Effect of Dietary Concentrate on Fungal Zoosporogenesis in Sheep Rumen

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ABSTRACT : Fluctuation of fungal zoospores on agar strips were observed in the rumen of sheep fed three different levels of dietary concentrate, timothy hay: concentrate = 3 : 0 (AF diet), timothy hay: concentrate = 2 : 1 (MC diet), timothy hay: concentrate = 1 : 2 (HC diet) respectively.

The number of zoospores on the strip was drastically decreased after morning feed with AF diet. The number was the highest at 0 h (1.34×10^2/cm^2), then declined to 2.0×10^3/cm^2 at 9 h after feeding. In the rumen of animals fed MC diet, the number of zoospores decreased with time after feeding, although the decrement was slower than that with AF diet. During 0-3 h after feeding, number of zoospores was 1.6×10^4/cm^2. Although the number slightly decreased at 6 and 9 h, relatively high levels were maintained. It seems that the inducers for zoospore-release were maintained at relatively high concentration throughout incubation period. The fluctuation pattern of number of germinated zoospores was different in the rumen of animals fed HC diet from those of AF and MC diets. The number of zoospores was constantly maintained at lower level (1.0×10^3/cm^2) than the other diets.

For MC diet, continuous high number of germinated zoospores may be due to the continuous release of zoospores by hemes in timothy hay and concentrate feed, and by unknown mechanisms. Unlike AF diet which promoted relatively rapid decline of zoosporogenesis, supplementation of concentrate feed to the timothy hay did not promote such rapid decline of zoosporogenesis. It was suggested that release of inducers for zoosporogenesis from concentrate feed persisted longer time than from timothy hay. HC diet promoted the lowest zoospore production, suggested the lowest fungal population size in this experiment.

These results show that an appropriate amount of concentrate may support fungal growth and stimulate zoosporogenesis in the rumen.

(Key Words: Anaerobic Rumen Fungi, Fungal Population Size, Dietary Concentrate, Agar Strip, Zoosporogenesis)

INTRODUCTION

Anaerobic rumen fungi have been isolated from the rumen and are considered to play important roles in plant cell wall digestion (Fonty and Joblin, 1991). Indeed, Gordon and Phillips (1993) showed a significant contribution from anaerobic fungi to acid-detergent fiber degradation in sheep rumen which were fed barley straw and lucerne. This contribution also improved feed consumption by the sheep. The removal of anaerobic fungi from the sheep rumen reduced feed consumption and re-inoculation of fungi recovered it sianificantly. Sheep with fungi ingested 40% more of a straw-based diet (high in fiber) than they ingested when without fungi (Gordon and Phillips, 1993). In general, feeding of the high fiber diet resulted in larger fungal populations (Bauchop, 1979, 1989; Grenet et al., 1989a, b, Orpin, 1984), while feeding of the high concentrate diets resulted in smaller fungal populations (Bauchop, 1979, 1989; Grenet et al., 1989b). However, supplementation of maize to sorghum silage resulted in a larger fungal population and stimulated fungal fiber digestion in the rumen (Akin and Windham, 1989). Roles of fungi in starch degradation were also emphasized (McAllister et al., 1993). Thus, effect of fibrous feed on fungal population size may be complicated by the presence of concentrate feed.

In the present report, we examined effects of concentrate diets on relative fungal population size in the rumen by a modified method of Ushida et al. (1989).

MATERIALS AND METHODS

Three rumen-fistulated wethers (BW; 55-63 kg, AV; 60 kg) were used in this experiment. Timothy (*Phleum pratense*) hay (NDF 59.2, ADF 34.1%) and commercial concentrate (TDN 70, DCP 12%, Coop Dairy 14, Kumiai-shiryo, Kobe, Japan) were fed to the animals. Three

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consecutive experimental periods were designed: first period, all forage (AF); second period, forage : concentrate = 2:1 (moderate concentrate, MC); third period, forage : concentrate = 1:2 (high concentrate, HC). Each period had a 14 d adaptation period followed by a 2 d sampling period. Equal amount of diets (1,200 g/d) was fed to animals at 09:00 and 21:00. The hay was substituted by concentrate at 100 g/d for 4 d in an adaptation period. Mineral block and water were fed to wethers *ad libitum*.

Agar strips were prepared according to Ushida et al. (1989), but agar concentration was increased to 4%. The agar strips were introduced into the rumen of sheep through fistula at 09:00 (0 h), 12:00 (3 h), 15:00 (6 h), 18:00 (9 h) and withdrawn after 3 h incubation. Fungal germinated zoospores developed on sections of the agar strips were counted after cotton-blue staining under appropriate magnification using a stereoscopic microscope (SMZ-U, Nikon, Tokyo, Japan) equipped with an eye piece grid. Because the zoospores survived for 2-3 h (Orpin, 1975, 1976) and the germinated zoospores could satisfactorily be observed after 3 h incubation time, measurements were made with 3 h intervals to estimate the number of viable zoospores in the rumen at the time of introduction.

Approximately 50 ml of rumen fluid was collected via fistula at the introduction or withdrawal of agar strip and measured pH immediately. The sample was strained through four layers of surgical gauze and stored at −20℃ until short chain fatty acid (SCFA) analysis. Five milliliters of rumen fluid were added 1 ml of 30% metaphosphoric acid and 1 ml of crotonic acid (8 g/l), and analyzed for SCFA by gas chromatography (GC4BM-PF, Shimadzu, Kyoto, Japan). Separation of SCFA was made by 5% polyethylene glycol (PEG) 6000 on Shimalite TPA (Wako Junyaku, Osaka, Japan) and quantified by C-R6A integrator (Shimadzu).

RESULTS AND DISCUSSION

Colonization of germinated zoospore of anaerobic rumen fungi on agar strip after 3 h- (a) and 24 h- (b) incubation in the rumen of animal fed MC diet is shown in figure 1. The germinating zoospore (G) on the agar strip were visualized by lact-phenol cotton blue. In figure 1-b, mature zoosporangia (M) also can be seen. In the method of Ushida et al. (1989), agar strips were incubated for 24 h. In our preliminary experiment, germinated zoospores on agar strips could be satisfactory detected at the end of 3 h-incubation. This incubation period was employed to observe detailed fungal fluctuation in the rumen. The numbers of germinated zoospores were considered as relative fungal population size at the time of introduction of agar strips into the rumen.

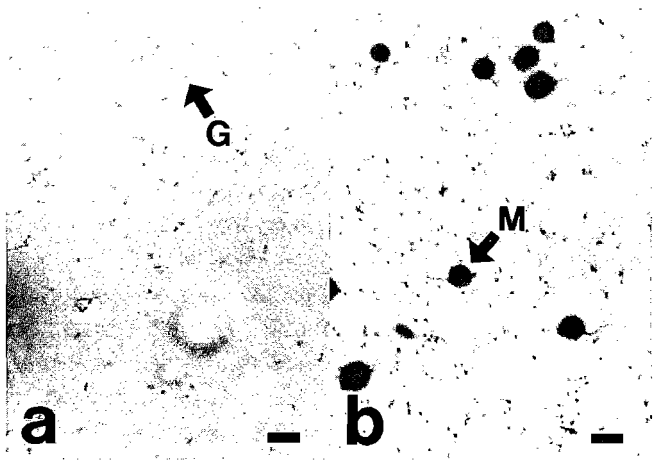

Figure 1. Micrograph showing colonization of anaerobic rumen fungi on agar strip after 3 h-incubation (a) and 24 h-incubation (b) in the rumen of animal fed MC diet (X 75). The germinating zoospores (G) and mature zoosporangia (M). on the agar strip were stained by lact-phenol cotton blue.
Bar shows 100 μm.

The numbers of the germinated zoospores and pH in the rumen of sheep fed varying amount of concentrate feed are shown in figure 2. The number of the zoospores on the strip was drastically decreased after morning feed with AF diet (figure 2-a). The number was the highest at 0 h ($1.3 \times 10^4/cm^2$), then declined to $2.0 \times 10^3/cm^2$ at 9 h after feeding. The average ruminal pH of animals fed AF diet at 0 h was 7.1. Then the pH declined and was maintained at lower levels (6.8-6.9). The correlation between number of zoospores and pH was not apparent. In the rumen of animals fed MC diet, the number of zoospores decreased with time after feeding, although the rate decrement was slower than that with AF diet. During 0-3 h after feeding, number of zoospores was $1.6 \times 10^4/cm^2$. Although the number slightly decreased at 6 and 9 h, the relatively high levels were maintained. It seems that the inducers for zoospore-release were maintained at relatively high concentration throught incubation period. At 0 h, the average ruminal pH of animals fed MC diet was 6.7, then the values declined to 6.4. The fluctuation pattern of number of germinated zoospores was different in the rumen of animals fed HC diet from those of AF and MC diets. The number of zoospores was constantly maintained at lower level ($1.0 \times 10^3/cm^2$) than the other diets. The ruminal pH was also placed at lower level (6.3

– 6.4) than the other diets. The numbers of zoospores at 0 h reflected the fungal population in the rumen.

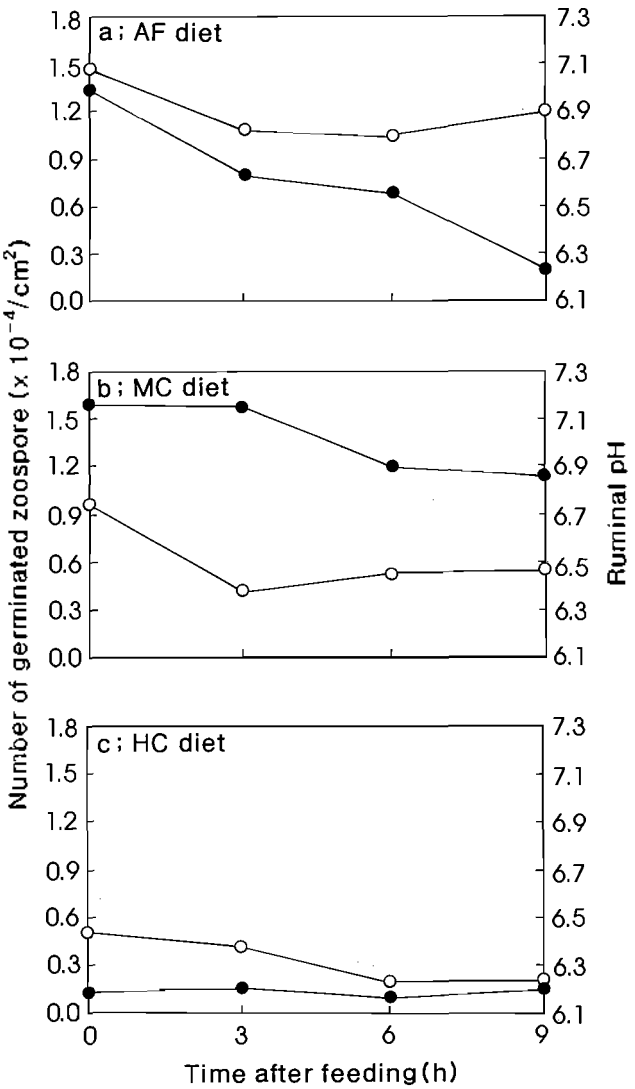

Figure 2. The effect of diet on number of germinated zoospores and ruminal pH.

○, Ruminal pH; ●, number of germinated zoospore.

Release of zoospores can be principally triggered by the introduction of hemes in plants into the rumen (Orpin and Greenwood, 1986b). For AF diet, high number of zoospores at 0 h was due to the release of zoospores from mature fungi induced by the hemes in timothy hay ingested by sheep. After that, other rumen microorganisms might utilize or destroy the hemes or the hemes might be washed out from the rumen, so that release of zoospore was decreased. Orpin and Greenwood (1986b) suggested that hemes acted on zoosporogenesis in synergism with other coponents of diet, or the responses of fungi were different according to the physiological state of fungi. The regulation of zoosporogenesis is indeed complex, since it could not be reproduced routinely *in vitro* (Orpin and Greenwood, 1986a). For MC diet, continuous high number of zoospores may be due to the continuous release of zoospores by hemes in timothy hay and concentrate feed, and by unknown mechanisms. Unlike AF diet which promoted relatively rapid decline of zoosporogenesis, supplementation of concentrate feed to the timothy hay did not promote such rapid decline of zoosporogenesis. It was suggested that production of inducers for zoosporogenesis from concentrate feed persisted longer time than from timothy hay.

Since life cycle of anaerobic fungi is relatively long (24-32 h) (Joblin, 1981; Lowe et al., 1987), fungi appeared to preferentially colonize on the ligno-cellulosic tissue which is tolerant to microbial attack and has longer retention time in the rumen (Akin, 1987; Akin and Benner, 1988; Akin et al., 1989; Bauchop, 1979; Bauchop, 1981; Bauchop, 1989). Fungal population size was increased in the rumen, when animals were fed alfalfa hay or meadow hay (Bauchop, 1981; Grenet et al., 1989a, b). On the other hand, the population size decreased when animals were fed sugar beat, soft leaf forage or grains (Bauchop, 1979; Bauchop, 1981; Gordon, 1985; Grenet et al., 1989a). Therefore the dependence of fungal development on the forage is obvious. HC diet promoted the lowest zoospore production, suggested the lowest fungal population size in this experiment. The present results were compatible with those earlier studies.

Concentrate feed usually promotes low ruminal pH. Low pH (less than 6.0) decreased release of zoospores in fresh rumen fluid (Orpin, 1975, 1976, 1977). Indeed, the pH was the lowest with HC diet in this experiment. Concentrate feed also often promotes high molar ratio of propionate. Propionate is known to have antifungal activity and is used as a food preservative (Horiguchi, 1982). Ushida et al. (1993) suggested that high rumen propionate level (27 mM) affected the composition of fungal microflora but not total population size. Since concentration of propionate ranged between 10 to 15 mM in the rumen of animals fed HC diet, the propionate level did not affect fungal population. Accordingly the smaller number of germinated zoospores was due to smaller supply of ligno-cellulosic materials and probably to low pH in the rumen.

Since concentrate feed mixture contains, proteins, microelements, vitamins and other nutrients, growth of fibrolytic microorganisms seems to be stimulated if physico-chemical condition such as pH is not critical.

The larger number of zoospores that reflected larger fungal population size with MC diet might be caused by this mechanisms, because amino acids, branched short chain fatty acids and vitamins stimulated growth of *Neocallimastix patriciarum* in pure culture, and acetate and soluble sugars stimulated zoosporic germination (Orpin and Greenwood, 1986a). Moreover, several isolates could not grow on cellulose without cellobiose suggesting the importance of soluble sugars (Matsui and Ushida, unpublished observation).

This result shows that an appropriate amount of concentrate may support fungal growth and stimulate zoosporogenesis in the rumen.

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REFERENCES

Akin, D. E. 1987. Association of rumen fungi with various forage grasses. Anim. Feed Sci. Technol. 16:273-285.

Akin, D. E. and R. Benner. 1988. Degradation of polysaccharides and lignin by ruminal bacteria and fungi. Appl. Environ. Microbiol. 54:1117-1125.

Akin, D. E., C. E. Lyon and L. L. Rigsby. 1989. Physical degradation of lignified stem tissues by ruminal fungi. Appl. Environ. Microbiol. 55:611-616.

Akin, D. E. and W. R. Windham. 1989. Influence of diet on rumen fungi. In: Nolan, J. V., R. A. Leng and D. I. Demeyer (eds). The Roles of Protozoa and Fungi in Ruminant Digestin. Armidale: Penumbul Books. p. 75-81.

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

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

Bauchop, T. 1989. Colonization of plant fragments by protozoa and fungi. In: Nolan, J. V., R. A. Leng and D. I. Demeyer (eds). The Roles of Protozoa and Fungi in Ruminant Digestion. Armidale: Penambul Books. p. 83-95.

Fonty, G. and K. N. Joblin. 1991. Rumen anaerobic fungi: Their role and interactions with other rumen microorganisms in relation to fiber digestion. In: Tsuda, T, Y. Sasaki, R. Kawashima (eds). Physiological Aspects of Digestion and Metabolism in Ruminants. San Diego, USA: Academic Press. p. 665-680.

Gordon, G. L. R. 1985. The potential for manipulation of rumen fungi. Rev. Rural Sci. 6:124-128.

Gordon G. L. R, M. W. Phillips. 1993. Removal of anaerobic fungi from the rumen of sheep by chemical treatment and the effect on feed consumption and *in vivo* fibre digestion. Lett. Appl. Microbiol. 17:220-223.

Grenet, E., A. Breton, P. Barry and G. Fonty. 1989a. Rumen anaerobic fungi and plant substrate colonization as affected by diet composition. Anim. Feed Sci. Technol. 26:55-70.

Grenet, E., G. Fonty and P. Barry. 1989b. SEM study of the degradation of maize and lucerne stems in the rumen of gnotobiotic lambs harbouring only fungi as celluloytic microorganisms. In: Nolan, J. V., R. A. Leng and D. I. Demeyer (eds). The Role of Protozoa and Fungi in Ruminant Digestion. Armidale, Australia: Penambul Books. p. 265-267.

Horiguchi, H. 1982. Bokin bobai no kagaku. Tokyo. Japan; Sankyo Shuppan. p. 144-147.

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

Lowe, S. E., G. W. Griffith, A. Milne, M. K. Theodorou and A. P. Trinci. 1987. The life-cycle and growth kinetics of an anaerobic rumen fungus. J. Gen. Microbiol. 133:1815-1827.

McAllister, T. A., Y. Dong, L. J. Yanke, H. D. Bae, K. -J. Cheng. 1993. Cereal grain digestion by selected strains of ruminal fungi. Can. J. Microbiol. 39:367-376.

Orpin, C. G. 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. J. Gen. Microbiol. 91:249-262.

Orpin, C. G. 1976. Studies on the rumen flagellate *Sphaeromonas communis*, J. Gen. Microbiol. 94:270-280.

Orpin, C. G. 1977. The rumen flagellate *Piromonas communis*: Its life-history and invasion of plant material in the rumen. J. Gen. Microbiol. 99:107-117.

Orpin, C. G. 1984. Role of ciliate protozoa and fungi in the rumen digestion of plant cell walls. Anim. Feed Sci. Technol. 10:121-143.

Orpin, C. G. and Y. Greenwood. 1986a. Nutritional and germination requirements of the rumen chytridiomycete *Neocallimastix patriciarum*. Trans. Br. Mycol. Soc. 86:103-109.

Orpin, C. G. and Y. Greenwood 1986b. The role of haems and related compounds in the nutrition and zoosporogenesis of the rumen chytridiomycete *Neocallimastix frontalis* H8. J. Gen. Microbiol. 132:2179-2185.

Roger, V., A. Bernalier, E. Grenet, G. Fonty, J. Jamot and P. Gouet. 1993. Degradation of wheat straw and maize stem by a monocentric and a polycentric rumen fungi, alone or in association with rumen cellulolytic bacteria. Anim. Feed Sci. Technol. 42:69-82.

Ushida, K., A. Okutani and Y. Kojima. 1993. Effect of sodium propionate on the population size and generic distribution of phycomycete fungi in sheep rumen. Anim. Sci. Technol. 64:250-253.

Ushida, K., H. Tanaka and Y. Kojima. 1989. A simple *in situ* method for estimating fungal population size in the rumen. Lett. Appl. Microbiol. 9:109-111.