Journal of Animal and Veterinary Advances 9 (19): 2466-2471, 2010
ISSN: 1680-5593

The Effect of Fat Content of Chemically Treated Sunflower Meal On *in vitro* Gas Production Parameters Using Isolated Rumen Microbiota

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Abstract: This experiment was conducted to investigate the effect of fat content (high: 165 g fat kg^{-1} DM and low: 25 g fat kg^{-1} DM) of sodium hydroxide (40 g kg^{-1} DM) or formaldehyde (3 and 6 g kg^{-1} DM) treated sunflower meal on *in vitro* gas production parameters used mediums containing isolated rumen microorganisms including total rumen microbiota (TM), bacteria (B), protozoa (P) or fungi (F). Results showed formaldehyde (both applied concentrations) caused a significant reduction in the rate and gas production from fermentable fraction of sunflower meal samples by the isolated microbial groups. Sunflower meal with high fat concentration treated with NaOH had the highest gas production ($p<0.05$) when fermented by the rumen isolated micro-biota (193, 33, 89 and 175 mL 500 mg DM sample for TM, B, P and F, respectively). Gas produced from the chemically treated or untreated high fat containing sunflower meal was more than the low fat content samples. Therefore, it was concluded both fat concentration and chemical treatments used in the present study may affect the fermentation potential of sunflower meal as evaluated by the applied in vitro procedure. In addition, *in vitro* gas production of high and low fat content sunflower meal by isolated rumen microbiota fractions are influenced by formaldehyde and NaOH treatments.

Key words: Sunflower meal, bacteria, protozoa, fungi, sodium hydroxide, formaldehyde

INTRODUCTION

Rumen microorganisms based on the degradation rate of plant cell walls and degrading enzyme activities could be categorized into 3 distinct groups; bacteria, protozoa and fungi (Lee *et al*., 2000) have a key role in the degradation of polysaccharides of plant cell walls but cellulolytic bacteria, such as *Ruminococcus albus*, *R. flavefaciens* and *Fibrobacter succinogenes* are a major microorganisms responsible for ruminal digestion of plant cell walls due to their metabolic diversity and numerical predominance (Cheng *et al*., 1991).

Dijkstra and Tamminga (1995) reported that the protozoa digest fiber in the rumen by 5-21%. Although, the protozoa make a significant contribution to fibre digestion by rumen microorganisms, their removal allows more bacteria to colonize the plant fibers (Newbold *et al*., 1989).

The role of fungi to the degradation of any feed fiber in the rumen is still unclear due to the difficulty in estimation of exact biomass of anaerobic fungi in the rumen contents. However, recently a quantitative competitive PCR assays for relative quantifying rumen anaerobic fungal populations in both *in vitro* and *in vivo* systems has been proposed (Sekhavati *et al*., 2009). Lee *et al.* (2000) concluded that fungal activity was mostly responsible for cell wall degradation. Fungi accompanying methanogenic bacteria might increase the digestion rate of cellulose or hemicellulose (Bauchop and Mountfort, 1981). However, it was reported that cellulolytic activity of certain species of fungi could be inhibited by some fibrolytic Ruminococci (Bernalier *et al*., 1992). The production of gas during the microbial fermentation of plant material by rumen contents has been used as an indirect measure of substrate digestibility (Menke and Steingass, 1988; Chaji *et al*., 2009).

The objective of present experiment was to determine the effect of fat content (high: 165 g fat kg^{-1} DM and low: 25 g fat kg^{-1} DM) of sodium hydroxide (40 g kg^{-1} DM) or formaldehyde (3 and 6 g kg^{-1} DM) treated sunflower meal on *in vitro* gas production parameters used mediums containing isolated rumen microorganisms including total rumen microbiota (TM), Bacteria (B), Protozoa (P) or Fungi (F).

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MATERIALS AND METHODS

Samples of Sunflower Meal (SM) containing different fat concentration (high: 165 g fat k g^{-1} DM (HSM) and low: 25 g fat kg^{-1} DM (LSM) were provided. They used as untreated or chemically treated using formaldehyde (37% solution of commercial grade formalin) at two levels of 3 and 6 g kg^{-1} DM of formaldehyde or one level of sodium hydroxide (40 g kg^{-1} DM).

The chemical reagents were sprayed on the samples and kept the mixture for 30 min at room temperature. Then samples were transferred into PVC bags, shacked for 5 min, sealed, evaporated and kept for 5 days at room temperature. Bags were then opened and spread the content in thin layers (4 mm) on a concrete floor to air-dried.

Experimental treatments were Untreated HSM (UHSM), NaOH treated HSM (SHSM), formaldehyde treated HSM [(F3HSM (3 g formaldehyde kg^{-1} DM treated HSM) and F6HSM (6 g formaldehyde kg^{-1} DM treated HSM)], Untreated LSM (ULSM), NaOH treated LSM (SLSM) and formaldehyde treated LSM [F3LSM (3 g formaldehyde kg^{-1} DM treated LSM) and F6LSM (6 g formaldehyde kg^{-1} DM treated LSM).

Rumen fluid was collected from four rumen fistulated sheep (body weight: 40 kg) prior to the morning feeding. Animals were daily fed 450 g concentrate, 550 g alfalfa hay and 180 g wheat straw once at day (09:00 a.m.). Rumen content was strained through four layers of cheese cloth.

The strained and free feed residual of rumen fluid was used as total rumen microbiota. Rumen isolated protozoa was obtained using centrifuge procedure (1000 RPM, 5 min). The isolation of ruminal fungi was carried out from non protozoa strained rumen fluid using antibacterial agent (streptomycin sulfate, penicillin G, potassium and chloramphenicol (0.1 mg m^{-1} each). Ruminal bacteria was isolated from non protozoa strained rumen fluid using antifungal agent (benomyle (500 ppm L^{-1}) and metalaxyle (10 mg L^{-1}). Antibiotics and the other chemical reagents applied to isolate rumen bacteria and fungi were added as 0.1 mL per 1 mL of *in vitro* gas production medium culture. *In vitro* method of Menke and Steingass (1988) was used to determine gas produced from each ruminal microbial fractions. Approximately, 500 mg of each sample (1.0 mm screen) were incubated with 35 mL buffered rumen fluid containing TM, B, P or F under continuous CO_2 reflux in a 100 mL calibrated glass syringes for 2, 4, 6, 8, 10, 12, 16, 24, 48, 72 and 96 h in a water bath msaintained at 39°C. Each sample in 4 replicates was incubated in 3 run together with three syringes containing only incubation medium (blank). After 96 h of incubation, the culture fluid of each sample was carefully removed into centrifuge tubes and pH values were immediately measured. Cumulative gas production data were fitted to the exponential equation:

$$Y = b (1-e^{-ct})$$

Where:

b = The gas produced (mL) from 500 mg DM of each sample
c = The gas production constant rate (h^{-1})
t = The incubation time (h)
Y = The gas produced at time t

Data of gas production parameters and pH were subjected to analysis as a completely randomized block design using the general linear model procedure by SAS (1996). Duncan's multiple range test was used to compare the means at $p<0.05$.

RESULTS

The gas production parameters (b and c) of chemically untreated and treated of low and high fat sunflower meal samples by total rumen microorganism, protozoa, bacteria and fungi over 96 h incubation are shown in Table 1. Formaldehyde at both concentration applied (3 and 6 g kg^{-1} DM) caused a significant decrease ($p<0.05$) in gas production from fermentable fraction. However, NaoH caused to increase the gas production parameters when samples were incubated with isolated ruminal mirobiota.

The highest and the lowest gas production by the rumen microbial fractions were obsereved when sunflower meal containing both levels of fat was treated with NaoH and 6 g formaldehyde, respectively ($p<0.05$). The highest gas production for all the samples evaluated was observed in medium containing TM which was followed by B whereas, a less gas volume was produced by F and P. The patterns of gas production parameters influenced by the fat content were similar between samples fermented by various rumen isolated microbial groups. The effect of formaldehyde, sodium hydroxide and fat content of the sunflower meal samples on *in vitro* pH of the medium pH containing rumen isolated microbial groups shown in Table 2. The lowest and the highest pH were detected in medium used sunflower meal treated with sodium hydroxide and 6 g formaldehyde, respectively ($p<0.05$).

Medium pH for low fat sunflower meal samples was higher than those of high fat sunflower meal ($p<0.05$). There was a significant difference in medium pH values when various rumen isolated microbial groups were considered. The lowest pH values were observed in medium of TM groups and the highest was for P.

Table 1: Gas production parameters of untreated or chemically treated (sodium hydroxide or formaldehyde) of low and high fat sunflower meal using isolated rumen microbial fractions

	Rumen microbial fractions**				Fat content effect**		Chemically processing effect**	
Treatments	Total microbiota	Bacteria	Protozoa	Fungi	SEM	p-value	SEM	p-value
Gas produced (mL^{-1} 500 mg								
ULSM	175.00	157.00	25.00	75.00	4.2	<0.05	2.3	<0.05
SLSM	190.00	170.00	32.00	87.00	-	-	-	-
F3LSM	137.00	116.00	20.00	53.00	-	-	-	-
F6LSM	86.00	70.00	15.00	40.00	-	-	-	-
UHSM	177.00	161.00	27.00	76.00	-	-	-	-
SHSM	193.00	175.00	33.00	89.00	-	-	-	-
F3HSM	146.00	123.00	20.00	56.00	-	-	-	-
F6HSM	108.00	91.00	18.00	45.00	-	-	-	-
Gas production constant rate (h^{-1})								
UlSM	0.08	0.02	0.03	0.02	0.003	<0.05	0.002	<0.05
SLSM	0.09	0.02	0.05	0.03	-	-	-	-
F3LSM	0.07	0.02	0.03	0.02	-	-	-	-
F6LSM	0.03	0.01	0.02	0.02	-	-	-	-
UHSM	0.06	0.02	0.03	0.03	-	-	-	-
SHSM	0.08	0.02	0.05	0.03	-	-	-	-
F3HSM	0.02	0.02	0.02	0.02	-	-	-	-
F6HSM	0.02	0.02	0.02	0.02	-	-	-	-

*Experimental treatments were untreated HSM (UHSM), NaOH treated HSM (SHSM), formaldehyde treated HSM [F3HSM (3 g formaldehyde kg^{-1} DM treated HSM) and F6HSM (6 g formaldehyde kg^{-1} DM treated HSM)], untreated LSM (ULSM), NaOH treated LSM (SLSM) and formaldehyde treated LSM [F3LSM (3 g formaldehyde kg^{-1} DM treated LSM) and F6LSM (6 g formaldehyde kg^{-1} DM treated LSM)]. **When mean±SEM of a treatment in each row and each column was greater than mean±SEM of the others, it is considered as significant ($p<0.05$)

Table 2: Main effect of fat content and chemically processing applied on *in vitro* gas production medium pH (96 h incubation) of sunflower meal using isolated rumen microbial fractions

	Rumen microbial fractions**				Fat content effect		Chemically processing effect	
Treatments*	Total microbiota	Bacteria	Protozoa	Fungi	SEM	p-value	SEM	p-value
Fat content								
High (165 g kg^{-1} DM)	6.52	6.93	7.30	7.11	0.001	<0.05	0.002	<0.05
Low (25 g kg^{-1} DM)	6.42	6.69	6.92	6.83	-	-	-	-
Chemically processing								
NaOH (40 g kg^{-1} DM)	6.24	6.69	6.92	6.83	-	-	-	-
Formaldehyde (3 g kg^{-1} DM)	6.52	6.96	7.40	7.17	-	-	-	-
Formaldehyde (6 g kg^{-1} DM)	6.65	7.07	7.47	7.30	-	-	-	-

*Experimental treatments were Untreated HSM (UHSM), NaOH treated HSM (SHSM), formaldehyde treated HSM [F3HSM (3 g formaldehyde kg^{-1} DM treated HSM) and F6HSM (6 g formaldehyde kg^{-1} DM treated HSM)], untreated LSM (ULSM), NaOH treated LSM (SLSM) and formaldehyde treated LSM [F3LSM (3 g formaldehyde kg^{-1} DM treated LSM) and F6LSM (6 g formaldehyde kg^{-1} DM treated LSM)]. **When mean±SEM of a treatment in each row and each column was greater than mean±SEM of the others, it is considered as significant ($p<0.05$)

DISCUSSION

The main objective of the present experiment is to evaluate the effect of fat concentration of untreated or chemically treated sunflower meal samples on *in vitro* gas production parameters when rumen isolated microbial groups used as inoculants. Previously, it was reported that *in situ* ruminal degradation of sunflower meal was influenced by fat content and chemically processing applied (Mohammadabadi *et al*., 2008). The amount of gas produced from rumen microbial fermentation of a feedstuff under *in vitro* condition is closely related to the digestibility and therefore to the energetic value of any feed for ruminants (Menke *et al*., 1979). Under the condition of the present experiment, NaOH caused an increase in the rate and gas production from fermentable fraction of the experimental samples when isolated microbial groups applied. Maximum gas volume was observed for rice straw treated with NaOH (Chen *et al*., 2007). Corn stover treated with 3% NaOH had higher ($p<0.05$) fiber and dry matter digestibility than did the untreated samples (Anderson *et al*., 1981). It was indicated that hemicellulose present in the untreated wheat straw was solubilized (32.5%) upon NaOH treatment (Lesoing *et al*., 1981). Bas *et al*. (1989) reported DM and NDF digestion of the straw treated with an alkali was greater than untreated straw (82 and 85%, respectively).

The alkali solution caused to hydrolyse the ester linkages between lignin and the cell wall polysaccharides mainly hemicellulose (Chesson, 1981). This makes carbohydrates and hemicellulose more accessible to the action of rumen microorganisms followed by significant improvements in organic matter digestibility and consequently increases the dry matter intake by animals (Nakashima and Orskov, 1989). Alkali solution caused to

cleave esterified bounds within cell wall structure, reduce the physical enmeshment of cellulose and solubilize the inhibitory phenolic compounds; thereby enhancing enzymatic saccharification and facilitates microbial colonization of plant cell walls and improve the ruminal degradation (Euna *et al*., 2006).

In addition any alkali solution provide a condition to increase the colonization of bacteria in cell wall, increase fungi and bacteria populations in rumen fluid or solid fractions, increase accessible locations for microbial attach and enzymes of carboxymethyle cellulolase, aviclase and xlylanase (Chen *et al*., 2007). Gould (1984) proposed that a dilute solution of alkali react with lignocellulosics to yield partially delignified products that are highly susceptible to enzymatic and microbial attach. The enhanced degradability has been ascribed to a solubilisation of total phenols (Chesson, 1981), arabinoxylans and cellulose (Lindberg *et al*., 1984) and arising from the cleavage of alkali-labile lignin-carbohydrate linkages (Alexander *et al*., 1987).

Under the present experiment condition, the protection of the experimental samples by formaldehyde decreased both b and c parameters. El-Waziry *et al.* (2005) reported a reduction in both b and c fractions for 3% tannic acid-protected soybean meal. Under present study condition, formaldehyde decreased the gas production parameters (b and c) when used at 6 g kg^{-1} compared with the less concentration. Present data show that the bacterial group generates a larger volume of gas production than fungal or protozoal group after 96 h fermentation. Rumen cellulolytic bacteria are believed to be responsible for most of the feed digestion in the rumen because of their numerical predominance and metabolic diversity (Cheng *et al*., 1991; Lee *et al*., 2000).

Concluded that fungal activity was responsible for most of cell wall degradation and degradation by anaerobic bacteria was significantly less than that of fungal. Anaerobic ruminal fungi interact with hydrogen-utilizing bacteria such as methanogens (Bauchop and Mountfort, 1981). The quantitative estimation revealed that a quarter to one-third of fiber breakdown in the rumen is of protozoal origin (Joblin, 1990).

Data of present experiment showed the highest gas production was observed in medium containing TM. Hidayat *et al*. (1993) reported that the highest rate of gas production was observed in fungi+protozoa. These controversial results about the relative contributions of rumen microbial groups to the degradation of plant cell walls may be associated with ways to isolate different microbial groups. Onodera *et al.* (1988) reported that the addition of protozoa to bacteria increased ruminal cellulose digestion but Newbold *et al.* (1989) demonstrated that ruminal cellulolytic activity of particle-associated populations was not affected by ruminal protozoa. Hidayat *et al*. (1993) reported that when the extent of hay and barley straw digestion by bacteria was maximal, the addition of either protozoa or bacteria did not further stimulate digestion.

Joblin (1990) concluded that *in vitro* examination of short-term protozoan-fungus incubations revealed that protozoa ingested fungal rhizoids and immature sporangia, therefore, *in vitro* studies that protozoa in the rumen probably interact negatively with anaerobic fungi (Joblin, 1990).

The results by Lee *et al.* (2000) showed that when the fungal fraction was incubated with the protozoal fraction, a steady decline (15.58%) in the degradation rate was observed at the end of the incubation period. Another possible explanation is that fungal sporangium can be degraded by protozoal chitinolytic enzymes (Morgavi *et al*., 1994).

Results of the present experiment, showed that sunflower meal treated with sodium hydroxide caused to decrease the medium pH; the highest pH was for sunflower meal treated with 6 g formaldehyde. Medium pH for low fat sunflower meal was more than those of the high fat sunflower meal samples incubated by all microbial fractions which confined the findings by Getachew *et al*. (2001) who reported that the addition of yellow grease and corn oil increased *in vitro* degradation and gas production but decreased pH. Machmueller *et al.* (1998) reported the reduction of protozoa by sunflower seed might decrease gas production. Results of the present study, indicated that the values of pH among various microbial groups were different.

CONCLUSION

It was observed that both *in vitro* gas produced from the fermentable fraction and constant rate of gas production were influenced by the fat content and the chemically processing applied to the samples evaluated.

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