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Full Length Research Paper

Influence of bacteria and protozoa from the rumen of buffalo on *in-vitro* activities of anaerobic fungus *Caecomyces* sp. isolated from the feces of elephant

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Anaerobic fungal isolates *Caecomyces* sp. from the feces of elephant and *Orpinomyces* sp. from buffalo rumen were co-cultured *in-vitro* with rumen bacterial and protozoal fractions collected from buffalo to observe the possible fate of these fungi in the rumen, if inoculated as microbial-feed supplements. When co-cultured together or separately with rumen bacteria and protozoa, *Caecomyces* sp. was adversely affected. However, bacterial and protozoal counts were higher, compared to the counts when grown alone. Similar patterns of results were observed when *Orpinomyces* sp. was grown in co-culture with bacteria and protozoa separately as well as together, indicating that it is possibly the inhibitory action of bacteria and protozoa, and not inter-species competition, that affects the growth of fungi preventing them from attaining their full fibre-degrading potential. Conversely, although fungal counts were lowered during their co-culturing with bacterial and protozoal fractions, their co-culturing increased the FPase activity of the co-cultured fraction which could be the apparent reason for enhanced fibre degradation.

Key words: Rumen microflora, anaerobic fungi, microbial interactions, fibre degradation.

INTRODUCTION

The rumen is a highly complex ecosystem that contains different microbial species. Ruminant's performance depends on the activities of their microorganisms to utilize the dietary feeds. The rumen microbial ecosystem comprised at least 30 bacterial (10^{10} to 10^{11}/ ml rumen fluid) (Stewart et al., 1997), 40 protozoa (10^5 to 10^7) (Williams and Coleman, 1997), and 6 fungal species ($<10^5$) (Ozkose et al., 2001; Nagpal et al., 2009b). Bacteria, fungi, and protozoa are responsible for 50 to 82% of cell-wall degradation (Lee et al., 2000). Although, substrate competition is high in the rumen, the synergism and symbiosis among different groups of microorganisms make the utilization of substrates more efficient. Many relationships are known to exist among microorganisms in the rumen (Lee et al., 2000), and it is well established that anaerobic fungi actively participate in degradation of plant materials in ruminants, as these penetrate plant tissues better than bacteria or protozoa (Orpin and Joblin, 1988). Therefore, a considerable potential exists for the manipulation of fungal activity in the rumen to benefit the utilization of poor quality roughages by domesticated ruminants for the increased production responses; and one potential mean may involve inoculation of efficient fungal strains into the ruminants (Paul et al., 2004; Dey et al., 2004; Lee et al., 2000; Thareja et al., 2006; Tripathi et al., 2007; Nagpal et al., 2009a, b; 2010). The interactions of anaerobic fungi with other rumen microbes can be positive, negative or neutral, depending on the microbial groups involved and the type of substrate used. Since rumen fungi produce appreciable amounts of H_2, they can interact with H_2 utilizers that in turn alter their metabolite production. Methanogens are the principal H_2 utilizers in rumen; and stable co-cultures of fungi and methanogens

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have been established *in-vitro* (Orpin and Joblin, 1997). In contrast, fibre degradation by *Neocallimastix frontalis* has been found to decrease in co-cultures with non-lactate utilizing *Selenomonas ruminantium*, a sugar fermenting H_2 consuming rumen bacterium, thus indicating the occurrence of interspecies hydrogen transfer (Richardson and Stewart, 1990).

The fungi release metabolites such as free sugars, which serve as energy sources for other bacteria. The fungi themselves may depend on the bacteria for vitamins, heme and amino acids (Williams et al., 1994). Co-culture of anaerobic fungi with rumen bacteria could also inhibit the activity of fungi (Dehority and Tirabasso, 2000), suggesting the role of bacteria in controlling fungal activities *in-vivo*. Moreover, since fungal zoospores are of small size, they are likely to be a prey for protozoa (Morgavi et al., 1994). Consequently, the rumen fungi do not appear to attain their full fibre-degrading potential in rumen due to the inhibition by other microbes. Therefore, the present investigation was aimed to study the effect of co-culturing with rumen bacteria and protozoa from buffalo on *in-vitro* activities of anaerobic rumen fungi *Caecomyces* sp. (from elephant feces), and was compared with that of *Orpinomyces* sp. (from buffalo rumen) to observe the possible fate of these fungi in the rumen, if exploited as direct-fed microbials/ animal feed additives.

MATERIALS AND METHODS

Anaerobic fungi *Caecomyces* sp. and *Orpinomyces* sp. were isolated from Indian elephant and Buffalo (Nagpal et al., 2009b, 2010), respectively, by following the method of Joblin (1981) with cellobiose as a carbon and energy source; and were characterized on the basis of number of flagella/ zoospore, thallus morphology (monocentric or polycentric), and rhizoid (filamentous or a vegetative cell) type (Trinci et al., 1994; Thareja et al., 2006; Nagpal et al., 2009b).

For co-culturing of fungal isolates with rumen bacteria and protozoa, a bacterial and protozoal fraction was prepared from rumen liquor of buffalo fed on a standard diet containing 10 kg green fodder maize, 1 kg concentrate mixture, and wheat straw *ad lib*, maintained at institute's cattle yard. Total rumen bacterial fraction was prepared by inoculating supernatant of strained rumen liquor in the basal anaerobic media containing cycloheximide (0.05 mg/ ml) and sodium lauryl sulpahte (0.01 mg/ ml). Total rumen protozoal fraction was prepared by inoculating the resuspended pellet of centrifuged rumen liquor into the media containing cycloheximide (0.05 mg/ ml), penicillin (0.10 mg/ ml) and streptomycin (0.10 mg/ ml). The rumen fungi and bacterial and protozoal fractions were grown anaerobically at 39°C in basal anaerobic media (Obispo and Dehority, 1992), in the presence of antibiotics, cycloheximide or sodium lauryl sulphate to inhibit bacteria, fungi or protozoa, respectively. In co-cultures of fungi and bacteria, fungal broth and bacterial fraction was inoculated to the media and sodium lauryl sulphate was added; while for co-cultures of fungi and protozoa, fungal culture broth and protozoal fraction were added along with penicillin and streptomycin. Fungal and bacterial counts were taken as thallus forming units (tfu/ ml) and colony forming units (cfu/ ml), respectively, using roll-tube method (Joblin, 1981). Protozoal counts were taken as direct microscopic counts (DMC/ ml) using methyl green as staining agent.

Filter paper cellulase (FPase) activities were estimated after incubation in Orpin's broth supplemented with 1% Whatman No. 1 filter paper (6 x 1 cm ≈ 50 mg) (Thareja et al., 2006; Tripathi et al., 2007; Nagpal et al., 2009b), keeping one un-inoculated set as control. Supernatants from incubated cultures were analyzed for estimation of reducing sugars (glucose) using dinitrosalicylic acid method (Miller, 1959). Reaction mixture, comprising 1.0 ml of 0.1 M phosphate buffer (pH 6.8), 0.5 gm of substrate and 0.5 ml of culture supernatant, was incubated at 39°C for 1 h. A similar reaction mixture was prepared for control. The enzyme activities were calculated as IU, that is, µmol of glucose released per hour per ml of culture filtrate. All the data were statistically analyzed as per the method of Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

When co-cultured with rumen bacteria and protozoa, the fungal population was found to be adversely affected (Table 1). During co-culturing of fungi and bacteria for 96 h, a reduction in fungal counts was observed. However, bacterial counts were found to be higher, compared to the counts when bacteria were grown alone (Table 1). Fungal counts were also found to be reduced, when isolate FE5 was co-cultured with protozoal fraction. On the other hand, when fungi, bacterial and protozoa were grown altogether, fungi could not survive after 48 h and even bacterial numbers were found to be negatively affected. Since the growth of isolate FE5 was found to be adversely affected during its co-culturing with bacteria and protozoa, it was assumed that, since the source of isolate FE5 was elephant, and rumen liquor for collecting bacterial and protozoal fractions, and for media preparation was taken from buffalo maintained at NDRI cattle yard, there could have been some inter-species interactions that were hampering the growth of fungi during its co-culturing with bacteria and protozoa. Hence, to verify this further, isolate RB_2, which was earlier isolated from buffalo, was also co-cultured with bacterial and protozoal fractions. In this case also, a similar effect was observed (Table 2).

Fungal counts were lower when co-cultured with bacteria and protozoa separately, and were further reduced when fungi, bacterial and protozoa were grown altogether. Therefore, it indicated that it was possibly because of the inhibitory action of bacteria and protozoa, and not inter-species competition, which was affecting the growth of fungi. Since fungi are slow-growers, this could have been the reason for their lowered counts during co-culturing with bacteria, because, by the time fungi started growing (72-96 h), bacterial population had already grown and produced enough metabolites that inhibited the growth of fungi.

Co-culturing of anaerobic fungi with rumen bacteria have been shown to inhibit the growth of the fungi (Bernalier et al., 1992; Roger et al., 1993; Dehority and Tirabasso, 2000). Stewart et al. (1992) and Bernalier et al.

Table 1. Counts of anaerobic fungus *Caecomyces* sp. (log tfu/ml), bacteria (log cfu/ml) and protozoa (log DMC) when grown alone and in co-cultures.

Treatment	Incubation period			
	0 h	48 h	72 h	96 h
Fungi, bacteria and protozoa grown alone				
Fungal counts	4.63±0.3^{a}	4.67±0.2^{a}	4.86±0.5^{b}	4.94±0.2^{c}
Bacterial counts	6.91±0.3^{a}	7.07±0.2^{b}	7.16±0.1^{c}	7.07±0.2^{b}
Protozoal counts	5.06±0.1^{a}	5.28±0.2^{b}	5.44±0.3^{c}	5.46±0.2^{d}
Fungi and bacteria grown in co-culture				
Fungal counts	4.63±0.3^{a}	4.62±0.1^{a}	4.70±0.2^{b}	4.76±0.4^{b}
Bacterial counts	6.91±0.3^{a}	6.98±0.1^{a}	7.21±0.3^{b}	7.12±0.5^{c}
Fungi and protozoa grown in co-culture				
Fungal counts	4.63±0.3^{a}	4.61±0.0^{a}	4.59±0.2^{a}	4.44±0.2^{b}
Protozoal counts	5.06±o.1^{a}	5.37±0.3^{b}	5.42±0.4^{c}	5.42±0.2^{c}
Fungi, Bacteria and protozoa grown altogether				
Fungal counts	4.63±0.3^{a}	4.59±0.5^{a}	ND	ND
Bacterial counts	6.91±0.3^{a}	6.96±0.3^{a}	6.90±0.5^{a}	ND
Protozoal counts	5.06±0.1^{a}	5.12±0.1^{b}	5.21±0.2^{c}	5.36±0.1^{d}

$^{a-d}$: values with different superscripts in same treatment at different incubation periods differ significantly (P≤0.05).

Table 2. Counts of anaerobic fungus *Orpinomyces* sp. (log tfu/ml), bacteria (log cfu/ml) and protozoa (log DMC) when grown alone and in co-cultures.

Treatment	Incubation period			
	0 h	48 h	72 h	96 h
Fungi, bacteria and protozoa grown alone				
Fungal counts	4.65±0.3^{a}	4.69±0.3^{a}	4.83±0.4^{b}	4.96±0.3^{c}
Bacterial counts	6.91±0.3^{a}	7.07±0.2^{b}	7.16±0.1^{c}	7.07±0.3^{b}
Protozoal counts	5.06±0.1^{a}	5.28±0.4^{b}	5.44±0.3^{c}	5.46±0.2^{d}
Fungi and bacteria grown in co-culture				
Fungal counts	4.65±0.3^{a}	4.66±0.2^{a}	4.76±0.5^{b}	4.89±0.2^{b}
Bacterial counts	6.91±0.3^{a}	7.01±0.5^{a}	7.18±0.6^{b}	7.12±0.2^{c}
Fungi and protozoa grown in co-culture				
Fungal counts	4.65±0.3^{a}	4.67±0.0^{a}	4.76±0.1^{a}	4.57±0.3^{b}
Protozoal counts	5.06±0.1^{a}	5.34±0.3^{b}	5.45±0.2^{c}	5.45±0.4^{c}
Fungi, Bacteria and protozoa grown altogether				
Fungal counts	4.65±0.3^{a}	4.61±0.3^{a}	4.49±0.4^{b}	ND
Bacterial counts	6.91±0.3^{a}	6.91±0.2^{a}	6.95±0.6^{a}	6.71±0.3^{b}
Protozoal counts	5.06±0.1^{a}	5.18±0.3^{b}	5.29±0.3^{c}	5.11±0.2^{d}

$^{a-d}$: Values (means ± SD; n = 3) with different superscripts in same treatment at different incubation periods differ significantly (P≤0.05).

(1993) also found an extracellular, thermo-labile protein produced by ruminococci, which inhibited the activities of anaerobic fungi. Dehority and Tirabasso (1993) also found that mixed rumen bacteria produce a heat stable compound *in-vitro*, which inhibits growth of the rumen fungi. Activities of *N. frontalis* were also found to decrease in co-cultures with *Selenomonas ruminantium*, a sugar fermenting H_2 consuming rumen bacterium, indicating the occurrence of interspecies hydrogen transfer (Richardson and Stewart, 1990). Thus, the rumen

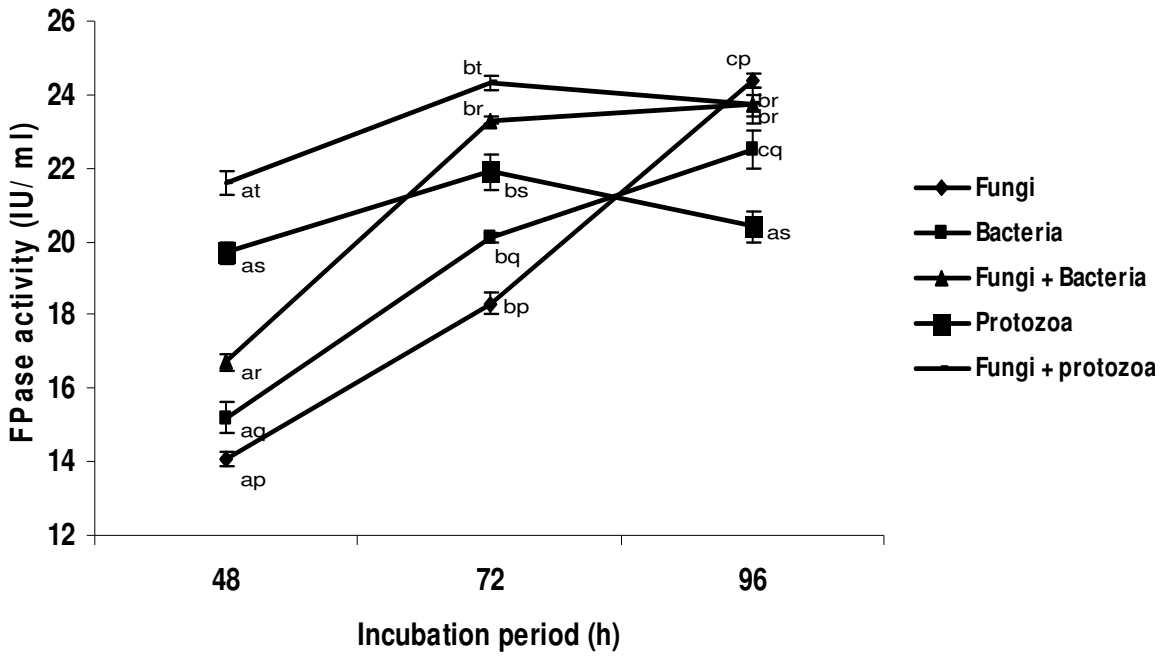

Figure 1. FPase activity of fungi, bacteria and protozoa grown alone and in co-cultures. $^{a-c}$: Values (means ± SD; n = 3) with different superscripts in same treatment at different incubation periods differ significantly (P≤0.05). $^{p-t}$: Values (means ± SD; n = 3) with different superscripts in different treatment at same incubation periods differ significantly (P≤0.05).

fungi do not appear to attain their full fibre-degrading potential in rumen due to the inhibition by bacteria. Since these fungi reproduce through small-sized zoospores which act as food for protozoa, hence, fungal counts were lowered during their co-culturing with protozoa due to the predatory action of protozoa over fungal zoospores. Co-incubation of protozoa with fungi have earlier also shown that the protozoa are able to ingest and digest fungi (Morgavi et al., 1994). The fungal growth was negatively affected by rumen protozoa, certainly because of protozoal predation on zoospores, or possibly due to the degradation of fungal sporangia by protozoal chitinolytic enzymes (Morgavi et al., 1994). Lee et al. (2000) also observed an inhibition of fungi as well as bacteria when co-cultured with protozoa. Moreover, since rumen is a continuous culture system, the co-culturing experiment carried under *in-vitro* conditions could not give clear picture of microbial interactions that take place inside the rumen.

On the other hand, when supernatant from co-cultured samples were analyzed for FPase activity using filter paper as substrate, FPase activity of co-cultured samples was found to be higher than that of samples from single fraction (Figure 1). FPase activity of isolate FE5 was 14.1, 17.8 and 19.3 IU/ ml after 48, 72 and 96 h, respectively; and in case of bacterial fraction, the activity was 12.3, 72.7 and 25.3 IU/ ml. When fungi and bacterial fraction were co-cultured, the FPase activity of the supernatant was 16.7, 23.3 and 23.7 IU/ ml after 40, 72 and 96 h, respectively. An increase in the rate and extent of cellulose degradation during co-cultures of fungi and methanogens has also been observed by Fonty and Joblin (1991); Orpin and Joblin (1997); Wood et al. (1986); Joblin (1989) and Bernalier et al. (1991). Similarly, FPase activity of protozoal fraction was 23.1, 21.9 and 19.3 IU/ ml after 48, 72 and 96 h, respectively; and it increased to 21.2, 24.3 and 23.7 IU/ ml, when protozoal fraction was co-cultured with isolate FE5. Onodera et al. (1988) and Lee et al. (2000) also reported higher cellulolytic enzyme in co-cultures of rumen protozoa and bacteria than in fungal monoculture. Hence, although fungal counts were lowered during their co-culturing with bacterial and protozoal fractions, their co-culturing was found to increase the FPase activity of the co-cultured fraction. And this could have been one of the reasons for enhanced fibre degradation/ utilization in the rumen when fungi are fed to the animal.

Conclusion

It is possibly the inhibitory action of bacteria and protozoa, and not the inter-species competition or cross-species adjustments which affects the growth of fungi and prevents them from attaining their full fibre-degrading potential in rumen. Furthermore, the enhanced

populations of protozoa and bacteria during co-culturing with fungi, and higher FPase activity could be the probable reason for enhanced fibre degradation in the rumen when fungi are fed to the animal.

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