Asian-Aust. J. Anim. Sci.
Vol. 20, No. 1 : 70 - 74
January 2007

www.ajas.info

Effect of Fungal Elimination on Bacteria and Protozoa Populations and Degradation of Straw Dry Matter in the Rumen of Sheep and Goats

D. B. Li and X. Z. Hou*

College of Animal Science and Medicine, Inner Mongolia Agricultural University, Hohhot 010-018, P. R. China

ABSTRACT : An *in vitro* study was carried out to investigate the differences in rumen microbes and fiber degradation capacity between sheep and goats. Three local male sheep and three Inner Mongolia male cashmere goats (aged 1.5 to 2 years; weight 25.0 to 32.0 kg) were each fitted with a permanent rumen cannula used to provide rumen fluid. Cycloheximide was used to eliminate rumen anaerobic fungi. The results showed that the quantities of fungal zoospores in the culture fluid of the control group were significantly greater in the sheep than in the goats; however, bacteria and protozoa counts were significantly higher in goats than in sheep. The digestibility of straw dry matter did not differ significantly between the two species before elimination of fungi, but tended to be higher for sheep (55.4%) than for goats (53.3%). The results also indicated that bacteria counts increased significantly after elimination of anaerobic fungi; however, the digestibility of straw dry matter significantly decreased by 12.1% and 8.6% for sheep and goats respectively. This indicated that the anaerobic fungi of the rumen played an important role in degradation of fiber. (**Key Words :** Anaerobic Fungi, Sheep, Goats, Bacteria, Protozoa, Digestibility)

INTRODUCTION

Comparative studies have shown differences between ruminant species in the utilization of roughage diets, most likely related to differences in their feeding behaviour and digestive processes (Dulphy et al., 1995). These differences may represent distinct feeding strategies resulting from survival adaptations in their characteristic natural environment. Sheep and goats are the most common ruminant species in China. Previous studies have reported differences between the two species regarding their voluntary intake of forages (Dulphy et al., 1994; Hadjigeorgiou et al., 2001), diet selection, fractional passage rate (Huston et al., 1986; Yanez Ruiz et al., 2004) and digestibility coefficients of forage (Domingue et al., 1991; Ramanzin et al., 1997). Microbial digestion of cellulose and other plant fiber components is essential to the utilization of natural diets by ruminant animals. It has been reported that fungi specifically colonize fibrous plant fragments in the rumen, and the apparent magnitude of their population suggests that they have a role in fiber digestion as initial colonizers in lignocellulose breakdown (Bauchop, 1981).

* Corresponding Author: X. Z. Hou. Tel: +86-471-431-7654, Fax: +86-471-430-1530, E-mail: Houxz05@sina.com.cn
Received December 2, 2005; Accepted April 13, 2006

Fazaeli et al., (2004) used five species of *Pleurotus* fungi (coded P-21, P-30, P-41, P-60 and P-90) to incubate soaked and pasteurised wheat straw. Their results indicated that fungal treatment significantly ($p<0.05$) increased the crude protein (CP) and reduced the cell wall components of the straw. In addition, other investigators showed that degradation of wheat straw DM significantly declined when cycloheximide (150 mg/L) was used to eliminate anaerobic fungi (Hillaire and Jouany, 1990). Anaerobic fungi have been extensively reviewed and studied, but their population and fiber digestion capacity in different ruminant species still remains unclear. So a fungal elimination method was used in our study to investigate the impact of *in vitro* anaerobic fungal elimination on bacteria and protozoa counts and degradation of straw dry matter in sheep and goats.

MATERIALS AND METHODS

Animals and diet

Three healthy local male sheep and three male cashmere goats were used as donor animals for rumen liquid. All animals were between 1.5 and 2 years old when the experiment started. Average live weight of the animals was between 25 and 32 kg. Each animal was fitted with a permanent rumen cannula. The animals were housed in

Table 1. Design of experiment

Periods	Cycloheximide dose*	Incubation period (h)** 0 2 4 6 12 24 48 72
Trial 1	C: 0.00 mg/ml	Fungal enumeration
	L: 0.15 mg/ml	72 h straw DM digestibility determination
	M: 0.25 mg/ml	Three repetitions in each group
	H: 0.50 mg/ml	
Trial 2	Treatment C	Enumeration of bacteria and protozoa
	Treatment M	Three replicates in each group

* C,L,M,H - Refers to control, low, medium, and high dose group respectively.

** Measurement items.

Table 2. Effect of different level of cycloheximide on total amounts of fungi in ruminal cultures of sheep and goats (TFU/ml, $\times 10^3$)

Treatment*/time (h)		0	2	4	6	12	24	48	72
Goat	C	2.53±0.48^{a}	1.38±0.32^{b}	1.09±0.32^{bc}	0.83±0.24^{c}	1.48±0.38^{a}	9.78±1.06^{a}	0.63±0.32^{b}	0.22±0.07^{b}
	L	1.25±0.24^{bc}	0.73±0.21^{c}	0.81±0.18^{c}	0.34±0.18^{d}	0.53±0.05^{b}	0.90±0.24^{b}	0.33±0.05^{bc}	0
	M	1.45±0.09^{b}	0.36±0.14^{d}	1.00±0.28^{bc}	0.10±0.00^{d}	0.40±0.12^{b}	0.36±0.17^{b}	0.10±0.00^{c}	0
	H	1.18±0.27^{bc}	0.45±0.21^{cd}	0.37±0.07^{d}	0.10±0.00^{d}	0	0	0	0
Sheep	C	2.06±0.31^{ab}	2.70±0.35^{a}	1.47±0.00^{a}	2.67±0.21^{a}	1.77±0.15^{a}	1.43±0.12^{b}	4.83±0.06^{a}	1.23±0.25^{a}
	L	0.15±0.10^{d}	0.41±0.00^{cd}	1.22±0.06^{ab}	1.33±0.12^{b}	1.41±0.12^{a}	1.23±0.10^{b}	0.57±0.06^{b}	0.32±0.26^{b}
	M	0.04±0.06^{d}	0	0	0	0	0	0	0
	H	0.46±0.00^{cd}	0.14±0.17^{dc}	0	0	0	0	0	0

"0" - no fungi were detected.

$^{a, b, c}$ Means within rows with different superscripts are significantly different.

* C,L,M,H - Refers to control , low, medium high dose group respectively.

individual pens and had free access to water and mineral blocks. The experimental diet was composed of hay and concentrate (forage:concentrate ratio 70:30). The feeding level of all animals was 1.2 times maintenance requirement. The diet was offered in two equal meals daily, at 07:00 and 19:00, throughout the experimental period.

Inoculum preparation

One day prior to the experiment, 400 mg straw and different doses of cycloheximide were administered to culture flasks fitted with rubber sleeves. On the following day equal amounts of rumen digesta were collected from the three sheep and the three goats 2hr after feeding at 07:00. Rumen digesta of the same species was put into a thermos which was prewarmed (39°C) and filled with CO_2. After returning to the lab, the digesta of each species was homogenized and then filtered through two layers of cheese cloth into a beaker. Immediately, 20 ml filtrate together with 40 ml buffer solution was added to culture flasks, and then the flasks were held at 39°C in a water bath. The buffer solution described by Chen and Russell (1988) was used with omission of the vitamins.

Enumeration methods

The fungal zoospores and the bacterial colonies were enumerated by the Hungate roll tube technique (Joblin, 1981). 1600IU/ml of antibiotics was injected into the fungal medium when the fungi were enumerated. Protozoa were counted in culture fluid dyed by methylgreen formalin salt (MFS) under a light microscope.

Design of the experiment

This experiment comprised two trials and the design is shown in Table 1.

Trial 1 : The aim of Trial 1 was to study the minimal doses of cycloheximide needed to eliminate anaerobic fungi after the shortest incubation period in sheep and goats. According to different doses of cycloheximide, four groups were generated (C: 0.00 mg/ml; L: 0.15 mg/ml; 11 M: 0.25 mg/ml and H:0.50 mg/ml, C, L, M and H represent the control, the low dose, the medium dose and the high dose group, respectively). Each group had three replicates. Fungal zoospores in each group were counted by the Hungate roll tube technique after 0, 2, 4, 6, 12, 24, 48 and 72 h incubation periods, and straw dry matter degradation was measured after 72 h incubation.

Trial 2 : The aim of Trial 2 was to study the effect of anaerobic fungal elimination on bacteria and protozoa counts in sheep and goats. The best cycloheximide dose (M: 0.25 mg/ml) established in Trial 1 was used. The quantities of bacteria and protozoa were measured after 0, 2, 4, 6, 12, 24, 48 and 72 h incubation periods in each species.

Statistical analyses

Statistical analyses were carried out by ANOVA procedures of the Statistical Analysis Systems Institute. A

Table 3. Effect of different level of cycloheximide on straw DM degradation (%) in 72 h ruminal culture of sheep and goats (%)

Species\treatment*	C	L	M	H
Goat	53.25±0.89[a]	46.54±1.80[bc]	48.09±1.62[b]	44.63±0.44[bc]
Sheep	55.40±0.18[a]	45.60±0.64[bc]	46.31±0.95[bc]	43.34±1.26[c]

[a, b, c] Means within rows with different superscripts are significantly different.
* C,L,M,H - Refers to control , low, medium high dose group respectively.

Table 4. Effect of elimination of fungi on total amounts of bacteria in ruminal culture of sheep and goats (unit/ml)

Treatment/time	0 h	2 h	4 h	6 h	12 h	24 h	48 h	72 h
Goat	($\times10^{11}$)	($\times10^{10}$)	($\times10^{10}$)	($\times10^{10}$)	($\times10^{10}$)	($\times10^{8}$)	($\times10^{7}$)	($\times10^{5}$)
C	0.64±0.15[c]	1.85±0.37[ab]	2.65±0.44[a]	2.59±0.11[b]	2.17±0.34[ab]	1.88±0.06[c]	1.45±0.07[a]	1.08±0.15[c]
M	1.03±0.09[b]	2.26±0.23[a]	3.46±0.36[a]	3.43±0.37[a]	2.62±0.37[a]	2.08±0.19[c]	1.07±0.21[ab]	1.25±0.22[c]
Sheep	($\times10^{10}$)	($\times10^{10}$)	($\times10^{10}$)	($\times10^{10}$)	($\times10^{10}$)	($\times10^{8}$)	($\times10^{8}$)	($\times10^{5}$)
C	1.08±0.79[ab]	0.90±0.12[b]	0.81±0.16[b]	0.69±0.06[c]	1.33±0.66[b]	2.66±0.44[b]	0.71±0.19[b]	2.97±0.58[b]
M	1.38±0.82[a]	2.35±0.40[a]	0.81±0.34[b]	2.90±0.10[ab]	1.70±0.41[ab]	3.28±0.93[a]	0.85±0.28[b]	3.57±0.51[a]

[a, b, c] Means within rows with different superscripts are significantly different.
"C" and "M" represent control group and medium cycloheximide dose group respectively.

Table 5. Effect of elimination of fungi on total amounts of protozoa in ruminal culture of sheep and goats (unit/ml, $\times10^{5}$)

Treatment/time		0 h	2 h	4 h	6 h	12 h	24 h	48 h	72 h	Average
Goat	C	1.30±0.05	1.08±0.13	1.15±0.05	1.33±0.08	1.73±0.13	1.28±0.09	1.27±0.06	1.25±0.22	1.32±0.22[a]
	M	1.30±0.41	1.35±0.10	1.25±0.05	1.10±0.20	1.20±0.19	1.42±0.09	1.37±0.09	1.33±0.12	1.30±0.15[a]
Sheep	C	1.40±0.21	0.90±0.05	1.25±0.10	1.20±0.15	1.06±0.11	1.10±0.08	0.91±0.08	0.75±0.02	1.07±0.20[b]
	M	1.52±0.06	1.05±0.09	1.35±0.12	1.3±0.06	1.35±0.07	1.25±0.30	0.95±0.13	0.90±0.05	1.21±0.20[ab]

[a, b, c] Means within rows with different superscripts are significantly different.
"C" and "M" represent control group and medium cycloheximide dose group respectively.

randomized block design was applied. The means were analyzed for significant differences by variance analysis. Where significant differences were found, Duncan's multiple range test ($p<0.05$) was used to separate the means. All data were expressed as the mean±SD.

RESULTS

Trial 1

The effect of different cycloheximide dose on fungal zoospore counts is shown in Table 2. During the incubation period, the quantities of fungal zoospores of sheep were significantly greater than of goats in treatment C, except at 0 h and 24 h. Table 2 shows that for each species there was a significant difference between the 0 h counts of fungal zoospores for treatments, and the number of fungal zoospores was greater in treatment C. As the level of cycloheximide increased, the number of fungi gradually declined in both species. When the cycloheximide dose reached 0.25 mg/ml (treatment M), fungal zoospores in culture fluid of both sheep and goat significantly decreased after the 2 h incubation period, as compared with treatment C. Therefore 0.25 mg/ml was selected as the most suitable supplement dose in the following experiment.

As shown in Table 3, there were significant ($p<0.05$) differences between cycloheximide levels in straw DM degradation which was greater in treatment C than in treatments L, M and H. There were no significant ($p>0.05$) differences between the two animal species in the straw DM degradation, but it tended to be higher for sheep than for goats in treatment C.

Trial 2

The number of bacterial colonies after 0, 2, 4, 6, 12, 24, 48 and 72h incubation periods is shown in Table 4. After elimination of fungi, the quantities of bacteria increased and the differences between treatments reached statistical significance ($p<0.05$) after 2, 6, 24, 72 h and 0 and 6 h of incubation for sheep and goats, respectively. From Table 4 it can also be seen that bacterial counts for goats were much higher than for sheep during the first 12h of incubation for both treatments C and M. However, after a 24 to 72 h incubation period, the bacterial counts markedly declined in both species, this decline rate was significantly faster for goats than for sheep ($p<0.05$).

Protozoa counts during the 72 h incubation period are shown in Table 5. There were no significant differences between treatments in protozoa counts (average values of the whole incubation period) within each species ($p>0.05$), although protozoa counts of sheep tended to be greater for treatment M than for C. Protozoa counts (average values) of goats were significantly greater than of sheep for treatment C ($p<0.05$). No significant differences between species in

protozoa counts were found for treatment M.

DISCUSSION

Comparative studies have shown that there were differences between sheep and goats in the utilization of roughage diets. Ramanzin et al. (1997) reported that with little possibility to choose dietary components, goats, as compared with sheep, showed lower food intakes, shorter rumen retention time and lower fiber apparent digestibility. Bauchop (1979) observed that quantities of fungal zoospores decreased when diets mainly comprised of leaves were offered; due to the rapid outflow rates of the diets there was not sufficient time for fungi to adhere to plant tissue and to reproduce. In trial 1, enumeration of fungal zoospores was greater for sheep than for goats for the following two reasons: First, goats have faster gastro-intestinal tract outflow rates than sheep (Huston, 1978; Ramanzin et al., 1997), which resulted in a shorter time for fungal adherance to plant tissue and reproduction and, consequently, lower counts of fungal zoospores. Second, the feeding behavior between sheep and goats may be different; goats select the more digestible and perhaps more palatable fractions of diets (Morand-Fehr et al., 1991) which results in lower fiber intakes, thus the conditions were not beneficial to fungal growth and reproduction (Bauchop, 1979; Akin and Rigsby, 1987). As shown in Table 2, there was a significant difference in the 0 h counts of fungal zoospores between treatments in each species. This may be caused by addition of cycloheximide.

As described in inoculum preparation, cycloheximide had been administered to the culture flask before incubation. When the filtrate of rumen digesta was added to the flask, cycloheximide began to exert its function on fungal elimination, and because of the high efficiency of cycloheximide, 0 h counts of fungal zoospores in treatments L, M and H decreased significantly as compared with the control group.

Ramanzin et al. (1997) observed that when forage: concentrate ratio was 90:10, apparent OM digestibility was higher for sheep than for goats (0.61 vs. 0.55); Huston, (1978) found dietary DM digestibility was 50% and 45% for sheep and goats respectively. The results of the present study indicated that straw DM digestibility *in vitro* was higher for sheep than for goats (55% vs. 53%). These results are consistent with the above studies; they may be attributed to differences between sheep and goats in rumen microflora, rumen pool and rumen fractional outflow rate. As shown in Table 3, straw DM digestibility markedly declined (declined by 12.1% and 8.6% for sheep and goats respectively) after elimination of fungi. Hillaire and Jouany (1990) reported that wheat straw DM degradation declined by 15% when cychloheximide (150 mg/L) was applied to eliminate anaerobic fungi in a semi-continuous culture system. A similar experiment was also conducted by Mao (2001); in his study fungi in goats were eliminated by the methods of Ford et al. (1987) and resulted in straw DM degradation declining from 38.9% to 28.9% after a 48 h incubation. Fungi can colonize sclerenchyma and penetrate into cell walls by rhizoids; as a result plant tissues are weakened by this process and are more easily reduced in particle size or made more fragile (Akin et al., 1983). In the present study, the process of bacteria adhering to plant tissues was probably influenced when rumen fungi were absent (treatments L, M and H), and thus straw DM digestibility after 72 h on these three treatments markedly declined. In addition, previous studies indicated that rumen fungi produced a series of hydrolytic enzymes, including the cellulases, hemicellulases, pectinases and phenolic acid esterases (Ho and Abdullah, 1999), and they were particularly proficient in producing xylanases (Akin et al., 1990). These enzymes enable fungi to invade and degrade the lignocellulosic plant tissues. After the elimination of fungi, straw DM digestibility declined because of the lack of these high activity enzymes.

Previous studies have shown that repression exists in co-culture of anaerobic fungi with cellulolytic bacteria (Richardson et al., 1986). As straw was used as the fermentation substrate in this investigation, cellulolytic bacteria were the major type of bacteria in the culture fluid. The repression between fungi and cellulolytic bacteria was nonexistant after the removal of fungi, therefore the number of bacteria increased rapidly for treatment M in both sheep and goats. As shown in Table 4, the bacteria counts were greater for goats than for sheep during the first 12 h of incubation. This difference was possibly due to the stronger repression of fungi on bacteria in the culture fluid of sheep than of goats. In addition, this difference may be attributed to feeding behaviour. Goats are more selective and their diets were greater in net energy and protein than the feed offered (Huston, 1978), which results in a more favorable rumen environment for bacterial growth. However, from 24 h to 72 h of incubation, the accumulation of fermentation end-products may have affected bacterial growth (Pirt, 1975), and thus bacterial counts declined rapidly for both sheep and goats.

Until now there is no direct evidence that fungal zoospores are ingested by rumen protozoa, but there are studies showing that fungal zoospore counts increase several fold after elimination of protozoa (Orpin, 1977). Bird and Leng (1985) reported when the diet of sheep was high in fiber and low in protein, the interaction of rumen fungi with bacteria was antagonistic and the removal of protozoa increased the fungal populations two to five times.

In this study, protozoal counts (average values) were greater for goats than for sheep, because a large number of bacteria in the culture fluid of goats served as an important nitrogen source for protozoa and promoted protozoal growth. As shown in Table 5, there were no significant differences between treatments C and M in protozoal counts after the fungal elimination. Jouany (1989) concluded that the number of zoospores present was not affected by protozoa, and *in vitro* studies have indicated that protozoa may prey on fungi (Alan G. and Williams, 1994). Conflicting results have also been found in other studies (Orpin, 1977). Therefore the relationship between fungi and protozoa is still unclear and further research is required in the future.

ACKNOWLEDGEMENTS

We are grateful to National Natural Science Foundation of China (project number: 30460095) and Inner Mongolia Science and Technology Bureau for financial support.

REFERENCES

Alan, G., N. Williams, Keith Joblin and G. Fonty. 1994. Interactions between the rumen chytrid fungi and other microorganisms. In: Anaerobic Fungi, 12nd (Ed. D. O. Mountfort and C. G. Orpin). Marcel Dekker, Inc. 207 Madison Avenue, New York. pp. 191-227.

Akin, D. E. and W. S. Borneman. 1990. Role of rumen fungi in fiber degradation. J. Dairy Sci. 73:3023-3032.

Akin, D. E. and L. L. Rigsby. 1987. Mixed fungal populations and lignocellulosic tissue degradation in the bovine rumen. Appl. Environ. Microbiol. 9:1987-1995.

Bauchop, T. 1979. Rumen anaerobic fungi of cattle and sheep. Appl. Environ. Microbiol. 38:148-158.

Bauchop, T. 1981. The anaerobic fungi in rumen fiber digestion. Agric. Environ. 6:339-348.

Chen, G. and J. B. Russell. 1988. Fermentation of peptides and amino acids by a monensin-sensitive ruminal peptostreptococcus. Appl. Environ. Microbiol. 54:2742-2749.

Domingue, B. M. F., D. W. Dellow and T. N. Barry. 1991. Voluntary intake and rumen digestion of a low-quality roughage by goats and sheep. J. Agric. Sci. Camb. 117:111-120.

Fazaeli, H., H. Mahmodzadeh, A. Azizi, Z. A. Jelan, J. B. Liang, Y. Rouzbehan and A. Osman. 2004. Nutritive Value of Wheat Straw Treated with Pleurotus Fungi. Asian-Aust. J. Anim. Sci. 17:1681-1688.

Hadjigeorgiou, I. E., I. J. Gordon and J. A. Milne. 2001. The intake and digestion of a range of temperate forages by sheep and fibre-producing goats. Small Rumin. Res. 39:167-179.

Hillaire, M. C., J. P. Jouany and G. Fonty. 1990. Wheat straw degradation, in rusitec, in the presence and absence of rumen anaerobic fungi. Proceed. Nutr. Soc. 49:127A.

Ho, Y. W. and N. Abdulan. 1999. The role of rumen fungi in fibre digestion. Asian-Aust. J. Anim. Sci. 12:104-112.

Huston, J. E., B. S. Engdahl and K. W. Bales. 1988. Intake and digestibility in sheep and goats fed three forages with different levels of supplemental protein. Small Rumin Res. 1:81-92.

Mao, S. Y. 2001. Isolation and purification of anaerobic fungi from the rumen and faeces of goats and comparison of fermentation activity between isolates *in vitro*. Master. Thesis, Nan Jing Agricultural University, Nan Jing, China.

Joblin, K. N. 1981. Isolation, enumeration, and maintenance of rumen anaerobic fungi in roll tubes. Appl. Environ. Microbiol. 42:1119-1122.

Joblin, K. N. and G. Naylor. 1989. Fermentation of woods by rumen anaerobic fungi. Fems Microbiol. Letters. 65:119-122.

Jouany, J. P. 1989. Effect of diet on populations of rumen protozoa in relation to fiber digestion. In: The role of protozoa and fungi in ruminant digestion (Ed. J. V. Nolan, R. A. Leng and D. I. Demeyer). Armidale, Australia: Penambul Books. pp. 59-74.

Martin, C. and E. Devillard. 1999. Influence of sampling site on concentrations and carbohydrate-degrading enzyme activities of protozoa and bacteria in the rumen. J. Anim. Sci. 77:979-987.

Orpin, C. G. 1977. Studies on the defaunation of the ovine rumen using dioctyl sodium sulphosuccinate. J. Appl. Bacteriol. 43:309-318

Ramanzin, M., L. Bailoni and S. Schiavon. 1997. Effect of forage to concentrate ratio on comparative digestion in sheep, goats and fallow deer. Anim. Sci. 64:163-170.

Richardson, A. J., C. S. Stewart and G. P. Campbell. 1986. Influence of co-culture with rumen bacteria on the lignocellulolytic activity of phycomycetous fungi from the rumen. XIV Intl. Congress Microbiol. 233:2-24 (Abstr).

Taylor, G. T. and S. J. Pirt. 1977. Nutrition and factors limiting the growth of a methanogenic bacterium (Methanobacterium thermoautotrophicum). Arch Microbiol. 113(1-2):17-22.

Van Os M., J. P. Dulphy and R. Baumont. 1995. The effect of protein degradation products in grass silages on feed intake and intake behaviour in sheep. Br. J. Nutr. 73:51-64.

Yanez Ruiz, D. R., A. Moumen, A. I. Martin Garcia and E. Molina Alcaide. 2004. Ruminal fermentation and degradation patterns, protozoa population, and urinary purine derivatives excretion in goats and wethers fed diets based on two-stage olive cake: effect of PEG supply. J. Anim. Sci. 82:2023-2032.