Reprod. Nutr. Dev. 44 (2004) 313–319

DOI: 10.1051/rnd:2004036

Original article

Effect of anaerobic fungi on in vitro feed digestion by mixed rumen microflora of buffalo

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(Received 4 August 2003; accepted 15 March 2004)

Abstract – Five strains of anaerobic fungi isolated from the faeces of wild (hog deer, *Cervus porcinus*; blackbuck, *Antelope cervicapra*; spotted deer, *Axis axis*; nilgai, *Baselophus tragocamelus*) and rumen liquor of domestic (sheep, *Ovies aries*) ruminants showing high fibrolytic enzyme producing ability were added to mixed rumen microflora of buffalo to study their effect on the digestibility of lignocellulosic feed (wheat straw and wheat bran in the ratio of 80:20), enzyme production and fermentation end products in in vitro conditions. Among the 5 isolates studied, FNG5 (isolated from nilgai) showed the highest stimulating effect on apparent digestibility (35.31 ± 1.61% vs. 28.61 ± 1.55%; $P < 0.05$), true digestibility (43.64 ± 1.73% vs. 35.37 ± 1.65%; $P < 0.01$), neutral detergent fiber digestibility (29.30 ± 2.58% vs. 18.47 ± 2.12; $P < 0.01$) of feed 24 h after inoculation compared to the control group. The production of carboxymethyl cellulase, xylanase, acetyl esterase and β-glucosidase was significantly ($P < 0.05$) higher in the FNG5 inoculated incubation medium. There was no improvement in the digestibility and enzyme production on the addition of the other 4 isolates. Total volatile fatty acid levels as well as the concentration of acetate, propionate, isobutyrate and valerate were significantly higher in the FNG5 added group as compared to the control group. The fungal isolate FNG5 from nilgai, a wild ruminant, was found to be superior to the other isolates tested and appears to have a potential to be used as a feed additive for improving fiber degradation in domestic ruminants.

rumen fungus / digestion / wild animals / enzyme / buffalo / rumen microflora / *Piromyces* sp.

1. INTRODUCTION

Attempts are being made to improve the digestibility of poor quality lignocellulosic feeds by the use of microbial or chemical feed additives. Manipulation of rumen fermentation by increasing the number or activity of lignocellulolytic micro-organisms in the rumen is one such step in this direction [1]. Among the rumen microbes, anaerobic fungi are considered to be important due to the production of highly active enzymes for lignocellulose degradation [2, 3] and have a unique ability to break and penetrate the fibrous feed particles through fungal mycelium, breaking the feed particles and providing more surface area for the action of other microbes [4]. Thus rumen

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fungi play an important catalytic role in the digestion of poor quality fibrous feeds. The administration of goat isolated anaerobic fungus with high fibrolytic activity to the rumen of a sheep resulted in increased digestibility and improved the intake of lignocellulosic feed [5]. Since there is a substantial variation among the fungal isolates from domestic as well as wild animals in their fibrolytic enzyme profile [6] and ability to degrade fibrous feeds, there exists a great scope to isolate efficient strains of fungi which produce higher levels of fibrolytic or lignin solubilizing enzymes so that they can be inoculated in the rumen of domestic animals in order to improve their utilization of fibrous feeds. The present investigation was made to evaluate the effect of the inclusion of different strains of anaerobic fungi to mixed rumen microflora of buffalo on in vitro feed digestion, fermentation characteristics and the fibrolytic enzyme profile.

2. MATERIALS AND METHODS

2.1. Fungal strains and preparation of the inoculum

The anaerobic fungi used in this experiment were isolated from the faeces and rumen liquor of different wild and domestic animals, as per the method of Joblin [7] under strict anaerobic conditions inside an anaerobic cabinet (Compact model; Don Whitely Scientific Co., Mumbai, India). The isolates were identified up to the genus level based on the type of thallus development and number of zoospore flagella [3]. The isolates FNG5 (*Piromyces* sp. isolated from faeces of nilgai), FBB1 (*Anaeromyces* sp. isolated from faeces of blackbuck), FHD1 (*Piromyces* sp. isolated from faeces of hog deer), FS1 (*Orpinomyces* sp. isolated from rumen liquor of sheep) and FSD4 (*Piromyces* sp. isolated from the faeces of spotted deer) used in this study, exhibited the highest carboxymethyl cellulase, microcrystalline cellulase, xylanase, acetyl esterase, p-coumaroyl esterase, feruloyl esterase and protease activity among 32 isolates from different wild (hog deer, *Cervus porcinus*; blackbuck, *Antelope cervicapra*; spotted deer, *Axis axis* and nilgai, *Baselophus tragocamelus*) and domestic (sheep, *Ovies aries*; goat, *Capra hircus*; buffalo, *Bubalus bubalis*) ruminants [8]. The fungal cultures were maintained by weekly transfer of 5% inoculum to fresh liquid medium [9] containing 0.02% cellobiose and 0.5% wheat straw only as a carbon source. The fungal inoculum for in vitro studies was prepared by transferring 5% of 6 day-old stock cultures to fresh medium containing 0.2% cellobiose as the carbon source and incubating it for a further 6 days.

2.2. In vitro digestibility

The fungal isolates were tested for their ability to improve the in vitro digestibility of feed by mixed rumen buffalo microflora [10, 11]. The fermentation medium included 37.5 mL of McDougall buffer [12], 10 mL of strained buffalo rumen liquor, and 2.5 mL of 6-day fungal culture (10^4 colony forming units·mL^{-1}) in a 100 mL conical flask containing 500 mg of feed (a mixture of wheat straw and wheat bran with an 80:20 ratio). The wheat straw and wheat bran had 90% and 92% dry matter (DM) and 85.5% and 47% neutral detergent fiber (NDF), respectively. Control flasks received 2.5 mL of autoclaved culture medium instead of live fungal culture. After bubbling oxygen free carbondioxide for 3–4 min, a cork fitted with a Bunsen gas release valve was tightly fitted on the flasks and these were incubated at 39 °C. All incubations were done in quadruplicates. At 0, 24, 48 h the contents were filtered through preweighed sintered glass crucible (grade 1) in order to estimate residual DM. The filtrate was centrifuged at 27 000 *g* at 4 °C for 15 min and the supernatant was used for enzyme assay and estimation of volatile fatty acids (VFA) (1.0 mL supernatant was preserved with 0.2 mL of 25% ortho-phosphoric acid for VFA estimation). After drying the residue at 80 °C

till a constant weight, the NDF content of the residue was also estimated. The percent true digestibility, apparent digestibility and NDF digestibility were calculated as follows [13].

True digestibility (TD) = (initial DM of feed taken for incubation – NDF residue) ×100/(initial DM of feed taken for incubation).

Apparent digestibility (AD) = (initial DM of feed taken for incubation – DM residue) × 100/(initial DM of feed taken for incubation).

NDF digestibility (NDFD) = (NDF at '0 h' – NDF at '24 or 48 h') × 100/NDF at '0 h'.

2.3. Estimation of enzymes

Carboxymethylcellulase (CMCase) and xylanase activities were estimated as described earlier [14–16]. The reaction mixture used for the estimation of these enzymes contained: 1.0 mL phosphate buffer (pH 6.8, 0.1 M), 0.5 mL substrate (1% carboxymethylcellulose and 0.25% xylan from oat spelt) and 0.5 mL enzyme; incubated at 39 °C for 1 h for CMCase and 30 min for xylanase. Glucose and xylose released during incubation were estimated as per the method described by Miller [17]. For the estimation of β-glucosidase activity, the assay mixture contained 0.1 mL enzyme, 0.9 mL substrate (0.1% p-nitrophenylglucopyranoside dissolved in phosphate buffer 0.1 M, pH 6.8) and 1.0 mL phosphate buffer (0.1 M, pH 6.8). After an incubation of 10 min at 39 °C the reaction was terminated by adding 2 mL of 2% sodium carbonate and the absorbance was measured at 410 nm [18]. The activity of acetyl esterase was estimated in an assay mixture containing 0.1 mL enzyme, 0.9 mL substrate (2 mM p-nitrophenyl acetate dissolved in a phosphate buffer of pH 6.0) and 2.0 mL phosphate buffer pH 6.0 [19]. After incubation of 10 min at 39 °C, the absorbance was recorded at 410 nm. The activities of carboxymethylcellulase and xylanase were expressed as μmole of glucose and xylose, released per min under the assay conditions. The activities of β-glucosidase and acetyl esterase were expressed as μmole of p-nitrophenol released per min under the assay conditions.

One international unit (IU) of the enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar (expressed as glucose or xylose), 1 μmol of p-nitrophenol per min. One mIU of enzyme activity = 0.001 IU.

2.4. Estimation of volatile fatty acids (VFA)

VFA were estimated using a Nucon-5765 gas chromatograph equipped with a flame ionization detector as per the method described by Cottyn and Boucque [20], using a glass column of 4 feet in length and 1.8 mm diameter packed with chromosorb 101, with gas flow rates for nitrogen, hydrogen and air as 30, 30 and 320 mL/min, respectively.

2.5. Statistical analysis

All the data were analyzed by one way ANOVA and the differences between the means were compared by the Duncan multiple range test [21].

3. RESULTS AND DISCUSSION

The results of the effect of fungal isolates on digestibility are presented in Figure 1. Among the 5 isolates tested, FNG5 (from nilgai) showed the highest stimulating effect on the apparent digestibility (35.31 ± 1.61% vs. 28.61 ± 1.55%; $P < 0.05$), true digestibility (43.64 ± 1.73% vs. 35.37 ± 1.65%; $P < 0.01$) and neutral detergent fiber digestibility (29.30 ± 2.58% vs. 18.47 ± 2.12%; $P < 0.01$) of lignocellulosic feed (a mixture of wheat straw and wheat bran in the ratio of 80:20). The FNG5 isolate also showed the highest ($P < 0.05$) level of CMCase (13.88 vs. 9.75 mIU·mL^{-1}), xylanase (30.73 vs. 23.29 mIU·mL^{-1}), acetyl esterase (308.8

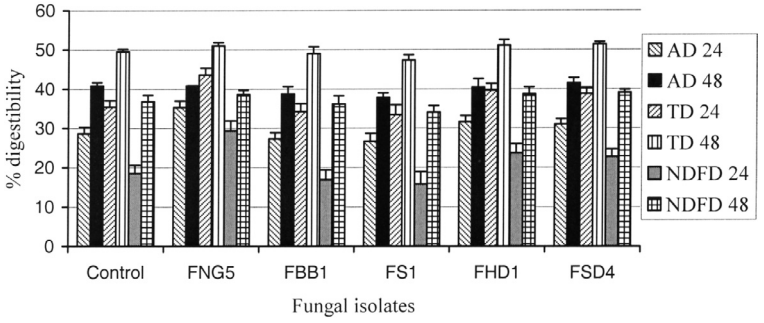

Figure 1. The effect of the addition of fungal isolates on in vitro nutrient digestibility (AD-apparat digestibility, TD-true digestibility, NDFD-NDF digestibility) 24 and 48 h following incubation (mean with SE, $n = 4$). FNG5, *Piromyces* sp. isolated from faeces of nilgai; FBB1, *Anaeromyces* sp. isolated from faeces of blackbuck; FHD1, *Piromyces* sp. isolated from faeces of hog deer; FS1, *Orpinomyces* sp. isolated from rumen liquor of sheep; FSD4, *Piromyces* sp. isolated from faeces of spotted deer.

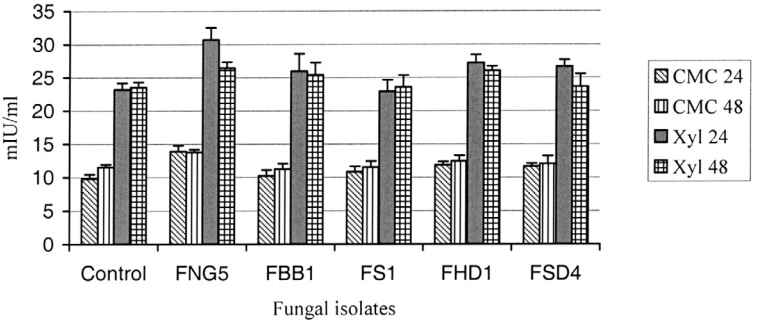

Figure 2. The effect of the addition of fungal isolates on in vitro enzyme activities (CMC, CMCase; Xyl, xylanase) 24 and 48 h following incubation (mean with SE, $n = 4$). FNG5, *Piromyces* sp. isolated from faeces of nilgai; FBB1, *Anaeromyces* sp. isolated from faeces of blackbuck; FHD1, *Piromyces* sp. isolated from faeces of hog deer; FS1, *Orpinomyces* sp. isolated from rumen liquor of sheep; FSD4, *Piromyces* sp. isolated from faeces of spotted deer.

vs. 234.6 mIU·mL^{-1}) and β-glucosidase (390.3 vs. 316.3 mIU·mL^{-1}) as compared to the control group (Figs. 2 and 3). Other four isolates had no effect on the enzyme level of the incubation fluid. The observed differences in digestibilities were also reflected in the levels of volatile fatty acids in the fermented medium (Tab. I). Total VFA was higher in the FNG5 inoculated group (6.35 vs. 5.68 mmol·100mL^{-1}) in comparison to that of the control. Similarly, there were significantly higher levels of acetate (4.39 vs. 3.86 mmol·100mL^{-1}), propionate (1.14 vs. 1.00 mmol·100mL^{-1}), isobutyrate (0.106 vs. 0.09 mmol·100mL^{-1}) and valerate (0.11 vs. 0.07 mmol·100mL^{-1}) in the FNG5 added group as compared to control group. The total VFA level was higher (6.02 vs. 5.68 mmol·100mL^{-1}) in the FHD1 inoculated group as compared to the control group. There was no effect of inoculation of the FBB1, FS1 or FSD4 on the total VFA level.

The enzymes tested in the present experiment are the major enzymes responsible for the degradation of lignocellulosic feed. Therefore the increased activity of fibrolytic enzymes resulted in a better in vitro

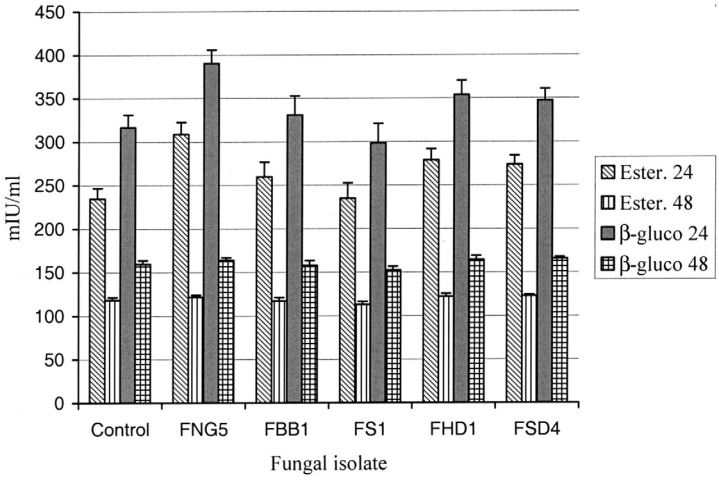

Figure 3. The effect of the addition of fungal isolates on in vitro enzyme activities (ester, acetyl esterase; β-gluco, β-glucosidase) 24 and 48 h following incubation (mean with SE, $n = 4$). FNG5, *Piromyces* sp. isolated from faeces of nilgai; FBB1, *Anaeromyces* sp. isolated from faeces of blackbuck; FHD1, *Piromyces* sp. isolated from faeces of hog deer; FS1, *Orpinomyces* sp. isolated from rumen liquor of sheep; FSD4, *Piromyces* sp. isolated from faeces of spotted deer.

feed degradability. The effect of the addition of *Piromyces communis* strain 22 to mixed rumen organisms on the in vitro degradation of cellulose has been evaluated in Korea [22]. They observed that on the addition of the fungal culture at the rate of 5% of rumen liquor inoculum and on incubation in semi-defined medium, there was a 26% increase in filter paper digestion and a considerable increase in enzyme activity after fermentation for 30 h. The higher concentration of VFA is an indication of a better fermentation which might be due to an improved degradation of the lignocellulosic feed. Similarly, a significantly higher digestibility of feed has been observed in wheat straw pre-treated with white rot basidiomycetes [23].

The results of the present experiment indicate that upon the addition of a fungal isolate FNG5, there was a significant effect on the digestibility of feed, level of fibrolytic enzymes and VFA production at 24 h of incubation, but at 48 h the differences among the treatments were not significant ($P > 0.05$). Although almost similar levels of major fiber degrading enzymes (CMCase and xylanase) were maintained at 24 and 48 h of incubation, there were only slightly higher levels of volatile fatty acids and digestibility parameters at 48 h. This might be due to the accumulation of certain metabolites which ceased the microbial activity as is apparent by slow feed degradability between 24 h and 48 h as compared to that between 0 h and 24 h in the control group. Similarly in another study at the authors' laboratory [24], there was a significant improvement in in vitro dry matter digestibility (IVDMD) of wheat straw by adding a probiotic, *Saccharomyces cerevisiae* at 24 h incubation, whereas, there was no effect at 48 h. It has also been reported earlier [25] that any change in lag phase and early fermentation patterns in in vitro condition are usually not evident at or beyond 48 h, which is in agreement with our results.

The findings of this experiment indicate that a fungal inoculum in the rumen liquor of buffalo accelerates the rate of fermentation, digestibility of lignocellulosic feeds and the synthesis of enzymes involved in

Table I. The effect of the addition of fungal isolates to buffalo rumen liquor inoculum on the levels of volatile fatty acids (VFA) (mmol·100 mL^{-1}) 24 and 48 h following incubation, mean ± SE ($n = 4$).

Items	Control	FNG5	FBB1	FS1	FHD1	FSD4	SEM	Sig.	*P*-value
				24 h post incubation					
Total VFA	5.68 ± 0.11^{c}	$6.35 \pm 0.10a$	5.25 ± 0.12^{c}	5.74 ± 0.24^{bc}	6.02 ± 0.26^{ab}	5.42 ± 0.17^{c}	0.11	**	0.002
Acetate	3.86 ± 0.07^{b}	4.39 ± 0.12^{a}	3.54 ± 0.14^{b}	3.99 ± 0.18^{ab}	3.90 ± 0.24^{b}	3.74 ± 0.11^{b}	0.08	**	0.006
Propionate	1.00 ± 0.04^{bc}	1.14 ± 0.01^{a}	0.85 ± 0.03^{d}	0.95 ± 0.04^{cd}	1.10 ± 0.07^{ab}	0.97 ± 0.05^{bc}	0.03	**	0.003
Butyrate	0.49 ± 0.02^{cd}	0.48 ± 0.03^{d}	0.61 ± 0.05^{b}	0.59 ± 0.03^{bc}	0.75 ± 0.03^{a}	0.46 ± 0.02^{d}	0.02	**	0.000
Isobutyrate	0.08 ± 0.00^{bc}	0.13 ± 0.01^{a}	0.06 ± 0.00^{d}	0.07 ± 0.00^{bc}	0.08 ± 0.00^{bc}	0.08 ± 0.01^{bc}	0.01	**	0.000
Isovalerate	0.09 ± 0.09	0.11 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.03	0.09 ± 0.02	0.01	ns	0.775
Valerate	0.07 ± 0.06^{ab}	0.11 ± 0.08^{a}	0.09 ± 0.08^{ab}	0.07 ± 0.01^{ab}	0.11 ± 0.02^{a}	0.06 ± 0.02^{b}	0.09	*	0.036
				48 h post incubation					
Total VFA	8.24 ± 0.62	7.73 ± 0.43	7.79 ± 0.42	7.49 ± 0.74	8.36 ± 0.51	7.80 ± 1.52	0.80	ns	0.931
Acetate	5.81 ± 0.44	5.3 ± 0.24	5.20 ± 0.28	5.04 ± 0.54	5.23 ± 0.47	5.27 ± 0.91	0.53	ns	0.919
Propionate	1.34 ± 0.18	1.36 ± 0.07	1.42 ± 0.03	1.23 ± 0.14	1.59 ± 0.07	1.39 ± 0.38	0.05	ns	0.62
Butyrate	0.64 ± 0.02	0.75 ± 0.07	0.82 ± 0.15	0.81 ± 0.04	1.00 ± 0.13	0.75 ± 0.24	0.05	ns	0.735
Isobutyrate	0.12 ± 0.00	0.10 ± 0.01	0.12 ± 0.01	0.14 ± 0.03	0.19 ± 0.04	0.14 ± 0.00	0.01	ns	0.432
Isovalerate	0.19 ± 0.02	0.12 ± 0.03	0.14 ± 0.02	0.17 ± 0.03	0.19 ± 0.02	0.17 ± 0.01	0.01	ns	0.456
Valerate	0.14 ± 0.00	0.09 ± 0.03	0.10 ± 0.02	0.10 ± 0.03	0.15 ± 0.01	0.09 ± 0.00	0.01	ns	0.458

[a,b,c,d,e] The values bearing different letters in a row differ significantly ($P < 0.05$).
FNG5, *Piromyces* sp. isolated from faeces of nilgai; FBB1, *Anaeromyces* sp. isolated from faeces of blackbuck; FHD1, *Piromyces* sp. isolated from faeces of hog deer; FS1, *Orpinomyces* sp. isolated from rumen liquor of sheep; FSD4, *Piromyces* sp. isolated from faeces of spotted deer.

fibre degradation and confirm the previous reports on the role of rumen anaerobic fungi in fibre degradation [2–4]. The fungal strain FNG5 identified as *Piromyces* sp. isolated from nilgai seems to have the potential to be used as a microbial feed additive to improve the utilization of lignocellulosic feed by domestic ruminants.

ACKNOWLEDGEMENTS

The authors are thankful to the Department of Biotechnology, Ministry of Science and Technology, Government of India for providing financial assistance for this project and Indian Veterinary Research Institute, Izatnagar, India for providing laboratory facilities for scientific work leading to this publication.

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