Influence of an Anaerobic Fungal Culture (*Orpinomyces sp.*) Administration on Growth Rate, Ruminal Fermentation and Nutrient Digestion in Calves

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ABSTRACT : The study was to see the effect of administration of ruminal fungi on feed intake, growth rate, rumen fermentation and nutrient digestion of calves (Tharparkar×Holstein-Friesian, average age: 10 months, average body weight: 130 kg). The 6 calves in first group were fed a mixture consisted of 50% wheat straw and 50% concentrate (Maize 62%, Groundnut cake 35%, Mineral mix. 2% and Common salt 1%) along with 1 kg green oats animal^{-1} day^{-1} while second group calves were fed the above-mentioned diet in addition to a dose of 160 ml (10^6 CFU/ml) fungal culture calf^{-1} week^{-1}. The average dry matter intake per day was slightly lowered in fungal fed calves yet feed conversion ratio was higher. The average daily weight gain was significantly higher (15.37%) in fungal administered group as compared to control. The nutrient digestibility was increased for crude fibre, NDF and ADF with fungal administration. Digestible energy value of straw-based diet in terms of percent TDN also increased. The pH and NH_3-N were lower whereas TVFA, total-N, TCA-N and number of zoospores were higher in rumen liquor in fungal administered group. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 6 : 820-824)***

Key Words : Anaerobic Ruminal Fungi, *Orpinomyces sp.*, Growth Rate, Nutrient Digestion

INTRODUCTION

To alleviate the problems associated with feedstuff utilization, attempts have been made in recent past to manipulate the rumen microbial ecosystem through dietary treatments (Ho et al. 1996; Gordon et al., 2000). The growing concern over the use of antibiotics and other growth stimulants in feed industry has increased attention in evaluating the effects of live microbial feeding on animal performance. Interests in the ruminal anaerobic fungi have been growing after their discovery by Orpin (1975), especially on their capacity for fibre digestion by preferentially colonizing highly lignified thick-walled sclerenchyma and vascular tissues. The fungal rhizoids penetrate deep into the recalcitrant tissues and digest cell wall components through enzymes, whereas bacteria act on peripheral areas. Rumen fungi have a strong fibrolytic activity that helps in degradation of low quality roughages by breaking the linkages between lignin and hemicelluloses (Akin et al., 1983; Borneman et al., 1992; Paul et al., 2003). However, the fibre degradation can be affected, as the fungal enzymes are inhibited by the addition of certain microbial inhibitory compounds within the plant structure (Chang and Calza, 2002). Gordon and Phillips (1993) reported that sheep with fungi present in the rumen ate more of a straw based diet because of increased digestibility. Lee et al. (2000) studied the influence of direct administration of *Orpinomyces* strain to rumen of sheep and reported the increased nutrient digestibility and nitrogen retention. Manikumar et al. (2002;2003) showed the ability of different fungal species on fibre digestibility *in vitro* and reported that *Orpinomyces sp.* is a most promising isolate for the degradation of cereal straws. Hence, the present study was conducted to observe the influence of *Orpinomyces sp.* administration on feed intake, growth rate, rumen fermentation and nutrient digestion in calves.

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MATERIALS AND METHODS

Fungal culture and inoculums preparation

Orpinomyces sp. (C-14), originally isolated and identified by Singhal (2000) was obtained from Fungal Biotechnology Laboratory. The culture was revived, maintained by roll tube technique (Hungate, 1969) and tested for identical morphology to the parent cultural characteristics including polycentric thallus and polyflagellated zoospores before being used as an inoculum. For culturing *Orpinomyces sp.* under strict anaerobiosis, the method adopted by Joblin (1981) was followed. Oxygen-free carbon dioxide gas was passed through the media from Biosystem Gassing Manifold to make the media completely free from oxygen. The bottles (160 ml) were shifted to the CO_2 gas incubator at 39±1°C for 7 d. The fungal colony count was done by Hungate's roll tube technique and 160 ml of *Orpinomyces sp.* culture at a concentration of 10^6 CFU/ml was drenched to each treatment animal.

Selection of experimental animals and growth studies

Twelve male crossbred (Tharparkar×Holstein-Friesian) calves of average age (10 months) were selected from the herd having average initial body weight of 130 kg. The

Table 1. Growth rate, total feed intake and feed conversion ratio in crossbred calves fed wheat straw based complete feed mixture without and with fungal (*Orpinomyces sp.*) culture administration

Particulars	Groups		t values
	Control	Fungal culture administered	
Average initial body wt. (kg)	131.0±23.71	128.7±12.75	0.158^{NS}
Average final body wt. (kg)	186.3±27.69	192.5±17.69	0.378^{NS}
Total body wt. gain (kg)	55.3±5.12	63.8±5.31	3.535*
Gain day^{-1} (g)	614.8±56.92	709.3±59.03	3.535*
Total DMI (kg)	366.78±46.38	363.79±30.93	0.088^{NS}
Av. DMI day^{-1} (kg)	4.08±0.52	4.04±0.34	0.088^{NS}
Feed conversion ratio (kg DM/ kg gain)	6.62±0.42	5.69±0.11	2.387^{NS}

NS=non-significant, * Significant ($p<0.05$).

animals were randomly allocated into two groups of six each in a completely randomized design. Calves were fed a diet consisting of roughage and concentrate on (50-50) DM basis. Roughage portion consisted mainly of wheat straw along with 1 kg green oats to each animal for meeting the vitamin A and other nutritional requirements of the growing calves as per NRC (1989) for a body weight gain of 600 g/d. The treatment group was offered the diet along with weekly administration of *Orpinomyces sp.* culture, orally.

The animals were housed in ventilated individual pens and were provided with fresh water free choice twice daily at 12:00 and 18:00 h. Throughout the experimental period, calves were maintained in an open asbestos sheeted shed with *Pucca* floor, having arrangement for individual feeding. Healthy surroundings and proper sanitary conditions were maintained. The calves were shifted to metabolic shed 4 d before the start of trial and were maintained there till the completion of 7 d trial period. The experimental calves were inspected daily for any disease and mortality.

After pre-adaptation (20 d) the calves were weighed in the morning before they were fed or watered, initially for two consecutive days to get the average body weight of animals. The weight of individual calf was recorded at weekly interval for 91 d. The absolute rate of growth was estimated as per Broody (1945). The daily feed intake was recorded by subtracting the feed refusal from daily feed offered and the percent feed conversion efficiency was calculated.

Digestibility trial

After 45 d of feeding, a digestibility trial of 7 d was conducted in a specially designed stall. Body weights of the animals were also recorded for 2 d consecutively before and after digestibility trial. A proper record of feed consumed, refusal and faeces voided by each calf was maintained during the trial period.

Rumen liquor was collected at zero hour from experimental animals through stomach tube (Lane et al., 1968) at the end of digestibility trial. After collection of rumen liquor, 1 to 2 drops of saturated mercuric chloride was added to stop the microbial activity. The samples of rumen liquor were kept in a refrigerator for further analysis.

Estimation of Proximate Principles and cell wall components

Ground samples of concentrate mixture, wheat straw, oats fodder, feed refusal and those of faeces were analyzed for proximate principles, viz., dry matter (DM), crude protein (CP), ether extract (EE), crude fibre (CF) and total ash as per standard procedures (AOAC, 1992). Cell wall components of feeds and faeces were estimated by the method of Goering and VanSoest (1970).

Analysis of rumen liquor

Immediately after collection of sample, the pH was recorded and TVFA was estimated by the method of Barnett and Reid (1956). Total nitrogen and TCA precipitable nitrogen were estimated as per Kjeldahl's method (AOAC, 1992). Micro-diffusion technique of Conway (1962) was used to estimate NH_3-N. The direct microscopic counts of fungal zoospores in rumen samples were made after appropriately diluting the samples in diluents (Bryant and Burky, 1953) using standard procedure and expressed per ml of rumen fluid. The statistical analyses were performed as per Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

The average daily weight gains were observed to be higher with treatment diet as compared to control and the differences were statistically significant. Since, the present study pertains only to the accelerating phase of growth, thus, the differences in growth performance among animals offered control and treatment diets, could be attributed to the better utilization of wheat straw by the latter group, because of the availability of more digestible energy from the breakdown of lignocelluloses bonds by '*Orpinomyces sp.*' (Table 1). Phillips and Gordon (1995) has applied a similar strategy to enhance the fibre digestion and fermentation in the rumen by anaerobic fungi in sheep and reported that production efficiency may be increased by 5 to 15%. Our results are in accordance as the growth rate in

Table 2. Digestibility coefficient (%) of various nutrients of wheat straw based complete feed mixture without and with fungal (*Orpinomyces sp.*) culture administration in crossbred calves

Particulars	Groups		t values
	Control	Fungal culture administered	
Dry matter	53.94±0.55	59.95±1.25	3.621*
Crude protein	59.76±0.63	65.82±1.53	3.669*
Ether extract	86.87±0.75	85.63±0.81	1.278^{NS}
Crude fibre	50.28±0.74	54.94±1.15	4.276**
Nitrogen free extractives	55.59±1.19	63.04±1.95	2.605*
Neutral detergent fibre	44.36±1.28	55.25±1.43	5.123**
Acid detergent fibre	42.94±1.02	51.98±1.68	3.686*
Cellulose	54.59±1.29	61.55±1.52	2.695*

NS=non-significant, * Significant ($p<0.05$), ** Significant ($p<0.01$).

Table 3. Nutritive evaluation, nutrient intake and efficiency of nutrient utilization in terms of DCP and TDN of wheat straw based complete feed mixture without and with fungal (*Orpinomyces* sp.) culture administration in crossbred calves

Particulars	Groups		t values
	Control	Fungal culture administered	
% DCP	9.11±0.14	9.75±0.29	2.289^{NS}
Total DCP intake (kg)	33.18±3.77	35.48±3.28	0.666^{NS}
DCP intake/day (g)	368.65±41.89	394.18±36.43	0.666^{NS}
DCP intake/kg gain (g)	601.51±33.43	556.04±23.66	1.111^{NS}
% TDN	55.33±0.47	60.79±1.13	3.709*
Total TDN intake (kg)	194.02±27.95	221.46±19.38	1.063^{NS}
TDN intake/day (kg)	2.16±0.31	2.46±0.22	1.063^{NS}
TDN intake/kg gain	3.51±0.32	3.47±0.11	0.132^{NS}

NS=non-significant, * Significant ($p<0.05$).

calves was found to increase by 15.37 percent with the fungal culture administration.

Gordon and Phillips (1993; 1998) reported an increase in the voluntary intake of straw based diet by 7 to 12% when sheep were dosed by mouth with monocentric fungi, originally isolated from herbivores other than sheep. Also an oral inoculum of a *Neocallimastix sp.* from sheep stimulated forage intake by 35% in early-weaned calves (Theodoru et al., 1990). In contrast, polycentric ruminal fungi dosed as a freshly grown culture at a weekly interval to calves showed no increase in voluntary feed intake (Table 1).

The average initial and final body weights of calves in treated and untreated group were found to be statistically non-significant. The average daily gains were observed to be higher in treated group in spite of the fact that calves ate equal amount of dry matter, thus clearly indicating that diet with weekly dosing of fungal culture was better utilized (Ha et al., 1994; Phillips and Gordon, 1995). Oka et al. (1999) also observed the average daily gain in male calves fed on high roughage basal diet as 750 g/d. Even though the DM intake was similar in both the groups, but due to the variation in body weight gains, the feed conversion ratio was found to be lower in treatment group than control, although the differences were statistically non-significant.

The digestibility coefficient of DM, CP, CF, ADF and NDF were significantly higher in treatment group compared to control, whereas, ether extract digestibility was similar in both the groups (Table 2). The improvement of digestion coefficients of nutrients by the administration of fungal culture would be expected from improved ruminal fermentation parameters (Lee et al., 2000;2001). The increase in the zoospore count with the dosing of *Orpinomyces sp.* in the rumen of calves might also be responsible for the breakdown of fibrous material of the straw due to which, there was a significant increase in the digestibility of different nutrients. Kostiukovoskii et al. (1990) isolated two anaerobic fungal strains from cattle rumen and reported that these fungi can utilize a wide spectrum of mono-, oligo- and poly- saccharides. Lee et al. (2000) administered *Orpinomyces* strain to the rumen of sheep and observed an increase in the nutrient digestibility resulting from an increase in the number of bacteria and fungi in the rumen by altering the pattern of VFA production.

Similarly, Akin et al. (1990) reported that polycentric fungi *Orpinomyces* increased the DM digestibility of Bermuda grass stems. Manikumar (2002; 2003) also reported the increased IVDMD of cereal straws after 48 h incubation with strained rumen liquor and *Orpinomyces sp.* (C-14). Contrary to this, Samanta et al. (2001) reported that the addition of *Piromyces sp.* to mixed rumen inoculum did not increase IVDMD significantly. The present study demonstrates that the introduction of *Orpinomyces sp.*

Table 4. Influence of anaerobic fungal (*Orpinomyces* sp.) culture administration on rumen fermentation parameters in crossbred calves

Particulars	Groups		t values
	Control	Fungal culture administered	
pH	7.18±0.03	7.01±0.03	3.068*
Total VFA (mM/100 ml)	11.57±0.36	13.02±0.58	1.828^{NS}
Total nitrogen (mg/100 ml)	84.00±3.83	109.20±2.39	4.538**
Ammonia nitrogen (mg/100 ml)	15.52±1.27	7.93±1.06	4.930**
TCA precipitable nitrogen (mg/100 ml)	57.50±3.09	87.97±1.96	6.969**
Average number of zoospores/ml	1.08×10^5	2.42×10^5	-

NS=non-significant, * Significant ($p<0.05$), ** Significant ($p<0.01$).

having superior fibrolytic activity into the rumen of crossbred calves can improve their nutrient utilization (Table 3) and that has a beneficial effect on the growth rate.

The percent DCP values for the control and treatment groups did not vary statistically, but the values for the treatment group were numerically high. The percent TDN values of the control and treatment groups varied significantly indicating higher energy availability to the calves in treatment group (Table 3). The higher energy availability to the calves of treatment group may also be due to the higher digestibility of nutrients (Table 2). The total DCP intake and DCP intake/d were recorded to be apparently more treatment group though the differences were statistically non-significant. The protein utilization measured as DCP intake/kg gain did not vary significantly between the two groups (Table 3). The total TDN intake and TDN intake/d were higher but statistically there was no difference in the efficiency of energy utilization of two groups while apparently the intake/kg gain was slightly less in treatment group. This provided more digestible energy to the animals for higher body weight gain due to which the percent energy utilization was found to be better (Table 3).

The DMI in growing calves averaged 2.8 kg/100 kg body weight with a range of 2.3 to 3.6 kg per 100 kg body weight on different diets (Punia and Sharma, 1988; Sehgal et al., 1999). The DMI/100 kg body weight value obtained in the present experiment on growing crossbred calves fed on treatment diet was in close agreement with the reported average value.

The pH of the rumen liquor varied significantly between two dietary regimes and was significantly less for the treatment group (Table 4). Johnson and Sutton (1968) reported that ruminal pH can vary from greater than 7.0 to less than 5.0 depending upon the type of diet. The lowering of pH in treatment diet might be due to the increased TVFA after administration of fungal culture as is evident from the data (Table 4), though statistically non-significant. The total-N was significantly high and NH_3-N was significantly low in the rumen liquor of treatment group, might be due to the higher TCA-N or maximum utilization of NH_3-N for the microbial protein synthesis. Also, the availability of more VFAs from in treatment group was able to incorporate almost all the NH_3-N into microbial protein causing a fall in pH. The total-N and NH_3-N concentrations in the SRL of cattle reported by Bhatia et al. (1982) are in close agreement with our values.

Mehrez et al. (1977) reported that the optimal NH_3 concentration for maximal rate of fermentation was high (23.5 mg/100 ml) in comparison with most published values for optimum NH_3 concentration (5-6 mg/100 ml) for maximal microbial protein synthesis. However, *in vivo* studies have shown the corresponding NH_3 concentration to be 8.8 to 13.3 mg/100 ml (Hume et al., 1970) or 28.9 mg/100 ml rumen fluid (Miller, 1973).

The 2.4 fold increased number of zoospores in the treatment group indicates its establishment in the rumen that may be responsible for lowering the pH and increased TVFA due to increase in nutrient digestibility leading to increased TDN. The increased count after administration of live anaerobic fungus may establish in the rumen and remain viable for a longer period. The additional availability of the energy is responsible for the increase in microbial protein synthesis and higher gain in body weight in calves. Administration of anaerobic fungal culture significantly improved digestibility and rumen fermentation parameters hence; it could improve the nutrient utilization in ruminants.

REFERENCES

Akin, D. E., G. L. R. Gordon and J. P. Hogan. 1983. Rumen bacterial and fungal degradation of *Digitaria pentzii* grown with or without sulphur. Appl. Environ. Microbiol. 46:738-748.

Akin, D. E., L. L. Rigsby, C. E. Lyon and W. R. Windham. 1990. Relationship of tissue digestion to textural strength in bermuda grass and alfalfa stems. Crop Sci. 30:990-993.

AOAC. 1992. Official Methods of Analysis. 16th edn. Association of official analytical chemists, Washington, DC.

Barnett, A. J. G. and R. L. Reid. 1956. Studies on the production of volatile fatty acids from the grass by rumen liquor in an artificial rumen. J. Agric. Sci. 48:315-321.

Bhatia, S. K., S. Prahalad, K. Pradhan and D. P. Sharda. 1982. Note on the effect of guar meals on intra ruminal metabolism of protein and microbial transaminase activity in crossbred cattle. Indian J. Anim. Sci. 52:800-802.

Borneman, W. S., L. G. Ljungdahl, R. D. Hartley and D. E. Akin. 1992. Purification and partial characterization of two feruloyl esterases from the anaerobic fungi in relation to plant cell wall

degradation. Appl. Microbiol. Biotech. 33:345-351.
Broody, S. 1945. Bioenergetics and growth. Reinhold Publ. Corp., New York, USA.
Bryant, M. P. and L. A. Burkey. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. 36:205-217.
Chang, J. S. and R. E. Calza. 2002 Effect of Cicer Milkvetch (Astragalus cicer) extracts on physiology of rumen fungus: *Neocallimastix frontalis* EB188. Asian-Aust. J. Anim. Sci. 15:1156-1164.
Conway, E. J. 1962. Micro-diffusion analysis and volumetric error. 5th edn. Crossby Lockwood and Sons Ltd., London.
Goering, H. K. and P. J. VanSoest. 1970. Forage fibre analysis. Agricultural Handbook No. 379, USDA, Washington, DC.
Gordon, G. L. R. and 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.
Gordon, G. L. R. and M. W. Phillips. 1998. The role of anaerobic gut fungi in ruminants. Nutr. Res. Rev. 11:1-36.
Gordon, G. L. R., M. W. Phillips, A. J. Rintout and S. W. White. 2000. Increased intake of fibrous feed by sheep orally dosed with a culture of and elite non-indigenous anaerobic gut fungus. Asian-Aust. J. Anim. Sci. 13 (Suppl) p. 13.
Ha, J. K., S. S. Lee, C. H. Kim, Y. J. Choi and H. K. Min. 1994. Effect of fungal inoculation on ruminal fermentation characteristics, Enzyme activities and nutrient-digestion in sheep. Proc. Soc. Nutr. Physiol. 3:197.
Ho, Y. W., N. Abdullah and S. Jalaludin. 1996. Microbial colonization and degradation of some fibrous crop residues in the rumen of goats. Asian-Aust. J. Anim. Sci. 9:519-524.
Hume, I. D., R. J. Moir and M. Somers. 1970. Synthesis of microbial protein in the rumen. 1. Influence of the level of nitrogen intake. Aust. J. Agri. Res. 21:283-296.
Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes. Methods Microbiol. 3B:117-132.
Joblin, K. N. 1981. Isolation, enumeration and maintenance of rumen anaerobic fungi in roll tubes. Appl. Environ. Microbiol. 42:1119-1122.
Johnson, V. W. and J. D. Sutton. 1968. The continuous recording of the pH in the bovine rumen. Br. J. Nutr. 22:303-306.
Kostiukovskii, V. A., O. N. Okunev and B. V. Tarakanov. 1990. Anaerobic cellulolytic fungi from cattle rumen. Mikrobiologiia 59:1067-1074.
Lane, G. T., C. H. Nellor, V. F. Colenbrander, K. R. Cummings and R. B. Harrington. 1968. Apparatus for obtaining rumino-reticular samples and the effect of sampling locations on pH and volatile fatty acids. J. Dairy Sci. 51:114-116.
Lee, S. S., J. K. Ha and K. J. Cheng. 2000. Influence of an anaerobic fungal culture administration on *in vivo* ruminal fermentation and nutrient digestion. Anim. Feed Sci. Technol. 88:201-217.
Lee, S. S., J. K. Ha and K. J. Cheng. 2001. Effects of LCFA on the gas production cellulose digestion and cellulase activities by the rumen anaerobic fungus Neocallimastix frontalis RE 1. Asian-Aust. J. Anim. Sci. 14:1110-1117.
Manikumar, B., A. K. Puniya and K. Singh. 2002. Influence of ruminal Fungi on degradation of wheat straw *in vitro*. Indian J. Microbiol. 42:133-136.
Manikumar, B., A. K. Puniya and K. Singh. 2003. Effect of ruminal fungi on *in vitro* degradation of rice straw. Indian J. Anim. Sci. 73:312-314.
Mehrez, A. Z., E. R. Orskov and I. McDonald. 1977. Rates of rumen fermentation in relation to ammonia concentration. Br. J. Nutr. 38:437-443.
Miller, E. L. 1973. Evaluation of food as source of N and amino acids. Proc. Nutr. Soc. 32:79-84.
NRC. 1989. Nutrient requirement of calves. National Academy of Science, Washington, DC.
Oka, A., T. Dohogo, S. Ohtagaki and M. Juen. 1999. Effects of roughage levels on growth, beef quality, ruminal contents and serum constituents in Japanese Black steers during the growing period. Anim. Sci. J. 70:451-459.
Orpin, C. G. 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. J. Gen. Microbiol. 91:249-262.
Paul, S. S., D. N. Kamra, V. R. B. Sastry, N. P. Sahu and A. Kumar. 2003. Effect of phenolic monomers on biomass and hydrolytic enzyme activities of an anaerobic fungus isolated from wild nil gai (*Baselophus tragocamelus*). Lett. Appl. Microbiol. 36:377-381
Phillips, M. W. and G. L. R. Gordon. 1995. Carbohydrate fermentation by three different polycentric rumen fungi from cattle and water buffalo in tropical Australia. Anaerobe. 1:41-47.
Punia, B. S. and D. D. Sharma. 1988. Effect of dietary energy source in complete feed on digestion and live weight gain in buffalo and crossbred cattle calves. Indian J. Anim. Sci. 58:715-720.
Samanta, A. K., T. K. Walli and K. K. Singh. 2001. Role of different groups of microbes on fibre utilization. Indian J. Anim. Sci. 71:497-498.
Sehgal, J. P., D. D. Sharma, M. K. Ghosh and K. K. Singhal. 1999. Fattening of male buffalo calves for veal production. Buffalo J. 2:235-239.
Singhal, A. 2000. Characterization of rumen fungi and their genetic analysis. M.Sc. Thesis, Dairy Microbiology Division, National Dairy Research Institute, Karnal, India.
Snedecor, G. W. and W. G. Cochran. 1980. Statistical Methods. 7th edn. The Iowa State University Press, Iowa, USA.
Theodorou, M. K., M. K. Gill, C. King-Spooner and D. E. Beever. 1990. Enumeration of anaerobic chytridiomycetes as thallus forming units: A novel method for the quantification of fibrolytic fungal populations from the digestive tract ecosystem. Appl. Environ. Microbiol. 56:1073-1078.