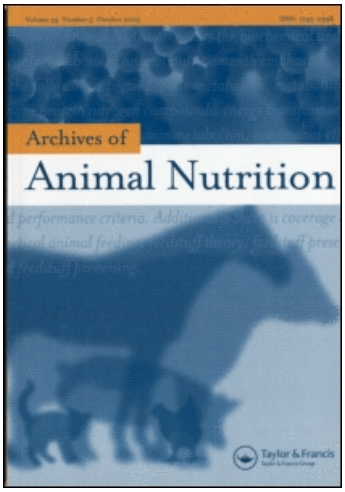

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In this investigation, the effects of feeding encapsulated cells (rhizomycelia and zoospores) of a fibrolytic isolate from an anaerobic fungus (*Neocallimastix* sp. CF 17) on nutrient digestion, ruminal fermentation, microbial populations, enzyme profile and growth performance were evaluated in buffaloes. In three *in vitro* studies, the true digestibility of wheat straw was increased after addition of CF 17 to buffalo rumen fluid ($p < 0.05$). In Exp. 1, three groups of six buffaloes each (initial BW [body weight] 148 $\pm$ 12.0 kg) were allotted to three dosing regimes: Group 1 received 200 ml of liquid culture of *Neocallimastix* sp. CF 17 (about 10^6 TFU [thallus-forming units]/ml); Group 2 received an encapsulated culture of the same fungi prepared from 200 ml liquid culture; Group 3: received 200 ml of autoclaved culture (Control). The supplementations were given weekly for four weeks (on days 1, 7, 14 and 21). During the dosing period, the average daily gain of Group 2 was higher than in the Control group (444 g/d compared with 264 g/d; $p < 0.05$). Furthermore, the digestibility of organic matter increased in Group 1 and 2 compared with the Control (64.8, 64.0 and 60.4% respectively; $p < 0.05$), resulting in an increase in the total digestible nutrient (TDN) percent of ration ($p < 0.05$). But these effects disappeared post-dosing. There were also an increase in concentration of volatile fatty acids, trichloroacetic acid precipitable N and number of fibrolytic microbes in the rumen during the dosing period ($p < 0.05$), but these effects declined post-dosing. Results of Exp 2., where the encapsulated culture was applied at intervals of 4 d or 8 d for 120 d, showed that a shorter dosing frequency did not improve growth performance or feed intake. However, independent of the dosing frequency the growth rate of both groups fed the encapsulated culture were about 20% higher than in the Control group ($p < 0.05$). The present study showed that encapsulated fungi have a high potential to be used as feed additive at the farmers' level and that weekly dosing can increase growth performance of wheat straw based diets.

Keywords: anaerobes; buffaloes; digestibility; feed additives; fibre; fungi; ruminants

1. Introduction

Recently emphasis has been given to enhancing the digestibility of poor quality lignocellulosic feed through the manipulation of rumen fermentation by increasing number or activity of ruminal lignocellulolytic organisms (Gordon et al. 1995).

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Among the rumen microbes, anaerobic fungi have the most potent plant cell wall degrading enzymes and have an unique ability to break and penetrate the fibrous feed particles through rhizoids, which help the access of other rumen microbes to the secondary cell wall of feed particles (Grenet et al. 1989). The administration of an anaerobic fungus, isolated from goats, to sheep resulted in an increased digestibility and intake of lignocellulosic feed (Lee et al. 2000). Similarly, other studies have also indicated a positive effect of liquid cultures of anaerobic fungi (Dey et al. 2004; Paul et al. 2004; Tripathi et al. 2007). There is substantial variation among fungal isolates from domestic as well as wild animals in their ability to degrade fibrous feeds, their lignocellulose degrading enzyme profiles and the ability to act in a different ecosystem. Hence, there is a need to isolate efficient strains of fungi, which produce higher levels of fibrolytic enzymes and can exert positive effects in co-culture with mixed rumen microbes of target hosts (domestic ruminants). Recently, it was shown that the addition of a specific fibrolytic fungi (isolated from the rumen of wild cattle) to the rumen fluid of buffalos resulted in a significant increase in fibre digestion, without increasing the methane emission per gram of truly digested wheat straw (Paul et al. 2010). However, being strict anaerobes, the culture, storage and feeding of anaerobic fungi is difficult. Even a brief period of exposure to air can kill these fungi. Hence, there is a need to develop and validate a suitable procedure for encapsulation or entrapment, which protect these fungi from brief periods of exposure to environmental stressors (e.g. oxygen, drying, osmotic stress, and mechanical injury) during storage or feeding so that a large scale use as feed additive can be possible. In an earlier study, we optimised a procedure for the encapsulation of anaerobic fungi to protect them from environmental stressors for a period sufficient for their use as a feed additive. The objectives of the present study was to evaluate the effect of feeding an encapsulated culture of a specific fibrolytic anaerobic fungus as a feed additive on growth, rumen fermentation characteristics and fibre digestion in growing buffaloes.

2. Materials and methods

2.1. Isolation, characterisation and preparation of inoculum

The anaerobic fungus used in this experiment was isolated from the faeces of wild cattle as described by Joblin (1981) under strict anaerobic condition using Hungate-type roll tubes (Bellco Glass Inc., Vineland, USA). The fungal isolate used in the present study exhibited the highest fibrolytic capacity (measured as the carboxymethyl cellulase [CMCase] and xylanase activity to degrade straw in pure culture) among the 165 anaerobic fungal isolates from various domestic (cattle, buffalo, sheep, goat and camel) and wild ruminants (wild cattle and blue bull). The selected isolate was assigned to the given genus (*Neocallimastix* sp.) of anaerobic fungi on the basis of their production of polyflagellated zoospores with a monocentric thallus and filamentous branching rhizoid (Mountfort and Orpin 1994). The fungal isolate was also characterised by molecular approaches, i.e. by polymerase chain reaction (PCR) amplification and sequencing of the ribosomal intergenic transcribed spacer (ITS) fragments and the alignment of the sequence with published sequence data. Genomic DNA was isolated (as per Pitcher et al. 1989) from fungal biomass harvested by centrifugation (7000 *g* for 5 min) from 3-day-old culture grown on a liquid broth. The ribosomal ITS region, defined by universal fungal primers (forward: 5́-TACACACCGCCCGTCGCTA-3́; reverse: 5́-TCCTCCGCTTATTGATATGC-3́),

was amplified from genomic DNA by PCR. The PCR was solution had a volume of 50 μl, comprising 25 μl x2 master mix (containing 3 mM Mg, 400 μM dNTP and 0.1 units/μl Taq polymerase; Quiagen, USA), 0.4 μl of 25 pmol of each primer, 2 μl of template DNA and 22 μl of molecular grade water. The amplification process was performed on the thermal cycler (model : PTC 200 G; Biorad, USA) with an initial denaturation of 94°C for 3 min followed by 30 cycles of 94°C for 40 s, 54°C for 1 min and 72°C for 1 min. The final extension was set at 72°C for 10 min. The amplified product, along with a 100 bp DNA ladder, was run in a 1% agarose gel electrophoresis and the amplified DNA was extracted from the gel using a Quiagen MinElute Gel Extraction kit. It was then sent for sequencing to a DNA sequencing service provider (Chromous Biotech, Bangalore, India). The obtained sequence was analysed by the basic local alignment and search tool (BLAST) against the GenBank® nucleotide database. The sequence showed a high homology with some of the published sequences of *Neocallimastix* genus.

The fungal isolate was maintained by a weekly transfer of 2% inoculum to fresh liquid medium containing 0.02% cellobiose, 0.25% wheat straw and antibiotics (filter sterilised [pore size 0.2 μm] ampicillin, chloramphenicol and streptomycin each at a 30 μg/ml final concentration in medium), and incubation at 39°C without shaking. The fungal culture was also preserved in liquid nitrogen after adding a suitable cryoprotectant.

The fungal culture inoculum for all studies was prepared by transferring 1% of 2-day-old stock cultures to an autoclaved anaerobic fungal medium. The cultures used as inocula were grown for 2 d at 39°C without shaking. The cultures used as inocula for the *in vitro* studies with rumen fluid were grown without antibiotics. In order to assess the number of fungal thallus-forming units (TFU) in an inoculum, 1 ml samples were serially diluted 10-fold in an anaerobic dilution solution (Bryant and Burkey 1953) contained in Hungate-type roll tubes. Small samples (0.2 ml) of 10^{-1} and 10^{-2} dilution were separately inoculated in to triplicate Hungate-tubes containing 2.8 ml of medium and 2% (w/v) agar kept molten at 50°C. The tubes were rolled on ice immediately after inoculation and were incubated for 5 d before the fungal colonies were counted through a microscope at a magnification of x40.

2.2. *Production of encapsulated fungi*

The encapsulation procedure was developed in an earlier research programme in the authors' laboratory and is under consideration for commercialisation (hence, details are not to be published). Briefly, for encapsulation, the basic method described by Johnsen and Flink (1986) was used after extensive modification to make it suitable for strict anaerobes. It was observed that the encapsulation as described by Johnsen and Flink (1986) cannot be directly used for strict anaerobes as it is highly permeable to oxygen and exposure to air for more than 2 h kills the fungus. Hence, the method of encapsulation was modified to make it impermeable to oxygen. This was achieved by the use of a suitable oxygen impermeable biopolymer and reducing agents. The encapsulation rendered protection from air exposure for up to 12 h and were easily solubilised in the rumen. The encapsulated fungi were partially dried and stored in an air-impermeable container in a carbon dioxide environment before feeding. *In vitro* studies indicated the production of a good level of enzymes when the stored encapsulated fungi were added to anaerobic medium and incubated anaerobically. The enzyme-producing ability of encapsulated fungi was not affected significantly up

to six months of storage. The shelf-life of the encapsulated fungal beads was found to be more than six months.

2.3. *Experimental design of the* in vitro *digestion trial*

Samples (500 mg) of wheat straw, ground to pass a 1 mm screen, were weighed into 100 ml Erlenmeyer flasks and incubated in quadruplicate in buffered rumen fluid with or without fungal inoculum for 24 h, largely as per the first of the two stages of *in vitro* rumen-liquor digestion procedure described by Tilley and Terry (1963). This was followed by treatment with a neutral detergent solution as described by Goering and van Soest (1970) (instead of the second stage of pepsin digestion of the Tilley and Terry [1963] procedure). Four growing female Nili-Ravi buffaloes (130 $\pm$ 5.2 kg BW [body weight]; age, 10 $\pm$ 0.2 months) were used as donors of rumen fluid. Buffaloes were fed on a total mixed ration comprising wheat straw, green jower (*Sorghum vulgare*) fodder and a concentrate mixture at a ratio of 75:5:25 (DM [dry matter] basis), respectively. The concentrate contained: maize grain, 300 g kg^{-1}; wheat bran, 370 g kg^{-1}; groundnut cake, 300 g kg^{-1}; mineral mixture, 20 g kg^{-1}; NaCl, 10 g kg^{-1}. Rumen fluid was collected via a stomach tube, 2 h after the morning feed (i.e. at 12:00 h). Samples from the four buffaloes were mixed in equal proportion, strained through two layers of cheesecloth and maintained at 39°C under O_2-free CO_2. At the end of the incubation, the flask contents were filtered through preweighed sintered glass crucibles (grade 1) for estimation of residual dry matter. The filtrate was cooled on ice, centrifuged at 27000 *g* for 15 min at 4°C and the supernatant was used to determine enzyme activities (CMCase and xylanase). After drying of residues at 60°C, NDF (neutral detergent fibre) content of residue was also estimated. The descriptions of *in vitro* true or apparent digestibilities were essentially according to van Soest and Robertson (1985) and were calculated as follows:

$$\text{True digestibility [\%]} = (\text{Initial DM of substrate [mg]} - \text{NDF residue [mg]}) \cdot 100/(\text{Initial DM of substrate [mg]})$$

$$\text{Apparent digestibility [\%]} = (\text{Initial DM of substrate [mg]} - \text{DM residue [mg]}) \cdot 100/(\text{Initial DM of substrate [mg]})$$

The *in vitro* experiment was repeated on two additional days to check the repeatability of the results. The activities of CMCase and xylanase were estimated as described earlier (Agarwal et al. 2000). The activity of CMCase and xylanase was expressed as micromoles of glucose and xylose respectively, released per min under assay conditions. One international unit (IU) of enzyme activity was defined as the amount of enzyme that released 1 μmol of the respective sugar per min. The protein content of enzyme sample was estimated after Lowry (1951).

2.4. *Experimental design of the* in vivo *digestion trial in growing buffaloes*

2.4.1. *Animals and feeding*

The design of the experiment was a completely random one, where three treatments (weekly dosing of liquid culture, encapsulated culture and autoclaved culture) each with six replicates were tested (initial BW of buffaloes: 148 $\pm$ 12.0 kg).

On the basis of the preliminary *in vitro* study and on the reported literature, a weekly administration of 200 ml culture (10^6 TFU/ml) was taken as expected minimum effective dose. Group 1, received 200 ml of fresh, liquid culture of *Neocallimastix* sp. CF 17 containing about 10^6 TFU/ml; Group 2, received encapsulated culture of the same fungi prepared from 200 ml liquid culture; and Group 3, received 200 ml of autoclaved culture (Control group). The supplementations were given weekly for four weeks (on days 1, 7, 14 and 21). For Group 1 and Group 3, the culture was administered by drenching. Group 2 received encapsulated fungi by mixing with concentrate feed, which was eaten within 20 min after offering. The buffaloes were fed individually on a ration comprising 1 kg concentrates, 1 kg green fodder and *ad libitum* wheat straw. The concentrate mixture contained per kg: 300 g of wheat grain, 370 g of wheat bran, 300 g of deoiled groundnut cake, 20 g of mineral mixture, and 10 g of NaCl. Per kg of DM the concentrate mixture contained 930 g of OM (organic matter), 189 g of CP (crude protein), 63 g of EE (ether extract), 272 g of NDF, and 137 g of ADF (acid detergent fibre). The wheat straw contained per kg DM: 910 g of OM, 27 g of CP, 11 g of EE, 850 g of NDF and 650 g of ADF. The green fodder (jower) contained per kg DM: 897 g of OM, 70 g o f CP, 20 g of EE, 700 g of NDF and 350 g of ADF. The feeding trial continued for 93 days, the feed intake of individual was recorded daily and the BW of animals were recorded at fortnightly intervals. Two digestibility trials of 6 d duration were conducted from days 11 to 17 (dosing period) and days 34 to 41 (post-dosing period). The offered feeds and residues of the previous day were recorded, samples of both were collected daily and pooled throughout the experimental periods for analysis. The amounts of faeces voided daily were weighed, thoroughly mixed in a pail and an aliquot (1 g per kg fresh faeces) was mixed with 15 ml of 20% H_2SO_4 and kept for N estimation. Another portion of the aliquot (3 g per kg fresh faeces) was kept for drying at 100°C in hot air oven for the estimation of dry matter and other proximate composition.

The chemical composition of representative samples of offered feed and residue and faeces were analysed to determine nutrient digestibility.

2.4.2. Sampling of rumen fluid

Rumen fluid was sampled daily via a stomach tube 2 h after the morning feed from day 18 to 20 (within dosing period) and day 42 to 44 (in post-dosing period) of the feeding trial to evaluate the effect of feeding fungal cultures on rumen fermentation patterns, enzyme activities and microbial population. Rumen fluid was strained through two layers of cheesecloth.

2.4.3. Biochemical analyses

The rumen fluid samples were analysed for trichloroacetic acid (TCA) precipitable N. Rumen fluid samples (5 ml) were mixed with 5 ml of 20% (w/v) TCA, allowed to stand overnight, centrifuged at 1000 *g* for 15 min and then the precipitate was analysed for N by AOAC (1990) procedure (ID No.988.05). To 1 ml of rumen fluid, 0.2 ml of metaphosphoric acid (25% w/v) was added and allowed to stand for 30 min at room temperature, centrifuged at 5900 *g* for 15 min and clear supernatant (1 μl) was used for analysis.

Volatile fatty acids (VFA) were estimated by a gas liquid chromatograph (Model 5765; Nucon Engineers, New Delhi, India) equipped with a double flame ionisation

detector as described by Cottyn and Boucque (1968), using a glass column of 1200 mm length and 1.8 mm diameter packed with chromosorb 101 with gas flow rates for nitrogen, hydrogen and air of 30 ml/min, 30 ml/min and 320 ml/min respectively. Peaks were identified by comparison with VFA standards of known composition.

2.4.4. Enumeration of microbes and estimation of enzyme activities in rumen fluid

Cellulolytic bacteria, hemicellulolytic bacteria, total bacteria and fungi were enumerated in rumen fluid by the most probable number (MPN) procedures described by Dehority et al. (1989).

For estimation of the enzyme activity, 5 ml of 0.4% lysozyme solution and 5 ml carbon tetrachloride were added to 30 ml of rumen fluid. The suspension was incubated for 3 h at 40°C and the reaction was terminated by keeping it in an ice bath. The suspension was vortexed and centrifuged at 27000 *g* for 30 min at 4°C to get the clear supernatant, which was used for assay of enzyme activities. Procedures for estimation of CMCase and xylanase were same as described earlier.

2.4.5. Chemical analysis of feed and faeces samples

Feed and faeces samples were analysed for dry matter (method 930.15), ash (method 942.05), crude protein (method 988.05), and fat (method 920.39) by procedures of AOAC (1990) and fibre fractions (NDF and ADF) according to van Soest et al. (1991). NDF was analysed with neutral detergent solution without dekalin, sodium sulphite and α-amylase and expressed with residual ash. The ADF was also expressed with residual ash.

2.5. Experimental design to measure the effect of dosing frequency in vivo

The design of the experiment was a completely random one, where three treatments (dosing of encapsulated culture every 4 or 8 days respectively, and dosing of autoclaved-encapsulated culture every 8 days) were tested each with six replicates (initial BW of buffaloes: 176 kg; SD ± 15.2 kg). Group 1 received encapsulated fungi (prepared from 200 ml of fresh culture of *Neocallimastix* sp. CF 17 containing about 10^6 TFU/ml) every 4 days; Group 2 received the same preparation as Group 1 every 8 days; Group 3 received 200 ml of autoclaved encapsulated culture every 8 day (Control group). The encapsulated fungi were fed by mixing with concentrate feed. The dosing period and feeding trial lasted for 120 days. The buffaloes were fed individually on a ration comprising 1.5 kg concentrate mix, 1 kg green jower fodder and *ad libitum* wheat straw. The composition of the concentrate mixture was same as used in the first feeding experiment. Daily intake of individual feed was recorded. The BW of animals was recorded at fortnight intervals.

2.6. Statistical analysis

To evaluate the effects of different treatments, the means of all parameters regarding the *in vivo* studies were subjected to one-way ANOVA. On detection of an overall

significant difference in ANOVA, multiple comparisons among means were carried out by Duncan's new multiple range test. Data on digestibility and enzyme profile in the *in vitro* studies were compared by Student's *t*-test. Repeatability was calculated from the ratio of the among group variance component to the among and within group variance component (Winer 1971) from one-way ANOVA of the combined data of three *in vitro* trials. Differences were considered to be significant at $p < 0.05$, whereas a trend was considered to exist if $0.05 < p \leq 0.10$.

Statistical analyses were performed using SPSS (2001).

3. Results

3.1. Characterisation of the fungal isolate

The selected isolate was assigned to the genus *Neocallimastix* sp. of anaerobic fungi on the basis of its morphological features. The morphology of colonies growing on the surface of cellobiose agar media has a central core of sporangia surrounded by radially growing rhizoids and colonies are large in size (ca. 12 mm diameter at 72 h of growth). The vegetative stage has a single sporangium borne on a single, branching rhizoidal system. Sporangia develop by enlargement of zoospore cyst. The shape of the sporangium is broadly subglobular. There is considerable variation in size of sporangia, the smallest being about 55 μm and the largest about 100 μm in diameter. Zoospores of the isolate are polyflagellate.

BLAST result of ITS sequence data indicated that the fungal isolate had high sequence homology to some of the members of *Neocallimastix* genus. Sequence data of the fungal isolate was deposited in GenBank® library (accession number GU055516).

A phylogram indicating phylogenic position of the fungal isolate is presented in Figure 1. The fungal isolate utilised in this study had phylogeny similar to that of *Neocallimastix* genus, but was slightly distantly related to other genera of anaerobic fungi.

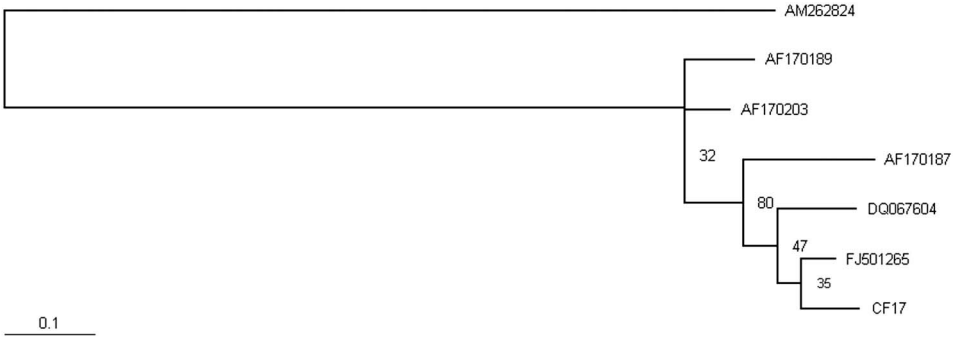

Figure 1. Phylogram showing phylogenic relationship of strain CF17 (GU055516) and members from some other genera of anaerobic fungi (AF 170189, *Orpinomyces* sp.; AF170203, *Piromyces* sp.; AF 170187, *Anaeromyces* sp.; DQ067604, *Sphaeromonas* sp.; and FJ501265, *Neocallimastix* sp.). *Saccharomyces cerevesiae* (AM262824) was used as outgroup reference. Phylogenic tree inferred from maximum-likelihood analysis of ITS sequences. Bootstrap values shown at bifurcating nodes were calculated by using 500 resampling and indicate statistical significance (percentage of outcome). The scale bar represents 10% estimated sequence divergence inferred from maximum-likelihood analysis.

The selected isolate showed higher fibrolytic ability among the 165 isolates of anaerobic fungi tested from different domestic animals (cattle, buffalo, sheep, goat, camel and horse) and wild ruminants (wild blue bull and cattle) from diverse locations of India. In a pure culture study, where the fungal isolates grew in anaerobic medium containing 0.5% wheat straw as sole carbon source for 5 d, the true digestibility of wheat straw amounted to 72.2% and a high concentration of extracellular fibrolytic enzymes was measured (CMCase, 54 mIU/ml and xylanase, 26.4 mIU/ml). The protein concentration of enzyme samples ranged from 105 to 162 μg/ml of enzyme sample.

3.2. *Effect of the fungal isolate on the* in vitro *digestion of wheat straw (*in vitro *trials)*

In all three *in vitro* trials the addition of CF 17 to the rumen fluid of buffaloes increased the true digestibility of wheat straw by 39.4, 22.3 and 75% respectively ($p < 0.05$) (Table 1). This indicates a clear positive effect and compatibility of the isolate with buffalo rumen microbes. Repeatability of apparent digestibility, true digestibility, CMCase and xylanase activities in *in vitro* experiments were 0.719, 0.804, 0.266 and 0.208 respectively. CMCase and xylanase activities were numerically higher in the CF 17-added group although the differences attained statistical significance in only one of three trials.

3.3. *Effect of dosing encapsulated vs. liquid culture in growing buffaloes (Exp. 1)*

From days 0 to 28 of Exp. 1 (which largely correspond to the dosing period), the average daily gain (ADG) was higher ($p < 0.05$) in the group fed encapsulated culture as compared with the Control group (Table 2). However, the ADG of the group fed the liquid culture was comparable with that of the Control group. In this first period, the intake of wheat straw was numerically higher in the supplemented groups. There were sharp rise in wheat straw intake in both supplemented groups

Table 1. Effect of addition of *Neocallimastix* sp. CF 17 to buffalo rumen-liquor inoculum on *in vitro* fermentation of wheat straw after 24 h incubation.

	Control	CF 17 addition	SEM	Significance (p-value)
First *in vitro* trial				
Carboxymethyl cellulose [mIU/ml]	9.94	11.50	0.525	0.263
Xylanase [mIU/ml]	3.88	6.12	0.479	0.095
Apparent digestibility [%]	21.7	33.3	1.42	0.038
True digestibility [%]	29.7	41.4	1.80	0.032
Second *in vitro* trial				
Carboxymethyl cellulose [mIU/ml]	7.56	14.28	0.280	0.001
Xylanase [mIU/ml]	4.52	6.28	0.525	0.231
Apparent digestibility [%]	19.8	27.2	1.38	0.092
True digestibility [%]	32.4	39.6	0.90	0.025
Third *in vitro* trial				
Carboxymethyl cellulose [mIU/ml]	16.3	22.1	1.57	0.163
Xylanase [mIU/ml]	9.0	14.9	0.93	0.037
Apparent digestibility [%]	18.0	25.2	1.04	0.005
True digestibility [%]	27.6	37.8	1.05	0.005

Table 2. Effect of weekly dosing (days 1, 7, 14 and 21) of *Neocallimastix* sp. CF 17 on growth rate and feed intake of buffaloes (Exp. 1).*

Parameter	Control	Liquid culture	Encapsulated	SEM	Significance (p-value)
Initial body weight [kg]	148.4	148.2	148.3	2.92	0.999
Body weight gain [g/d]					
Day 0 to 28	264[a]	333[ab]	444[b]	42.0	0.026
Day 29 to 93	269	303	309	33.9	0.566
Day 0 to 93	266	313	353	26.0	0.093
Wheat straw intake [kg DM/d]					
Day 0 to 28	1.72	1.94	1.97	0.117	0.307
Day 29 to 93	2.29	2.45	2.36	0.071	0.313
Day 0 to 93	2.09	2.27	2.22	0.085	0.303

Notes: *Constant concentrates intake of 0.90 kg DM/d for all periods, green fodder intake 0.18, 0.20 and 0.19 kg DM/d for periods days 0 to 28, days 29 to 93 and period days 0 to 93 respectively; Means not sharing the same superscript differ significantly ($p < 0.05$).

around the days of dosing and wheat straw intake in these groups were slightly higher during the dosing period, but the difference were reduced after dosing was withdrawn.

The effect of experimental treatment on *in vivo* whole-tract digestibility and the nutritive value of the fibre-rich diet is presented in Table 3. Compared with the Control group, the digestibility of DM in Exp. 1 was significantly higher in the group fed the liquid culture, whereas it tended to be higher in the group fed encapsulated culture. Furthermore, there was a significant increase in OM digestibility in both supplemented groups in Exp. 1 (dosing period) resulting in a significant increased in TDN percent of the ration, but the effect declined post-dosing (Exp. 2). During the dosing period (Exp. 1), the digestibility of NDF also increased in the group fed the encapsulated culture compared with the Control ($p < 0.05$), but declined to the level comparable with that of the Control in the post-dosing period (Exp. 2). The digestibility of CP and EE was not changed after addition of fungal cultures.

The effects of experimental treatment on rumen liquor parameters are presented in Table 4. The concentration of total VFA and TCA-precipitable nitrogen increased significantly after feeding the liquid culture or the encapsulated culture. In the dosing period this effect was always significant. Within the dosing period, the population density of fungi in the group received liquid culture were by 2.5-fold higher and maintained an increased level in the post-dosing period, although a decline was evident. However, the population density of fungi in the two other groups (Control and encapsulated culture) did not increase in both experimental periods. In the supplemented groups, the population density of cellulolytic bacteria increased 3-fold during the dosing period and maintained the increased level even in the post-dosing period, although a substantial decline was evident. A similar trend was observed for the total bacteria and hemicellulolytic bacteria. The levels of CMCase and xylanase were significantly higher in both supplemented groups during the dosing period. Increased levels of CMCase persisted in the group fed with the liquid culture in the post-dosing period, but not in the group fed the encapsulated culture. The increase in level of xylanase observed during the dosing period did not persist in the two treatment groups in the post-dosing period.

Table 3. Effect of weekly dosing (days 1, 7, 14 and 21) of *Neocallimastix* sp. CF 17 on digestibility and nutritive value of the diet (Exp. 1).

	Control	Liquid culture	Encapsulated	SEM	Significance (p-value)
Exp. 1*					
DM digestibility [%]	56.1[b]	60.5[a]	59.5[ab]	1.19	0.049
OM digestibility [%]	60.4[b]	64.8[a]	64.0[a]	1.05	0.024
CP digestibility [%]	77.8	77.9	73.3	3.14	0.519
EE digestibility [%]	52.4	51.3	59.8	3.00	0.130
NDF digestibility [%]	49.7[b]	53.5[ab]	54.7[a]	1.30	0.041
ADF digestibility [%]	45.7	47.1	49.2	1.78	0.402
Nutritive value of ration					
TDN [%]	55.8[b]	59.8[a]	59.1[a]	1.00	0.033
CP [%]	9.39	9.94	9.50	0.63	0.812
Exp. 2[#]					
DM digestibility [%]	48.2	53.4	48.2	1.88	0.113
OM digestibility [%]	52.8	58.0	52.0	1.77	0.063
CP digestibility [%]	49.3	55.3	52.9	1.61	0.056
EE digestibility [%]	76.8	81.5	78.3	2.51	0.415
NDF digestibility [%]	39.7	46.4	39.6	2.12	0.064
ADF digestibility [%]	37.5[b]	44.9[a]	36.5[b]	2.35	0.048
Nutritive value of ration					
TDN [%]	49.2	54.0	48.6	1.62	0.064
CP [%]	7.72	7.67	7.88	0.15	0.609

Notes: *Within dosing period (days 11 to 17 of Exp. 1); [#]Post dosing period (days 34 to 41 of Exp. 1); Means not sharing the same superscript differ significantly ($p < 0.05$).

3.4. *Effects of different dosing frequencies on the performance of buffaloes (Exp. 2)*

The effects of feeding the encapsulated fungal isolate at different intervals on growth rate and feed intake are presented in Table 5. During the experimental period of 120 d, the growth rate in both groups receiving the live encapsulated culture was significantly increased by about 20% compared with the group receiving an autoclaved culture (Control). Because this effect was independent from the dosing interval, the feeding trial indicated that there is no additional benefit of dosing live encapsulated fungi at 4 d intervals as compared to dosing at 8 d intervals. The intake of wheat straw was comparable among the groups.

4. Discussion

4.1. *Effect of the fungal isolate on the* in vitro *digestion of wheat straw*

The main purpose of conducting the *in vitro* studies was to check the compatibility of the fungal isolate (isolated from the faeces of wild cattle) with the rumen fluid microbes of buffaloes. Our earlier studies have indicated that some of the exogenous fungal isolates (isolated from different hosts) are not able to exert a positive effect on rumen fermentation, probably due to poor compatibility of the introduced fungi with the existing rumen microbes (Paul et al. 2004, 2010). The present study indicated *in vitro* a high repeatability of the apparent and true digestibility estimates after addition of the fungal isolate. This indicated a consistency in stimulation of digestibility and good compatibility of the fungal isolate with the rumen fluid microbes of buffaloes. However, the repeatability of enzyme activities was relatively low, which may be attributable to inherent lower accuracy of the assay methods for enzymes.

Table 4. Effect of weekly dosing (days 1, 7, 14 and 21) of *Neocallimastix* sp. CF 17 on rumen liquor parameters during the dosing and the post dosing period (Exp. 1).

	Control	Liquid culture	Encap-sulated	SEM	Significance (p-value)
Dosing period (days 18–20)					
Total volatile fatty acids [mM/100ml]	10.2[a]	12.4[b]	12.2[b]	0.488	0.021
TCA-precipitable N [mg/100 ml]	43.0[a]	49.3[b]	48.2[b]	1.46	0.027
Fungi [10^4/ml]	9.3[a]	23.5[b]	11.3[a]	3.13	0.021
Cellulolytic bacteria [10^7/ml]	11.8[a]	38.0[b]	35.0[b]	2.97	0.021
Hemicellulolytic bacteria [10^8/ml]	20.3[a]	34.7[b]	32.5[b]	3.21	0.025
Total bacteria [10^9/ml]	24.8[a]	54.0[b]	52.2[b]	3.95	0.001
Carboxymethyl cellulase [mIU/ml]	49.0[a]	64.5[b]	63.2[b]	4.07	0.045
Xylanase [mIU/ml]	30.8[a]	52.2[b]	53.5[b]	4.98	0.017
Post-dosing period (days 42–44)					
Total volatile fatty acids [mM/100 ml]	7.47[a]	9.50[b]	7.42[a]	0.305	0.001
TCA-precipitable N [mg/100 ml]	36.9[a]	43.5[b]	38.2[a]	1.49	0.028
Fungi [10^4/ ml]	9.25[a]	17.3[b]	11.5[a]	1.68	0.022
Cellulolytic bacteria [10^7/ml]	13.3[a]	20.8[b]	12.8[a]	2.25	0.058
Hemicellulolytic bacteria [10^8/ml]	26.8	34.8	28.8	2.51	0.062
Total bacteria [10^9/ml]	27.3[a]	41.5[b]	30.8[a]	2.96	0.019
Carboxymethyl cellulase [mIU/ml]	37.3[a]	51.5[b]	38.0[a]	3.39	0.026
Xylanase [mIU/ml]	26.0	32.5	36.3	3.97	0.235

Note: Means not sharing the same superscript differ significantly ($p < 0.05$).

Table 5. Effect of dosing of encapsulated anaerobic fungi on growth rate and feed intake of buffaloes (Exp. 2, duration 120 d).*

	Dosing of encapsulated fungi				
Parameters	Live 4-d interval	Live 8-d interval	Autoclaved 8-d interval (Control)	SEM	Significance (p-value)
Initial BW [kg]	176.3	176.2	176.5	6.61	0.999
ADG [g/d]	475[a]	479[a]	403[b]	21.3	0.040
Intake of wheat straw [kg DM/d]	2.73	2.68	2.54	0.08	0.308

Notes: *Constant intake of concentrates and green fodder of 1.35 and 0.24 kg DM/d respectively. Means not sharing the same superscript differ significantly ($p < 0.05$).

Information on the effect of anaerobic fungal culture to mixed rumen micro-organism on the *in vitro* feed digestibility, fibrolytic enzyme activities or fermentation pattern is scant. Lee et al. (1998) evaluated the effect of the addition of the *Piromyces communis* strain 22 to mixed rumen organisms on *in vitro* degradation of cellulose. They observed that with the addition of the fungal culture at a rate of 5% of rumen-liquor inoculum, followed by 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 addition of the fungal culture also increased the number of total bacteria, cellulolytic bacteria and anaerobic fungi by 40%, 35% and 350% respectively,

compared with the Control group. Previously, Paul et al. (2004) reported that one isolate of anaerobic fungi (*Piromyces* sp. FNG5) out of five tested isolates from wild ruminants, significantly increased the lignocellulose digestion by mixed rumen microflora of buffalo. Also in the present study, the fungal isolate showing a high straw-degrading ability in pure culture exerted a positive effect on fermentation of straw when added to mixed rumen microflora. The extent of the positive effect was inversely related to the strength of the rumen-liquor inoculum. Our preliminary *in vitro* studies indicated that the addition of the fungal culture to buffalo rumen fluid either in liquid culture or encapsulated form produce comparable results.

4.2. Effects of dosing encapsulated vs. liquid culture in growing buffaloes

In contrast with the effects of feeding liquid anaerobic fungal cultures, data on dosing encapsulated fungi are not available in literature. To our knowledge, this is the first report on dosing encapsulated anaerobic fungi.

The effectiveness of feeding a liquid culture of anaerobic fungi on rumen fermentation and growth performance has already been demonstrated in several *in vivo* studies. Lee et al. (2000) reported that daily intraruminal dosing of 200 ml of a liquid culture of *Orpinomyces* strain (isolated from goats) to sheep increased nutrient digestibility (DM digestibility from 71.5 to 75.2%; NDF digestibility from 65.1 to 68.9%; and ADF digestibility from 57.3 to 62.9%). Gordon et al. (2000) reported that in sheep receiving a nonindigenous anaerobic fungus (*Piromyces* sp. CS15 isolated from cattle rumen), having higher cellulolytic activities than those normally isolated from sheep, the voluntary DMI was increased in by 12%. In previous studies (Harrison et al. 1988; Frumholtz et al. 1989) the direct feeding of microbial cultures increased the growth of ruminal bacteria significantly, which improved feed efficiency. Several other studies (Paul et al. 2004; Tripathi et al. 2007) demonstrated that dosing of a liquid culture of anaerobic fungi increases the digestibility of fibrous feeds and growth performance of recipients. The improvement in nutrient digestion by the feeding of superior isolates of anaerobic fungal culture, which has higher cellulolytic activities than those normally isolated from the host animals, was attributed to increased microbial number and enzyme activities in the rumen. The present study demonstrated that encapsulated fungi can exert a similar effect on digestibility of fibre and growth performance as observed with a liquid culture.

The concentration of total VFA and TCA-precipitable nitrogen increased significantly with fungal feeding, either as a liquid or encapsulated culture (Table 4). Higher fermentation of fibre components, resulting in increased availability of energy and increased microbial population in the fungus-administered group, would be expected to increase the microbial protein production in the fungus-fed group. Surprisingly, there was no increase in fungal population in the group fed encapsulated fungi, although there was an increase in cellulolytic, hemicellulolytic and total bacterial population, rumen enzymes, VFA and TCA-precipitable nitrogen. It may be possible that encapsulated fungi remain entrapped in capsular polymer, and although they continue to produce and release enzymes, they fail to propagate freely in the rumen. A similar observation was noted in our earlier *in vitro* studies with pure culture (data not shown). When encapsulated fungi were incubated in anaerobic medium or autoclaved rumen liquor, there were no free fungi in the medium, but fungi continued to grow in entrapped capsular material and produced a

high level of enzyme. It is known that encapsulated microbes behave differently than their free counterparts (Mattiasson and Hahn-Hägerdal 1982).

In terms of feed intake, digestibility of nutrients and the growth performance of buffaloes, the encapsulated culture had similar effects as liquid culture. Although the results indicated that positive effects (VFA production, microbial population and enzyme level) of fungal dosing subsided relatively rapidly in encapsulated culture as compared with liquid culture (Table 4). This may be attributed to differences in fungal population dosing liquid vs. encapsulated culture. Nevertheless, the usefulness of the encapsulated fungi has been conclusively demonstrated. This technology will enable us to use anaerobic microbes as a feed additive at the farmers' level and on a commercial scale. The dosing of liquid culture of strict anaerobes on a large scale is not practically feasible, as unavoidable exposure to oxygen during preparation, handling and feeding kills the anaerobic microbes. However, these difficulties can be overcome by encapsulation of the culture.

5. Conclusions

The results of the present study indicated that feeding buffaloes with an encapsulated culture of anaerobic fungi (*Neocallimastix* sp. CF 17; isolated from faeces of wild cattle), which has a high lignocellulolytic activity, resulted in increased digestibility of fibrous feed, production of VFA and microbial population. Administration of encapsulated culture increased the growth performance of buffaloes significantly. The effects of encapsulated culture were largely comparable with those of liquid culture, although effects were slightly less persistent in case of encapsulated culture. The feeding of encapsulated culture has the potential to be applied at the farmers' level and on a commercial scale, as it protects the culture from environmental stressors to which the strict anaerobes are inevitably exposed when they are used as feed additive.

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