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Full Length Research Paper

Evaluation of the effect of fat content of sunflower meal on rumen fungi growth and population by direct (quantitative competitive polymerase chain reaction) and indirect (dry matter and neutral detergent fibre disappearance) methods

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The major aim of this study was to evaluate the effect of fat content of sunflower meal (150 and 30 g fat /kg dry matter, high and low fat, respectively) on population, growth and activity of rumen anaerobic fungi by using direct (quantitative competitive polymerase chain reaction, QC-PCR) and indirect (dry matter (DM) and neutral detergent fibre (NDF) disappearance in rumen fungi media culture for 12 days) methods. The results of QC-PCR showed that rumen anaerobic fungi population in the medium containing high fat sunflower meal was greater as compared to low fat sunflower meal (+0.14 *vs.* +0.10) (P<0.05). Also, disappearance of dry matter after 12 days incubation with rumen fungi will be 36.1 and 35.7 g/100 g DM for high and low fat sunflower meal, respectively) (P>0.05). High fat of sunflower meal caused increase in natural detergent fibre disappearance 12 days after culturing as compared to low fat sunflower meal (145.2 *vs* 139.2 mg/g dry matter, respectively) (P<0.05). Therefore, it appears that fat content of sunflower meal does not negatively affect the population, growth and activity of rumen fungi.

Key words: Fat, sunflower meal, rumen fungi, quantitative competitive polymerase chain reaction, disappearance.

INTRODUCTION

Sunflower meal is a source of supplemental protein for livestock feeding. The amount of hull or fibre in sunflower meal is the major source of variation in nutrient content of this feed, and is entirely adequate as the sole source of supplemental protein in dairy rations. The high fat sunflower meal eliminates the need for some or all the fat supplement requirements (Hamilton et al., 1992). This fat is rich in polyunsaturated fatty acids, and a source of linoleic acid (66% of the total fatty acids) that increases the proportion of unsaturated fatty acids and conjugated linoleic acid (CLA) in milk (Schingoethe et al., 1996). It was reported that unsaturated fatty acids have negative effects on rumen microorganisms and reduces digestibility (Palmquist and Jenkins, 1980). Fats added to ruminant diets can disrupt fermentation in the rumen, and result in reduced degradation of plant cell wall components (Jenkins, 1993). Getachew et al. (2001) showed that the addition of yellow grease and corn oil increased *in vitro* rumen degradation and gas production.

Rumen fungi produce a wide range of polysaccharide degrading enzyme and are the primary colonizers of fibrous plant materials that degrade lignin-containing

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plant cell walls (Akin and Borneman, 1990). Encysted zoospores of rumen fungi produce a rhizoidal system capable of penetrating plant tissues, thereby allowing access of fungi to fermentable carbohydrates that are not accessible to other rumen micro-organisms (Bachuap, 1981). Factors such as diets, time and frequency of feeding; all influence fungi population within the rumen (Denman et al., 2006). Zoospore enumeration and marker techniques (hydrogen or formic acid produced by the culture) have been developed for the estimation of fungal populations in the rumen, but these methods are time consuming and cumbersome (Denman and Mcsweeney, 2006). Chitin as a component of the cell wall of anaerobic fungi, is as a marker of fungal populations (Rezaeian et al., 2004). Disappearance in rumen fungi media culture is used to determine fungi growth. With the advancement of molecular enumeration methods, in particular 18S rDNA gene probing methods, researchers were able to monitor fungal species within the rumen (Stahl et al., 1988). Quantitative competitive polymerase chain reaction (QC-PCR) technique plays an important role in nucleic quantification because of their significant lower cost of equipment and consumables (Franz et al., 2001). Available information on the effect of fat content of sunflower meal on growth and population of rumen fungi is scarce. Therefore, the objective of this study was to evaluate the effect of fat content of sunflower meal (165 and 30 g/kg dry matter (DM), high and low fat respectively) on rumen anaerobic fungi growth and population by using direct (QC-PCR) and indirect (DM and neutral detergent fibre (NDF) disappearance in rumen anaerobic fungi media culture for 12 days) methods.

MATERIALS AND METHODS

Culturing of rumen anaerobic fungi

Rumen anaerobic fungi were isolated from wheat straw residues, which were incubated using polyester bags in the rumen of Holstein fistulated steers (420 ± 12 kg BW) that were feed by 30:70 concentrate : forage diet (corn grain, barley grain and wheat bran : sugarcane silage, corn silage, alfalfa hay and wheat straw). The method of Joblin (1981) was used to grow fungi using 100 ml serum bottles under continuous CO_2 reflux. The inoculated bottles were incubated at 39°C for three days. The isolated anaerobic fungi of rumen were then used as a source of inoculum for further culture. Serum bottles containing 45 ml of anaerobic fungi culture medium, and 1 g of high and low fat sunflower meal (SMh and SMl, respecttively) were used to culture the rumen isolated anaerobic fungi at 39°C with three times subculturing (three replicates per treatment).

DNA extraction and quantitative competitive PCR

Genomic DNA of rumen anaerobic fungi was isolated from pure culture using Guanidine Thiocyanate-Silica Gel method. About 0.5 ml aliquot was taken from the 200 ml sample using a wide-bore pipette, so as to ensure that a homogeneous sample containing plant particles and liquid was obtained. For rumen fungi pure cultures, genomic DNA was extracted from biomass harvested by centrifugation (2000 ×g, 10 min) from broth (Fliegerova et al., 2006). Universal PCR primer pair GAF was used to amplify a specific region of 18S rDNA from rumen anaerobic fungi. The non-homologous competitor (standard control) was designed to contain GAF-universal primer binding sequence of ~25 bp at ends encompassing a phage sequence (phage lambda sequence location 46775-46797 and 46891-46912, respectively, according to Gen-Bank accession no. NC_1416) (Table 1). After purification of competitor DNA, target DNA (fungi culture) and serial dilution of competitor DNA (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) was used in the QC-PCR and was shown to amplify under the same reaction condition and the same amplification efficiency as the target DNA. The PCR was performed in a final volume of 25 µl sealed in a capillary tip, and thermocycling was carried out in a model 2000 (Biometra). The PCR mixture contained 50 ng of template DNA, 2 µl 10X PCR buffer, 2.5 mM $MgCl_2$, 200 µM each dNTPs, 10 pM of each primer and 1 U Taq DNA polymerase. The PCR amplification condition was as follows: denaturation at 94°C for 4 min followed by 35 cycles of 94°C for 30 s; 56°C for 30 s; and 72°C for 1 min followed by a final extension at 72°C for 5 min.

Quantification of PCR products

The PCR products were quantified by photographing agarose gels with Polariod 665 film (Polaroid, St. Albans, England), which produce a negative image of the photograph. The negative was scanned with a GS-670 image densitometer (Bio-Rad, Hercules, Calif) and analyzed with ImageJ 1.38x software (National Institutes of Health, USA). Differences in the fluorescence of different sized ethidium bromide-stained PCR fragments was corrected by multiplying by a ratio of 110/191 obtained by comparison to the amplified standard (Piatak et al., 1993).

Measurement of DM and NDF disappearance of rumen fungi culture

Samples of sunflower meal used as the substrate of culture media were collected from each bottle after washing twice with distilled water followed by filtration using sintered glass crucibles (grade 1, porosity G1). They were then freeze dried to constant weight for DM determination. The dry matter disappearance of each sample was calculated as the difference between initial and the residual weight of the dried substrate. Content of NDF of samples were determined from the freeze-dried samples using the method of Van Soest et al. (1991), and losses of each sample were calculated as the difference between initial and the residual weight of the dried substrate.

Statistical analysis

The effect of fat on the changes of fungal populations was analyzed as a completely randomized design by of ANOVA and ANCOVA using the GLM procedure of SAS, and data of DM and NDF disappearance of medium were analysed as repeated measurement. When a significant ($P< 0.05$) F test was detected, means were compared by Duncan test.

RESULTS AND DISCUSSION

The reaction of competitive PCR for DNA extracted from medium of rumen anaerobic fungi is shown in Figure 1. The result of competitive PCR in direct method shows that the growth and population of rumen anaerobic fungi in medium containing high fat sunflower meal was more

Table 1. PCR primers for amplifying DNA of target and competitor primers for amplifying target and non-homologous competitors.

Anaerobic rumen fungi (target)
GAF1: 5´-GAGGAAGTAAAAGTCGTAACAAGGTTTC-3´
GAF2: 5´-CAAATTCACAAAGGGTAGGATGATTT-3´
Lambda phage (competitor)
LaGAF1:5´- gaggaagtaaaagtcgtaacaaggtttcGAAGTTCGCAGAATCGTATGTG-3´
LaGAF2:5´- caaattcacaaagggtaggatgatttGCTGTGGACATAGTTAATCCG-3´

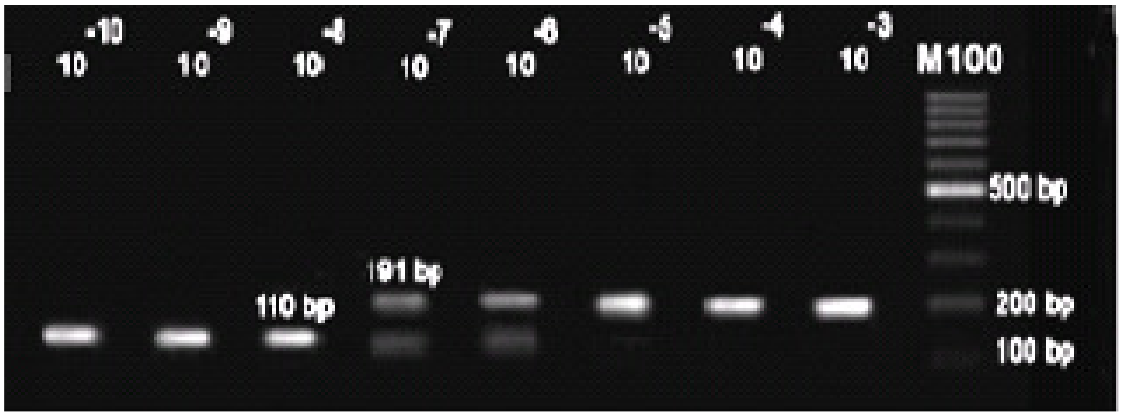

Figure 1. Competitive PCR reaction for DNA extracted from fungi medium. 110 bp, rumen fungi; 191 bp, competitor (standard).

Table 2. Competitive PCR results for DNA extracted from fungi medium.

	Rumen fungal of competitor in different dilutions (Log T/C)				
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
SM_h	0.14^{b}	-0.24^{b}	-0.69^{b}	-1.32^{b}	-1.62^{b}
SM_l	0.10^{a}	-0.29^{a}	-0.76^{a}	-1.37^{a}	-1.65^{a}
S.E.M	0.01	0.02	0.03	0.02	0.01

SM_h, High sunflower meal; SM_l, low sunflower meal; S.E.M, standard error of mean, means within each column with different letters are significantly different ($P<0.05$). Log (T/C), the log ratio of intensities of amplified target (fungi) DNA to standard was plotted against the dilution of the standard control (competitor).

than that of low fat sunflower meal (Table 2 and Figure 2) ($P<0.05$). Also, according to the result of this study in indirect method, fungi growth and disappearance of DM and NDF of high fat sunflower meal in the cultures was higher than that of low fat sunflower meal, and there was a high decrease in DM and NDF of substrates over the three days of the growth of anaerobic fungi (Tables 3 and 4) ($P<0.05$).

The result of the current study indicates that fat content of sunflower meal had no negative effect on anaerobic fungi growth in media culture. This result supports the suggestion of Machmuller (1998) that showed increased microbial growth when fat was fed. Getachew (2001) concluded that addition of yellow grease and corn oil increased *in vitro* fermentation and gas production by rumen microbes (0.35 and 0.38 ml gas/g DM, respectively). These results agree with the results of Grummer et al. (1993), in which increasing tallow up to 5% of the diet DM did not adversely affect forage DM degradability in the rumen though Withney et al. (2000) reported linear decline for DM disappearance by inclusion of soybean oil in the diet. Also, Palmquist and Jenkins (1980) and Pantoja et al. (1994) reported that unsaturated fatty acids have negative effects on rumen microorganisms and reduces digestibility and degradation of plant cell wall component. Decreased *in vitro* fermentation and fiber digestibility by supplementation of soybean oil was observed by Broudiskov (1990) and Betman and Jenkins

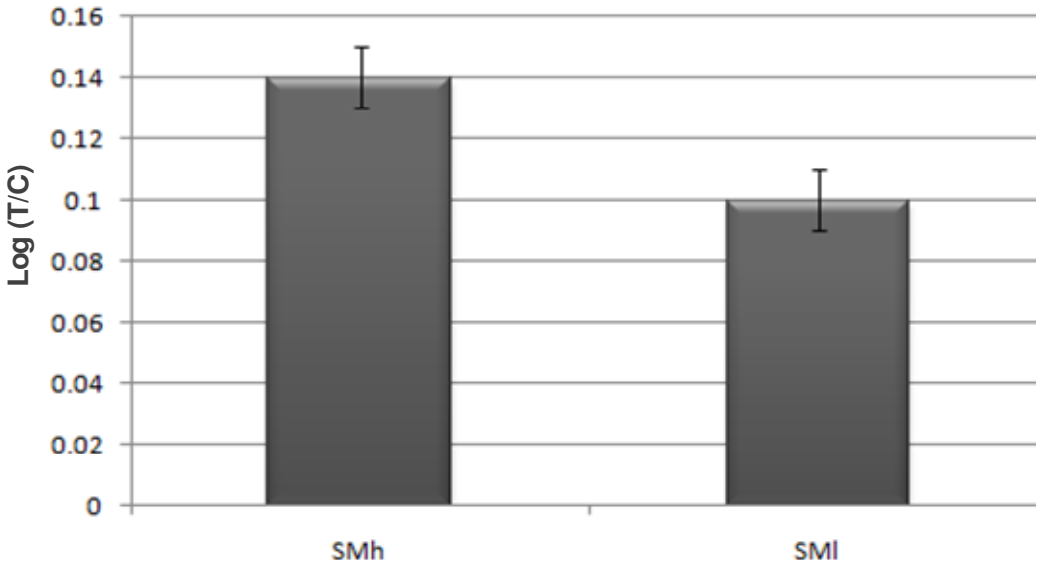

Figure 2. Quantitative changes of rumen anaerobic fungal population (by QC-PCR) with different treatments. SM_h, High sunflower meal; SM_l, low sunflower meal; Log (T/C), the log ratio of intensities of amplified target (fungi) DNA to standard was plotted against the dilution of the standard control (competitor).

Table 3. The effect of fat content on dry matter disappearance of sunflower meal by anaerobic rumen fungi culture.

IT	DM disappearance (g /100g DM)		
(day)	SM_h	SM_l	s.e.m
1	4.4[a]	3.9[b]	0.01
3	22.5[a]	21.7[b]	0.1
9	33.9	33.6	0.5
12	36.1	35.7	0.3

IT, Incubation time; SM_h, high sunflower meal; SM_l, low sunflower meal; S.E.M, standard error of mean, means within each column with different letters are significantly different ($P<0.05$).

Table 4. The effect of fat content on neutral detergent fibre disappearance of sunflower meal by anaerobic rumen fungi culture.

IT	NDF disappearance (mg/g DM)		
(day)	SM_h	SM_l	S.E.M
1	5.6[a]	5.1[b]	0.1
3	88.7[a]	78.5[b]	0.4
9	.134.2[a]	129.1[b]	0.3
12	145.2[a]	139.2[b]	0.6

IT, incubation time; SM_h, high sunflower meal; SM_l, low sunflower meal; S.E.M, standard error of mean, means within each column with different letters are significantly different ($P<0.05$).

(1998). The study of Pantoja et al. (1994) showed a linear reduction in ruminal NDF digestion with increasing degree of fat unsaturation. Fats added to uminant diets can disrupt fermentation in the rumen, causing reduced digestibility of nonlipid energy sources (Jenkins, 1993).

The reduction in fermentation was about four percent which corresponded to the average dietary increase in fat of 4 to 5%, but other researchers reported similarly, that the high fiber concentration in the diet (54.4% NDF) may have reduced effects of fat on fiber digestion. Researchers showed that free fatty acid associated with fibre particles in the rumen can prevent the close attachment of cellulolytic microbes, which is a prerequisite for fibre degradation (Jenkins, 1993). Tackett et al. (1996) observed an interaction between the fibre content in the diet and fat supplementation on ruminal fermentation. Addition of fat led to disruption of ruminal fermentation only in a low fibre diet, whereas in a high fibre diet, this effect did not occur. Feed particles from hay compete with microbial surfaces for binding of fatty acid, helping to reduce the negative effects on ruminal microorganisms (Maczulak et al., 1981).

Therefore, the results of the present experiment show that the rumen anaerobic fungi growth and population determined by QC-PCR (direct method) and disappearance of DM and NDF (indirect method) in the medium containing high fat sunflower meal was greater as compared to the low fat sunflower meal. It means fat content of sunflower meal had no negative effect on anaerobic fungi growth in media culture.

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