 h of incubation and its kinetic was estimated using model: $GP = A[1 - e^{-c(t-L)}]$. To investigate ruminal and post ruminal digestion, the *in situ* and Ankom daisy-II incubation techniques were performed. Although enzyme supplementation levels had no significant effects on gas production, quadratic response to enzyme levels was observed after 12 h post incubation and continued up to 96 h post incubation. Microwave irradiation did not show significant impact on cumulative gas production. Interaction of enzyme and microwave irradiation was also not significant. While, there was no significant difference in potential gas production parameter (A) due to the effects of exogenous enzyme levels, microwave irradiation times or their interactions, gas production rate (c) was higher than control for all enzyme treatments. Significant effect of feed enzyme and microwave irradiation interaction was observed on *guar* DM digestibility. Post-ruminal digestibility of CP in compound treatment of no enzyme and 2 min microwave irradiation has the lowest digestibility (905.4 g/kg). Moreover, interaction between enzyme and microwave irradiation on *guar* meal post-ruminal CP digestibility was significant. It was concluded that heat processing of high protein oil seed meals followed by exogenous feed enzyme supplementation might lessen the possible Maillard reaction effects of heat processing. In conclusion, according to present study such order of compound treatments might shift protein digestion from rumen to post-ruminal sites. Consequently, this will improve protein and amino acids profile of followed digesta to small intestine, which is crucial in animal productivity performance.

Key words: *Guar* meal, Microwave irradiation, Enzyme pre-treatment

The most important *guar* growing area centres on Jodhpur in Rajasthan, India (Harris 2012). *Guar* (*Cyamopsis tetragonoloba*) meal, main by-product of *guar* gum production (Lee *et al*. 2002), is a protein-rich material containing about 33–60% protein and used as a feed ingredient for poultry and ruminants both, but requires processing to improve palatability and remove anti-nutritional factors. Feedstuffs high in rumen-undegradable protein (RUP) have a more gradual release of ammonia (Veen 1986). Heat-processing produces a palatable and digestible *guar* meal that can be used instead of soybean and cottonseed (Rahman and Leighton 1968).

Microwaves the non-ionizing electromagnetic waves (Tatke and Jaiswal 2011) are being investigated in ruminant nutrition (Sadeghi and Shawrang 2006, 2008, Maheri-Sis *et al*. 2012) because of the less startup time, faster heating, energy efficiency, space savings, precise process control, selective heating and final products with improved nutritive quality (Sumnu 2001). Release of reducing sugars by exogenous enzymes may increase available carbohydrates in rumen required to shorten the lag time needed for microbial colonization and also enhance rapid microbial attachment and growth (Forsberg *et al*. 2000). Availability of reducing sugars and heat treatment at the same time can accelerate Maillard reaction. Heat-xylose treatment might decrease ruminal protein degradation that occurs via Maillard reaction between aldehydes group of xylose and amino acid (Van Soest 1989). The objective of this study was to investigate the effects of different levels of fibrolytic enzymes and microwave irradiation, alone and in combinations, on *in vitro* gas production, fermentation characteristics, dry matter and crude protein ruminal and post-ruminal disappearance of *guar* meal.

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MATERIALS AND METHODS

Samples preparation and treatments: Guar meal was purchased commercially. The moisture content of *guar* meal samples was adjusted to 30% and determined according to AOAC (2002). Triplicate 500 g samples of moistened *guar* meal were placed in a Pyrex pan (28×28×6 cm) with 1 to 2 cm height and were subjected to microwave irradiation at a power of 900 W (1.8 w/g microwave energy) for 2 and 4 min. During the microwave irradiation samples were automatically rotated slowly to decrease in temperature gradients and to fix uniform temperature distributions.

The exogenous enzyme was a powdered multi-enzyme commercially available feed additive product containing cellulase 5,000,000 u/kg, xylanase 10,000,000 u/kg, beta-glucanase 1,000,000 u/kg, pectinase 140,000 u/kg, phytase 500,000 u/kg, α-amylase 1,800,000 u/kg activities, as indicated by the manufacturer. This product also contains hemicellulase, amyloglycosidase, pentosanase, pectinase and phytase activities. A 0.25% stock solution of enzyme was prepared by dissolving 5 g Natuzyme in 200 ml distilled water.

In vitro *gas production incubation:* Rumen liquor samples were obtained from the 3 adult Ghezel×Arkhar-merino crossbred male sheep (38±1.5 kg) that were fed twice a day on a diet comprising 800 g DM alfalfa hay and 200 g DM commercial concentrate was offered to the animals twice daily at 09.00 AM and 16.00 PM in equal sized meals at maintenance level (NRC 1985). The animals had access to freshwater and mineral lick *ad lib*. Rumen fluid, obtained 2 h after morning feeding. Rumen fluid was pumped with a manually operated vacuum pump and transferred into pre-warmed thermos flask, combined, filtered through four layers of cheesecloth and flushed with CO_2.

Four replicate 65 ml serum vials were prepared for each of 9 treatments (3 × 3 factorial arrangement of microwave irradiation for 0, 2 and 4 min and enzyme at 0, 0.25 and 0.50 g/kg DM) and blank for *in vitro* gas production. Untreated and microwave treated *guar* meal was dried, ground to pass a 2.0-mm screen used as substrates, loaded (300 mg) into serum vials and treated 24 h before incubation with calculated exact volumes of 0.25% enzyme stock solution to supply the final concentration of enzyme which needed (0, 0.25, 0.50 g/kg DM).

Buffered (McDougall 1948) rumen liquor solution (2: 1, buffer: liquor) was added (20 mL) to each vial. All vials were purged with oxygen-free CO_2 and then sealed firmly with a butyl rubber stopper again, placed on a rotary incubator-shaker at 39°C. At each incubation time (2, 4, 6, 8, 12, 16, 24, 36, 48, 72 and 96 h), 4 vials were removed from the incubator and gas production (GP) was measured using a water displacement technique (Fedorak and Hrudey 1983).

Three-step in vitro *procedure:* Three ruminally cannulated sheep of first experiment were used to ruminal *in situ* degradation of *guar* meal DM and CP on maintenance feeding. Animals fed a diet containing 60% alfalfa hay and 40% commercial concentrate at maintenance level (NRC 1985). Animals were fed twice a daily at 09.00 and 18.00 h (with free access to water) and were adapted to 3 wk before the incubation. Approximately 5 g of untreated and treated *guar* meal in 10 replications placed into 10 × 5 cm heat sealed nylon bags and incubated in the rumen for 12 h with rumen liquor (Gargallo *et al.* 2006).

Upon ruminal removal, the bags were squeezed and rinsed for 5 min 3 times in an automatic washing machine (or until the runoff is clear) and stored at "18°C. Residues were adjusted to chemical analysis and the rest were pooled. Residuals were analyzed for dry matter and N content. Calculate the pepsin-pancreatin digestion of protein as the amount of the sample N (rumen-exposed residue) minus the N remaining after pepsin-pancreatin incubation divided by the amount of sample N (Gargallo *et al*. 2006).

Chemical analysis: Guar meal dry matter (DM, method ID 934.01), ash (method ID 942.05), ether extract (method ID 920.30) and crude protein (CP, method ID 984.13) were determined as per AOAC (2002). Neutral-detergent fiber (NDF) and acid detergent fiber (ADF) were determined with fiber analyzer using the manufacturer recommended reagents and filter bags. Analysis of NDF was conducted with a heat stable α-amylase and without sodium sulphite and expressed exclusive of residual ash as ADF.

Curve peeling and statistical analysis: Gas production kinetic parameters were estimated using Marquardt method with NLIN option of GLM procedure of SAS 9.1 (SAS 2003) according to the exponential equation of France *et al*. (2000). The analysis of variance was performed using the GLM procedures of SAS (2003) using the model:

$$Y_{ij} = ¼ + ±_i + ²_j + \mu_{ijk},$$

where Y_{ij} is the value of each individual observation for the dependent variables, *¼* the overall mean, α_i the effect of *i* level of enzyme (*i=0, 1, 2*), β_j the effect of *j* level of microwave irradiation (*j=0, 1, 2*) and μ_{ijk} the random residual error. The effects of the levels of enzyme and microwave irradiation were assessed using orthogonal polynomial contrasts to test for linear and quadratic effects of the level of them, separately. Differences among treatments within incubation were determined PDIFF statement of LSMEANS option of SAS (SAS 2003).

RESULTS AND DISCUSSION

Chemical composition: Chemical components (Table 1) are similar to those reported by Jahani-Azizabadi *et al*. (2010). Observed difference for CP, NDF and ADF content

Table 1. Chemical composition of *guar* meal (gr/kg DM)

Dry matter	955.00
Ash	51.00
Crude protein	496.20
Neutral detergent fiber	151.10
Acid detergent fiber	66.20
Hemicellulose [1]	84.90
Ether extract	4.70

[1] Hemicellulose,Neutral detergent fiber, Acid detergent fiber.

of *guar* meal in literature can be due to variety differences, type of oil extraction process (mechanical, semi mechanical or using chemical solvents) during *guar* meal production.

In vitro *gas production:* Cumulative gas production data of 2, 4, 6, 8, 12, 16, 24, 36, 48, 72 and 96 h incubation times and gas production kinetics parameters are shown in Table 2. We have preferred 96 h of incubation period for amplification of kinetics' data (Orskov and McDonald 1979). The impact of consideration an appropriate interaction time in enzyme supplementation is to create a stable enzyme-feed complex that protects free enzymes from proteolysis in the rumen (Kung *et al*. 2000). However, some studies demonstrated that fibrolytic enzymes may be resistant to rumen proteolysis for a significant period of time (Hristov *et al*. 1998, Morgavi *et al*. 2000, 2001). Although enzyme supplementation levels had not significant effects of gas production throughout incubation times (P>0.05; Table 2), quadratic response of gas production to enzyme levels was observed 12 h post incubation and continued up to 96 h post incubation (P<0.01). This suggested that there is not linear relationship between enzyme levels and cumulative gas production of *guar* meal in presence of microwave irradiation. Morgavi *et al.* (2000) showed that low levels of enzyme from *T. longibrachiatum* stimulated the adhesion of *Fibrobacter succinogenes* to corn silage and alfalfa hay though this effect was lost at high levels. They concluded that at high levels the fibrolytic enzymes competed with the rumen bacterium for available binding sites on cellulose. Microwave irradiation has not significant impact on cumulative gas production (P>0.05). Also, interaction of different enzyme levels and microwave irradiation times was not significant (Table 2). Although microwave treatment causes less thermal damages to the test material than general heating methods such as hot water heating, it causes biochemical reactions (Banik *et al*. 2003) and changes the molecular conformation of starch (Lewandowicz *et al*. 2000) and protein (Gross *et al*. 1988) and physicochemical properties, such as the solubility and gelatin temperature (Lewandowicz *et al*. 2000), of feed products. Hence, such molecular structure change can affect cumulative gas production of *guar* meal that exist at current study.

Up to our knowledge, most part of feed enzyme products, studied at recent researches consisted of carbohydrase enzymes that originated from ruminal microorganism. The xylanases are the main enzymes involved in degrading xylan core polymer to soluble sugars. In general, endoglucanases hydrolyze cellulose chains at random to produce cellulose oligomers of varying degrees of polymerization; exoglucanases hydrolyze the cellulose chain from the non-reducing end, producing cellobiose, and ß-glucosidases hydrolyze short chain cellulose oligomers and cellobiose to glucose (Beauchemin *et al*. 2003). One of the main ingredients of current study feed enzyme is xylanase and ß-1,4 xylosidase. Bhat and Hazlewood (2001) described that major enzymes involved in degrading the xylan core polymer to soluble sugars are xylanases and

ß-1,4 xylosidase. Cumulative gas production (ml/g DM) values were increased by enzyme addition, which suggested that the total amount of fermentable material (core polymer to soluble sugars) was changed by enzyme addition (Table 2).

Analysis of the fitted data revealed that there were not significant differences in potential gas production (A) between treatments and effects of enzyme levels, microwave irradiation times or their interactions were not significant. In contrast, the gas production rate (c) was higher than control for all enzyme treatments (P < 0.01). The 2 min microwave irradiation treatment resulted in the numerically highest potential gas production parameters among other treatments (Table 2) as well as this, the highest amounts of this parameter were observed in moderate concentration of enzyme treatment (253.6, 265.6 and 267.8 ml/ g DM for 0, 2 and 4 min of microwave irradiation

Table 2. Effects of different compound treatments on *in vitro* gas production of *guar* meal

	No enzyme			Enzyme (250 g/ton)			Enzyme (500 g/ton)				*P-values* [1]			*Contrasts* [2]			
Microwave	No	2 min	4 min	No	2 min	4 min	No	2 min	4 min	S.E.M	E	M	E×M	EL	EQ	ML	MQ
Gas production (ml/g DM)																	
2 h	24.4	19.8	24.6	22.1	23.0	20.2	24.3	23.5	22.9	1.44	NS	NS	NS	**	NS	NS	NS
4h	54.9	47.6	50.3	53.4	50.6	45.6	51.6	51.9	51.3	2.54	NS	NS	NS	NS	NS	NS	NS
8 h	101.9	97.9	88.7	95.9	103.4	96.3	92.3	100.3	97.3	5.06	NS	NS	NS	NS	NS	*	NS
12 h	146.4	136.0	129.1	120.6	138.5	132.5	115.5	132.4	131.0	6.59	NS	NS	NS	*	NS	NS	NS
24 h	216.8	202.9	193.9	199.4	206.9	205.7	175.8	191.7	196.8	8.87	*	NS	NS	NS	*	NS	NS
48 h	255.3	235.2	225.9	237.9	247.9	247.4	204.4	229.6	237.5	12.03	NS	NS	NS	NS	*	NS	NS
96 h	271.7	255.0	245.5	254.4	272.3	273.8	220.4	255.5	265.3	12.25	NS	NS	NS	NS	*	NS	NS
Parameters of gas production [3]																	
A	268.8	248.6	241.5	253.6	265.6	267.8	216.8	247.8	258.4	12.36	NS	NS	NS	NS	*	NS	NS
c	0.069	0.072	0.070	0.060	0.064	0.061	0.068	0.063	0.060	0.0039	*	NS	NS	NS	NS	NS	NS
lag	0.679	0.941	0.490	0.295	0.542	0.801	0.043	0.182	0.333	0.1512	***	NS	NS	NS	*	NS	NS

[1] E, Enzyme effect; M, microwave irradiation effect; E×M, interaction effect between E and M. [2] EL, linear response of enzyme levels; EQ: quadratic response of enzyme levels; ML, linear response of microwave irradiation times; ML, quadratic response of microwave irradiation times. [3] A: ml/ g DM; c: h^{-1}; h: h

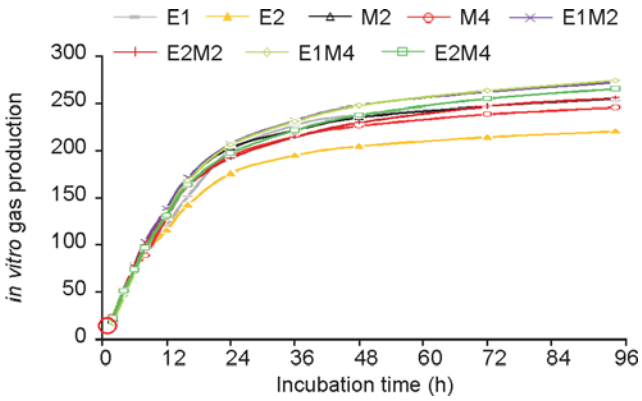

Fig. 1. Cumulative gas production curves of exogenous enzyme (E) and microwave (M) treated and untreated guar meal overall incubation times {E1: enzyme (250 g/ton); E2: enzyme (500 g/ton); M2: microwave treated for 2 min; M4: microwave treated for 4 min; E1M2: enzyme (250 g/ton) + microwave treated for 2 min; E2M2: Enzyme (500 g/ton) + microwave treated for 2 min; E1M4: enzyme (250 g/ton) + microwave treated for 4 min; E2M4: enzyme (500 g/ton) + microwave treated for 4 min}.

respectively in 250 g/ ton enzyme application rate). The fractional rate of gas production (c) increased with increasing enzyme in all microwave treatments, suggesting that enzymes degraded complex substrates to simpler ones, allowing a faster ruminal colonization and fermentation, as observed also by Colombatto *et al*. (2003). Also, Lag time parameter of gas production was affected significantly by different enzyme levels (P<0.001; Table 2).

Three-step in vitro procedure

Rumnail digestibility: Guar meal treated by compound treatment of 500 g/ton fees enzyme + 2 min microwave irradiation had the maximum 12 h ruminal digestibility and the lowest amount (Table 3) was observed with no enzyme + 4 min microwave irradiated treatment. Significant effects of enzyme and microwave irradiation interaction on *guar* DM digestibility were observed (P<0.001). Whereas use of compound treatment of 2 min of microwave irradiation + 250 g/ton enzyme resulted in the highest CP digestibility, no enzyme + 2 min of microwave irradiation treatment has the lowest CP digestibility (Table 3). Likewise, observation for DM, raw *guar* meal has moderate digestibility of CP (791.9 g/kg) in comparison with other treatments (Table 3). Ruminal digestibility of CP and DM data are in agreement with the mentioned logics for gas production because in absence of feed enzymes, microwave irradiation of high moisture *guar* meal induces reduction in DM and CP ruminal digestibility due to intensification of Maillard reaction, formation of disulphide bands and finally increase of acid detergent insoluble nitrogen fraction (Jahani-Azizabadi *et al.* 2010, Parnian *et al.* 2013).

Post-ruminal digestibility: Microwave irradiation for 2 min and enzyme addition @ 250 g/ton resulted in the highest post-ruminal DM digestion (Table 3) while lowest digestibility was observed for no microwave irradiation and 500 g/ton treatment. In CP post-ruminal digestibility, no enzyme + 2 min microwave irradiation treatment has the lowest digestibility. The highest post-ruminal digestibility was belonging to raw *guar* meal (no enzyme + no microwave treatment). In addition, enzyme supplementation on ruminal digestibility and effects of interaction between enzyme and microwave treatment were significant (Table 3). Jahani- Azizabadi *et al.* (2010) remarked that heat treatment and heat-xylose treatment effectively impacted reduction of DM and CP ruminal disappearance and increased ruminaly undegradable protein fraction of *guar* meal's protein. Moreover Fathi Nasri *et al*. (2006) reported that heat processing of soybean meal, declined its DM and CP ruminal degradability as well as increased intestinal digestibility. In agreement with other reports, Broderick *et al.* (2006) proposed that heat treatment of linseed meal decreased ruminal CP and DM disappearance and increased bypass protein amounts (rumen undegradable protein) and its intestinal digestibility. They concluded that such increment of bypass protein in response to heat treatment might be result of blocking active and reaction sites of feed with microbial proteolytic enzymes in the rumen.

To explain the observed change in ruminal degradability of *guar* meal protein, this may proceed by unraveling of α-helical chains through the heating process, their alignment and reorganization into the ß-sheet conformation (Parnian *et al.* 2013). In other word, heat treatment under certain conditions reduces ruminal degradable starch fraction by trapping and making the protein matrix more resistant to proteolysis (Ljøkjel *et al*. 2003). Release of reducing sugars

Table 3. Effects of different compound treatments on ruminal and post-ruminal degradability of *guar* meal

	No enzyme			Enzyme (250 g/ton)			Enzyme (500 g/ton)				*P-values* [1]			*Contrasts* [2]			
Microwave	No	2 min	4 min	No	2 min	4 min	No	2 min	4 min	S.E.M	E	M	E×M	EL	EQ	ML	MQ
12 h in situ ruminal digestion (g/kg)																	
DM	848	754	698	895	918	937	915	950	980	12.5	****	NS	****	NS	NS	****	NS
CP	792	737	593	845	886	917	885	935	972	17.1	****	NS	****	NS	NS	****	*
Post-ruminal digestion (g/kg)																	
DM	763	811	791	761	835	815	733	796	799	7.6	**	****	NS	NS	****	*	**
CP	953	905	930	948	940	930	943	918	927	2.86	***	****	****	***	****	***	****

[1]E, Enzyme effect; M, microwave irradiation effect; E×M, interaction effect between E and M.[2] EL, linear response of enzyme levels; EQ: Quadratic response of enzyme levels; ML, linear response of microwave irradiation times; ML, quadratic response of microwave irradiation times.

by exogenous enzymes is probably an important mechanism by which exogenous enzymes operate. This may therefore increase available carbohydrates in the rumen required to shorten the lag time needed for microbial colonization and also enhance the rapid microbial attachment and growth (Forsberg *et al.* 2000). It was concluded from the study that disappearance characters of feedstuff achieved by both ruminal and post-ruminal incubation of feedstuff in nylon bags, could reveal feed additives and different processing methods as a potential and feasibility at current age of animal nutrition. Microwave irradiation following by feed enzymes treatments in high protein diets that including oilseed meals, might change protein digestion from rumen to post-ruminal sites as well as lessen extra effects of Maillard reaction. Application of microwave irradiation to process oilseeds can use extensively regarding its environment friendly, economical, clean and more available technology.

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