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Final report

project

Increasing the productivity of cattle in India and Australia with rumen fungal treatments

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1 Acknowledgments

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2 Executive summary

Ruminants remain an important part of agricultural production in both Australia and India, mainly because they provide food in the form of meat and milk, but also by providing draft power, fuel and fertiliser.. In northern Australia, cattle are used mainly for the production of beef, whereas in India, cattle and buffalo are used more for milk production, which is an important protein source for Indians. The common factor limiting ruminant production in both locations is the quality of the feed available for production. In both India and northern Australia ruminants depend mainly on crop residues, stubble and native pastures which are high in lignocellulose. Feed with a high proportion of lignocellulose is poorly digestible making it difficult for the animals to derive the nutrients they require for production. The dairy industry in India and the beef industry in northern Australia would both benefit from new technologies that could improve the utilisation of low quality roughages. CSIRO in Australia had developed research which was aimed at improving productivity of sheep grazing poor quality feed by stimulating rumen fungal populations. This project extended that research by developing two new technologies that have the potential to improve the digestibility of the fibre component of the diet of ruminants so that milk and meat production will keep pace with the growth of the human population. These technologies involved the use of a nutritional supplement to selectively enhance fibre degrading activity of anaerobic fungi in the rumen and a living fungal supplement.

The project was undertaken by scientists at CSIRO Livestock Industries in Brisbane, Australia and at the National Institute of Animal Nutrition and Physiology (NIANP) in Bangalore, India which is a research institute of the Indian Council of Agricultural Research (ICAR). The Australian group is recognised internationally for their expertise in ruminant microbiology while the Indian group have a strong nutrition background and continues to seek opportunities in rumen biotechnology research and development.

Fungal-specific nutrients which had been identified previously by CSIRO for use in sheep were evaluated in the laboratory and the field for their beneficial effects on the rumen microbiology and production responses in cattle and buffaloes. In particular, two organic sulfur nutrients mercapto-1-propionic acid (MPA) and 3-mercapto-1-propanesulfonic acid (MPS) were tested in three different cattle trials and compared to an inorganic sulfur supplement which is inexpensive and readily available. There was consistent improvement in nitrogen utilisation and microbial protein production in the rumen of these animals but surprisingly this was due to a general improvement in the efficiency of microbial fermentation of lignocellulose and not from specific stimulation of fungi. The benefits observed in animals supplemented with organic compounds were over and above inorganic S and the mechanism behind this improvement warrants further investigation. One major cattle trial was undertaken in India to examine the effect of the organic S supplement MPS on milk production. Cattle were fed a standard diet used for dairy cattle in India and supplemented with MPS, inorganic S or given no supplement (control). The response in milk production, fat content and rumen fermentation parameters were measured after peak lactation. MPS improved milk production more than the inorganic supplement, which was also higher than the control. There appears to be a real effect on milk production that is most likely due to the improved rumen fermentation but this needs to be examined in animals over an entire lactation. Because of these positive responses in milk production further animal trials will be undertaken following this project in India using cattle at the start of their lactation curve to get an accurate estimate of the size of the total response in milk production. This work has practical implications for the cattle industry in northern Australia where a cheap form of organic sulfur could improve productivity when poor quality pastures are low in sulfur.

To develop a living fungal supplement for improving lignocellulose utilisation, more than 200 different fungi were obtained from domestic and wild ruminants in India and stored at NIANP. Because of this unique biological resource, NIANP has been nominated by ICAR

as the site of a central collection and database of fungal cultures and central source of expertise in India. NIANP, in part because of the efforts of this project, arguably holds the most diverse rumen fungal collection in the world. Elite fungi from this collection were tested in several cattle trials but the impacts on productivity were inconsistent. It is clear from the work in both India and Australia that this approach for improving productivity is not currently practical or cost effective because of the time and effort involved to isolate, characterise and culture enough of the fungal cocktail for delivery, and the complicated nature of fungal ecology in the rumen.

The training, communication and collaboration between project teams were a highlight of this project. Four Indian scientists were trained in rumen microbiology and molecular biology at the CSIRO laboratories in Australia. They are now in a position to undertake work of a very high quality and in a research field that is critical to countries where a large proportion of ruminant production is on low quality feed. The team has communicated their work to the external scientific community through a number of good quality peer reviewed journal articles as well as conference proceedings.

3 Background

Ruminants are an important part of agricultural production in both Australia and India, mainly because they provide food in the form of meat and milk, but they also provide draft power, fuel and fertiliser in developing countries. Both India and Australia have large cattle populations (India, 196 million; Australia, 27 million; FAO, 1997) which depend upon fibre utilisation for sustainable productivity of milk and meat. In addition, India has a large population of buffaloes (80 million) that are important as a source of milk and to supply draught power and which consume significant quantities of poor quality forage. Production efficiency is low for Indian livestock and there is a heavy reliance on poor-quality fodders such as crop residues. Generally, a lactating cow receives so little digestible fodder that the All-India annual milk yield per cow is in the order of 1,000 kg (FAO, 1997). Clearly, improvements in the utilisation of straws and stovers to provide additional nutrients to the animal has the potential of raising the overall milk production by these animals and contributing to the income of households running small farms. Improving livestock production in developing countries is seen as means of raising the wellbeing of the rural poor (Delgado et al., 1999). In northern Australia cattle are used mainly for the production of beef, whereas in India, cattle and buffalo are used more for milk production, which is an important protein source for Indians since little meat is consumed.

In Australia, there are increasing numbers of cattle finished in feedlots and the industry relies on bovines raised on diets high in fibre for the supply of animals for the feedlots. Increasing the efficiency of production of those animals is an important factor in the continued success of the beef industry. Chronic shortages of feed and fodder coupled with poor nutritive value of available feeds have lowered the productive capacity and fertility of India's livestock. The availability of feed and fodders has not changed over the past two decades since there has been no change in the cultivated area devoted to fodder production in India, being 4.4% of the total cropped area. Consequently, the livestock population mostly depends on dry fodder from fodder crops and crop residues, with nutritional supplements in the form of concentrates. Therefore the common factor limiting ruminant production in both locations is the quality of the feed available for production. In India, the problem stems from the need to feed a large human population, which means the majority of high energy/quality cereal grains are used to satisfy human requirements and the ruminants depend mainly on crop residues or stubble, which are high in lignocellulose. In northern Australia the quality of feed is seasonal and cattle depend on low quality feed, high in lignocellulose, for a significant proportion of the production cycle. Feed with a high proportion of lignocellulose is poorly digestible making it difficult for the animals to derive the nutrients, particularly energy, they require for production; often these feeds do not provide enough energy even for maintenance. The dairy industry in India and the beef industry in northern Australia would both benefit if the utilisation of low quality roughages could be improved.

Over the past twenty years in India, several initiatives in the field of animal nutrition and husbandry have in total seen the doubling of the per caput milk availability. Principal among these has been improvements in the genetic potential of livestock through concerted efforts in artificial insemination'. Other initiatives have been the introduction of urea-mineral-molasses lick blocks (supported by ACIAR) for large ruminants which provide an efficient means for delivering additional nitrogen and trace minerals to livestock (see Leng, 1990). Improved formulations have been developed for and tested in different regions of the country (e.g. Srinivas and Gupta, 1997; Garg et al., 1998). The introduction of a cold manufacturing process has resulted in cheaper blocks with a longer life (Garg et al., 1998) which will encourage greater use of this effective technology. Therefore, the overall productivity of lactating cows and buffalo has increased, but is now approaching a production ceiling which is imposed by the availability of energy from fodder for the livestock.

CSIRO has for the past several years been developing technology to enhance fibre digestion and fermentation in the rumen by increasing the activity of the anaerobic rumen fungi (Gordon and Phillips, 1993; Gordon et al. 1996; Phillips and Gordon, 1991). The work has successfully identified anaerobic fungi that are not indigenous to sheep and a fungal-specific feed additive (mercaptopropionate; MPA) that significantly increases the dietary intake and the digestion of fibre by sheep. Production efficiency is estimated to be increased by 5-10% from the additional nutrients available to the animal. Feed intake of treated sheep was stimulated by 10-20% and improvements in production efficiency of 5-10% were observed. When sheep grazing poor quality feed were given these treatments they maintained liveweight, whereas untreated sheep lost weight. There appears to be an additive effect of combining the two types of fungal treatment in sheep, but none of this work has been undertaken using cattle or buffalo. The results from this work are contained in Patent Application No. PCT/AU98/00075 entitled "Fungal Sulfur Source and Method using Same", which does not include India and so no licence was required to use the IP.

This project was developed by scientists at CSIRO Livestock Industries in Brisbane, Australia and at the National Institute of Animal Nutrition and Physiology (NIANP) in India, a research institute of the Indian Council of Agricultural Research (ICAR). The Australian group is recognised internationally for their work in molecular microbial ecology in gut systems and the Indian group have a strong nutrition background, with good facilities for nutrition studies in large and small ruminants, but no expertise in rumen microbiology or molecular biology. The idea was that if similar results to those found in sheep could be obtained in cattle and buffalo then there was a very real opportunity to improve meat and milk production in Australia and India. The aim of the project was to develop a cost effective fungal nutrient treatment for cattle and buffalo in India and Australia. The hypotheses for the project were developed based on the work of CSIRO (Dr Geoff Gordon) with sheep and the project was structured around the 4 objectives which are outlined below.

Australia and India share similar climates in the tropical / subtropical regions and a reliance on the productive capacity of large ruminants from poor quality feed or fodder (at least on a seasonal basis in Australia) for the provision of human food. This provides a comparative advantage to Australia over other developed nations in undertaking a project of this kind in relation to India. CSIRO has the background intellectual property to conduct this project. Also, the scientists and technical staff at CSIRO who conducted the project have extensive research experience in rumen microbiology and ecology including anaerobic fungi. Anaerobic fungi seem to be the rumen micro-organisms of first choice for increasing the supply of nutrients from poor quality feed and fodder to large ruminants because of the results with sheep and the advanced nature of the research compared with the manipulation of other rumen microbial groups (e.g. fibre degrading bacteria, for instance

Livestock production in India, particularly from large ruminants, bears several similarities to production systems in neighbouring and regional countries. Large ruminants are expected to provide protein for human consumption, draught power and fertiliser for crop production, and finally as a valuable asset for wealth accumulation, from diets consisting of crop residues as the major component. Therefore, the results obtained in this project are relevant to several other countries and the ‘spillover’ of fungal treatments for large ruminants will benefit production there. Indeed, the low average milk yields reported for Sri Lanka and Vietnam together with an adequate domestic supply of crop residues suggest that fungal treatments to improve nutrient intake for milk production would be relevant to these two countries. There is also scope to improve the efficiency of milk production per animal in Pakistan. The calculated availability of crop residues per ‘bovine’ in Thailand, Vietnam and China together with the low availability of ‘bovine’ meat in these countries suggests that improved conversion of the nutrients in straws to liveweight would be beneficial.

Before this project commenced there were nearly 200 million cattle and 80 million buffalo in India, and about 2 million diary cattle while Australia had 25 million beef cattle. Therefore, the potential market size or number of animals is very large in both India and Australia. Adoption by even a small percentage would have a significant impact upon the overall industry in either country.

4 Objectives

Specific Objectives

1. To obtain superior fibre-degrading strains of anaerobic fungi in India and Australia that have the potential to persist in the rumen of cattle and buffaloes.
2. To evaluate the fungal-specific nutrient developed as a supplement for sheep in cattle and buffaloes under conditions where they receive poor-quality feeds
3. To evaluate an oral fungal inoculum for increased voluntary intake and digestion of poor quality feed (crop residues) by cattle and buffaloes.
4. To improve milk production from poor quality feed by use of cost effective fungal treatments (fungal specific nutrient and/or fungal inoculum) in India.

Expected benefits and technologies to be developed

- Feed supplement (cost effective) to promote anaerobic fungal activity will be available.
- Different inocula of elite anaerobic fungi will be available for use in India.
- Productivity and efficiency of production from large ruminants fed on poor-quality roughages will be improved in India and Australia.
- Improved milk production on small holder farms in India to assist in greater food security and advance the process of poverty alleviation.
- Expertise to initiate further studies in rumen microbial manipulation will be developed by Indian scientists

Technology expected to be developed through this project has the potential to increase the efficiency in terms of milk in cattle and buffaloes. It was anticipated that an enhanced ability for fibre digestion would prove to be very effective under Indian conditions of milk production. An increase in daily milk yield of 1 kg for 10% of India's 100 million milking buffaloes and cows would translate to an increase in annual production of around 10 million tonnes. This additional production would be sufficient to provide milk to an additional 40 million people at the present per caput availability of around 220 g/d. The technology would also be beneficial to production from Australia's 26 million cattle, particularly those in the pasture-based beef industries in the tropical and subtropical regions. Around 54% of the beef herd are in the northern regions of seasonally poor pasture.

Consultations between ACIAR and India identified the following priority issues:

- • Address increased demand for livestock and fish products arising from increased incomes
- • Embrace the opportunities offered by collaboration on applications of biotechnologies and new information technologies

Rumen biotechnologies have the potential to improve small-holder production where cattle and buffaloes form an integral part of the system (ACIAR-ICAR India Consultation, 2001). The proposed use of occasional dosing of ruminants with superior strains of anaerobic fungi together with the daily provision of a cheap fungal-specific nutrient for the purpose of increasing the utilisation of the available poor quality feed would be highly suited to small holder farming systems in rural villages. Also, improvement in milk production by crossbred cows is expected to have a significant influence on fresh milk supply for human consumption, as they produce about 23% of bovine milk in India.

Implementation of this project would bring together NIANP India and CSIRO Australia in a collaboration which aimed to deliver an advanced but simple to apply feeding technology

to small-holders. The project has the potential to impact on the availability of high quality food protein for many millions of people in the Indian subcontinent as well as those in other parts of East and Southeast Asia.

5 Methodology

The project essentially followed the original proposal plans except for the following changes which were agreed at the mid-term review in 2005 as follows:

It was agreed that MPA (mercaptopropanoic acid) is unlikely to be approved for use in animals as a feed additive due to potential hazards associated with handling the compound as a feed formulation. Future work should focus on MPS and sulphate as increased productivity from crop residues should result from sulfur and urea supplementation. Future work in Australia would concentrate on studying diet-host interactions and fungal populations in order to develop an inoculum where fungi persist in the rumen. This should lead to more predictable results in terms of colonisation of the rumen with superior strains of rumen fungi.

Future work in India would concentrate on animal production trials. In the first study intake and efficiency of rumen microbial protein synthesis in cattle fed either paddy straw or millet straw supplemented with sodium sulphate or MPS would be compared. Each experimental group would act as its own control. Further studies would then involve growth or milk production in animals fed crop residues supplemented with urea and either sulphate or MPS.

Australian resources would also concentrate more on training of visiting Indian scientists in molecular ecology and analysing the DNA from their fungal isolates

5.1 Objective 1

To obtain superior fibre-degrading strains of anaerobic fungi in India and Australia that have the potential to persist in the rumen of cattle and buffaloes

This objective was addressed by:

5.1.1 SUB-PROJECT 1.1

The isolation and purification of new strains of both monocentric and polycentric anaerobic fungi from rumen fluid and/or faeces of indigenous (wild) and domesticated ruminants in India and Australia

Isolation methods

Anaerobic fungi were isolated from rumen contents and/or from fresh faeces of large herbivores by following the methods described by Phillips and Gordon (1988, 1989) for monocentric fungi and Phillips and Gordon (1995) for polycentric strains. Roll tubes were used for the monocentrics whereas agar plates in an anaerobic chamber were used for the polycentrics; the rumen-fluid medium described by Caldwell and Bryant (1966) was used. Isolates were maintained by regular subculture until they were pure and then were stored in liquid nitrogen with a cryoprotectant (Phillips and Gordon, 1988).

Animal sources

Australian fungal isolates were obtained from a range of domesticated and exotic ruminants held under grazing conditions in northern Australia. India has several species of large native ruminant from which new strains of anaerobic fungi were sought. Isolations of anaerobic fungi were made from rumen fluid and fresh dung. Fungi typical to indigenous cattle and water buffalo were isolated to establish the baseline of fungal diversity and fibre-degrading ability present in domesticated large ruminants. In Australia, use was made of an existing collection of anaerobic fungi from large ruminants. In the past, water buffalo have yielded some new types of anaerobic fungi (Phillips and Gordon, 1995) and isolates were obtained from these animals.

5.1.2 SUB-PROJECT 1.2

The in vitro assessment of the fibre degrading abilities of the fungal isolates and fungal specific nutrients

Screening method

Isolates of anaerobic fungi were tested for their rates of cellulose and plant fibre degradation in vitro and compared with the rates found for other strains of fungi commonly found in cattle and/or buffaloes. Rates and extent of Neutral detergent fibre (NDF) degradation in plant samples (eg. Spear grass, Rhodes grass and paddy straw) were determined. Measurements of gas pressure generated in closed tubes of fungi cultured on plant substrate (e.g. milled local hay) was also used for screening different anaerobic fungi Menke et al., (1979). This activity was undertaken both in India and in Australia, using a small number (c. 5-6) of 'reference' strains of anaerobic fungi. The absolute value of the cellulose/fibre degradation rate is not of major importance; it is the rank of a strain relative to the other strains being tested (including the 'reference' strains) which is important in selecting potentially elite anaerobic fungi. The ranking can be achieved by any of the methods described above. Stimulation of fibre degradation and induction of enzymes by addition of fungal-specific nutrient (MPA) to cultures of individual fungal strains was also assessed. Using similar methods, other sulfur compounds (approximately 10) with similar chemical structure to MPA were screened for ability to stimulate fungal growth while not being readily utilised by ruminal bacteria.

5.1.3 SUB-PROJECT 1.3

The further development and use of modern molecular biology techniques to detect the presence of specific anaerobic fungi in recipient ruminant animals

DNA extraction.

Genomic DNA was isolated from pure fungal and bacterial species and rumen samples by employing the FastDNA kit and FastPrep instrument (Q-BIO gene, Quebec, Canada) as per the manufactures instructions. In essence the method involves resuspending the microbial cells in a lysis buffer in tubes containing garnet beads. The samples are then homogenised in the FastPrep instrument for two by 30 second intervals at a speed setting of 5; samples were incubated on ice for two minutes between disruption cycles. After centrifugation of the sample the supernatant was mixed with the supplied glass milk solution and washed before a final elution step, which releases the DNA from the glass milk. For rumen samples a 1.5 ml aliquot was taken from the 500 ml sample using a wide bore pipette so as to ensure an homogeneous sample containing fluid and digesta was obtained. The concentration of purified DNA is determined by absorbance at 260 nm and the samples are stored frozen.

Real-time PCR primer design and assay conditions.

The designed primers used for real time PCR are described in table1.

Table 1. PCR primers for real-time PCR assay

Target Species	Forward primer/Reverse primer
General bacteria	CGGCAACGAGCGCAACCC*/ CCATTGTAGCACGTGTGTAGCC
General anaerobic fungi	GAGGAAGTAAAAGTCGTAACAAGGTTTC/ CAAATTCACAAAGGGTAGGATGATT
Fibrobacter succinogenes	GTTCGGAATTACTGGGCGTAAA/ CGCCTGCCCCTGAACTATC
Ruminococcus flavefaciens	CGAACGGAGATAATTTGAGTTTACTTAGG/ CGGTCTCTGTATGTTATGAGGTATTACC

** modified from Lane 1991*

The primers for detecting total bacterial 16S rDNA sequences were designed by firstly modifying the existing universal bacterial primer 1114f (Lane 1991) to increase the Tm to 60^{o}C. The reverse primer was designed at a distance of 130 bp 3' of the forward primer based on the ARB 16S ribosomal sequence multiple alignments downloaded from the ribosomal database project (RDPII). Primers were analysed using primer express (Applied Biosystems Foster City, CA) and designed for an optimal Tm of 60^{o}C. Primers were then compared with sequences available at NCBI via a BLAST search to ascertain primer specificity (Altschul et al., 1990) and against the RDP II and ARB databases using the probe match analysis function (Cole, et al., 2003, Ludwig et al., 2004). A similar regime was followed for the design of the two fibrolytic bacterial primer sets to target *F. succinogenes* and *R. flavefaciens* species. The general anaerobic fungal primer set was designed from multiple alignments of fungal 18S ribosomal and ITS1 gene sequences, which included all available anaerobic fungal sequences and representatives of closely and distantly related fungal species.

Conventional PCRs for the validation of the designed primers specificity against target genes were performed in 30 µl reactions with the addition of 2.5 mM $MgCl_2$ and employing Platinum Taq (Invitrogen Carlsbad, CA). Reactions were performed using a Bio-Rad iCycler thermal cycler under the following conditions: one cycle at 94^{o}C for 2 min, 40 cycles of 94^{o}C for 30 sec, 60^{o}C for 15 sec and 68^{o}C for 1 min. The PCR products were analysed by running on 2% agarose gels containing ethidium bromide.

Real time PCR assays were performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems Foster City, CA). Assays were set up using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen Carlsbad, CA). Optimisation of assay conditions was performed for primer, template DNA and $MgCl_2$ concentrations. An optimal primer concentration of 300 nM and a final MgCl2 concentration of 3 mM were finally chosen for the assay under the following cycle conditions: one cycle of 50^{o} C for 2 min and 95^{o}C for 2 min for initial denaturation, 40 cycles at 95^{o}C for 15 s and 60^{o}C for 1 min for primer annealing and product elongation. Fluorescence detection was performed at the end of each denaturation and extension step. Amplicon specificity was performed via dissociation curve analysis of PCR end products by raising the temperature at a rate of 1^{o}C/ 30 sec from 60^{o}C to 95^{o}C. Total microbial rumen DNA was diluted to 1:10 prior to use in real time PCR assays.

Amplification efficiencies for each primer set were investigated by examining a minimum 6 point dilution series of total microbial rumen DNA template. In addition amplification efficiencies for *F. succinogenes, R. flavefaciens* and total anaerobic fungal primer sets were also performed on template from genomic DNA dilutions of *F. succinogenes* S85, *R. flavefaciens* Y1 and *Neocallimastix* LM1 respectively.

DGGE.

Denaturing gradient gel electrophoresis (DGGE) offers the means to rapidly assess the diversity of microbial species in an ecosystem (Muyzer and Smalla, 1998). Further, White et al. (1999) suggested the applicability of this technique to the rumen microbial population and thus this method was applied to fingerprint microbial populations in the rumen. Other molecular methods of discriminating populations of rumen fungi based on ribosomal ITS 1 and 18S rRNA were under development at the commencement of the project by Dr Brookman and Dr Theodoreau in the UK and were adopted using the methods described by Brookman et al (2000) and Tuckwell et al (2005).

ARISA

The presence of a length polymorphic region within the ITS 1 region of gut fungi was first exploited for phylogenetic analysis by Brookman and colleagues (2000). PCR amplification of the length polymorphic region using anaerobic fungal specific primers, followed by separation of the PCR products on high-resolution gels was shown to be a useful tool for quickly identifying fungal members from faecal samples. As members of the

same genera tended to have a similar length polymorphic region, it was possible to discriminate fungal genera based on mobilities of the ITS1 amplicons in high-resolution gels. In this project we further developed this method to analyse diversity of gut fungal populations from cattle rumen samples using an automated ribosomal intergenic spacer region (ARISA) method. The technique involves the labelling of the forward primer with carboxyfluorescein (FAM) and then separation of the products using an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA) for identifying gut fungi. A modification of the previously described anaerobic gut fungi specific primer MN100 through the addition of FAM labelling at the 5' end (5'TCCTACCCTTTGTGAATTTG) was used in conjunction with the reverse primer MNGM2 (5'CTGCGTTCTTCATCGTTGCG) (Tuckwell et al., 2005)

For all samples a template concentration of 50 ng was used in a PCR reaction using Platinum Taq (Invitrogen, Carlsbad, CA) with the following cycling conditions: 1 cycle at 94^{o}C for 3 min, 30 cycles at 94 ^{o}C for 10 s, 58 ^{o}C for 20 s 72 ^{o}C for 1 min. After amplification, products were diluted 1:100 with sterile H_2O and 1 μl was mixed with 10 μl highly deionized formamide (Applied Biosystems, Foster City, CA) and 0.1 μl GeneScan 500 LIZ (Applied Biosystems). Samples were denatured at 94oC for 2 min prior to loading onto a 3130xl genetic analyser (Applied Biosystems). Analysis of gel files was performed using Genemapper ver. 4.0 (Applied Biosystems).

5.2 Objective 2

To evaluate a fungus-specific nutrient in cattle and buffaloes under conditions where they receive poor-quality feeds

This objective was addressed by:

The in vitro and in vivo assessment of the fungal-specific nutrient (MPA) for Indian large ruminants and Australian cattle

5.2.1 Fungal specific nutrient

Mercaptopropionate (MPA; the nutrient chemical) was prepared in Australia (the commercially available acid is converted to the dry sodium salt) and tested both in Australia in cattle and in India in cattle and buffalo.

Other fungal specific nutrients (eg. Mercaptopropanesulfonate; MPS) and any new sulfur compounds identified in sub-project 1.2 were also subjected to in-vivo evaluation if considered non hazardous and cost effective.

In vitro expt

Initially the response of rumen fungi and selected cellulolytic bacteria to fungal specific nutrient compared with sodium sulphate were assessed in vitro to determine its specificity for fungi and to confirm the optimum dose for large ruminant feeding trials. Measurements of NDF digestibility or gas pressure generated in closed tubes of a rumen inoculum cultured on plant substrate (e.g. milled local hay) were also used to assess rate of fermentation. The culture medium (minerals) for these studies was sulfur deficient.

In vivo expt

Animal trials were conducted to compare (1) fungal specific nutrient supplementation with (2) unsupplemented controls and (3) a negative control supplemented with sodium sulphate. These studies will determine whether fungal specific nutrient has additional benefits to sodium sulphate supplementation on S deficient diets. The chemicals were placed as a component in the regular concentrate mixture offered to the animals. The daily amounts of fungal nutrient given for two-week periods to the animals (300 kg liveweight) was approximately 6.0 g/d, which was a projection from a similar trial

previously performed with sheep in Australia. Animals were also supplemented with non-protein nitrogen (urea) to correct any nitrogen deficiency of the roughage diet and the sulfur supplements were provided so that the final ratio of N:S was approximately 10:1.

The animals were initially adapted to the roughage for three weeks in pens during which dry matter intake (DMI) was measured before transferring to metabolism cages for a nine-day digestion trial (period 1). The animals were then allocated equally on feed intake to three treatments. Feed intake, faecal output and urine production were determined for a six-day period during each digestion trial and rumen samples collected for three days at approximately three, seven, 11 and 23 hours after the start of feeding each day. Digesta samples were taken directly from three sites within the rumen (anterior, central and posterior) and passed through screen with a mesh size of 2 X 1.5 mm. Sub-samples of liquor were taken for analysis of sulfide, short chain volatile fatty acids (VFA), ammonia and MPA. The pooled filtrate, in excess of 500 g per sampling, which contained rumen fluid and plant particles, was then stored at -80^{o}C until DNA was extracted for microbial population analysis. During the six-day collection periods, samples of feed offered and any residues were taken daily, pooled for each animal and stored at –20^{o}C. A constant daily aliquot (10% w/w) of faeces from each animal was also pooled for the six days. Total daily urine production was collected into 100 mls of 20% v/v sulfuric acid to give a final pH of less than three and a daily proportion (0.4% v/v) of each urine collection was diluted one to five with water and pooled for the six day period and stored at –80^{o}C until analysed.

Immediately after collecting rumen liquor, a sub-sample (3.5 ml) was taken for sulfide analysis with a sulfide-specific ion electrode (Model 9616 Sure-Flow Combination Silver/Sulfide Electrode; Thermo Orion, Beverly, MA) connected to a specific ion meter (pH/ISE Meter Model 710A, Orion) following the manufacturer's instructions and the method of Khan et al.(1980). The sub-sample was stabilised by mixing with an equal volume of freshly prepared SAOB aqueous solution (8% NaOH, 7.16% Na_2EDTA, add 3.52% ascorbic acid prior to use) and centrifuged (16,000 x g; 10 min) prior to analysis.

5.2.2 Volatile fatty acid

Rumen fluid samples were centrifuged (12,000 x g; 10 min) and the supernatant (1.6 ml) mixed with 400 µl metaphosphoric/internal standard solution (20% meta-phosphoric acid/ 0.24% 4-methyl valeric acid), recentrifuged and analysed using a Varian 3400 Gas Chromatograph fitted with an 8035 autosampler (Varian Australia Pty., Ltd.) on a packed glass column (2 metres x 6 mm OD x 2 mm ID) containing 10% FFAP/1% H_3PO_4 on Chromasorb WAW 100/120 mesh. The C2-C5 acids were separated over 16 min using nitrogen as a carrier at 12 ml min-1. Peaks were detected by Flame Ionization and integrated using Delta 5.0 Chromatography Data System (Digital Solutions Brisbane, Qld). An external standard (2 ethyl butyric) was added to samples prior to injection, together with meta-phosphoric to prevent fatty acids binding to free surfaces. The short chain volatile fatty acids (SCVFA) detected were acetate, propionate and butyrate and branched chain acids (BCVFA) were isobutyrate and isovalerate.

5.2.3 Ammonia.

Rumen ammonia levels were estimated spectrophotometrically at 630 nm by the indophenol method of Chaney and Marbach (1962).

5.2.4 Urinary purines

Microbial protein flow from the rumen was estimated by the urinary purine method (Chen et al. 1990; Chen and Gomes 1992). Urine was collected daily for six days from each animal into a container with approximately 100 ml of 10 % H_2SO_4. A sub-sample (50 ml) was taken each day and stored at –20^{o}C. Urine was analysed for allantoin, uric acid, xanthine and hypoxanthine to determine total purine. The urinary purines, allantoin and

creatinine were separated by HPLC using a Luna Amino (NH2) column (250 x 4.6 mm; 5 µm particles) with a mobile phase A (90% acetonitrile: 10% water) for 10 min, then mobile phase B (60% acetonitrile: 40% water) for 30 min, followed by phase A for 15 min at a flow rate of 1 ml min-1. PDA detection (Waters996 Photodiode Array Detector) enabled separate integrations for creatinine and allantoin at 235 nm and 214 nm, respectively. Uric acid was determined by the enzymatic UV-absorbance method described by Chen and Gomes (1992).

5.2.5 Ruminal DNA Extraction and Quantitative PCR Analysis of Rumen Micro-organisms

Total microbial genomic DNA was isolated from rumen sub-samples by employing the FastDNA kit and FastPrep instrument (Q-BIO gene, Quebec, Canada) as mentioned previously (Denman and McSweeney 2006). A rumen sub-sample (1.5 ml) was taken from the 500 ml rumen sample using a wide bore pipette so as to ensure a homogeneous sample containing fluid and digesta.

Real-time PCR assays were performed on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, California) for monitoring of anaerobic rumen fungi and the cellulolytic bacteria, F. succinogenes and R. flavefaciens as described by Denman and McSweeney (2006). PCR primers used for each microbial group are given in Table 1. The population sizes of specific bacterial groups were expressed as a percentage relative to the estimated abundance of total bacterial 16S ribosomal gene. The relative quantification assay is particularly useful when tracking multiple targets within many samples and is particularly useful for estimating differences between treatments and monitoring shifts in discrete microbial populations at different intervals. Biomass of rumen fungal populations was estimated from quantitative real-time PCR results using a conversion factor of 1 mg of total rumen fungal biomass per 13 µg fungal DNA (Denman and McSweeney, 2006). Assays were performed using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, California). An optimal primer concentration of 300 nM and a final MgCl2 concentration of 3 mM were employed for each assay under the following cycle conditions: one cycle of 50^{o}C for 2 min and 95^{o}C for 2 min for initial denaturation, 40 cycles at 95^{o}C for 15 s and 60^{o}C for 1 min for primer annealing and product elongation. Fluorescence detection was performed at the end of each denaturation and extension step. Amplicon specificity was performed via dissociation curve analysis of PCR end products by raising the temperature at a rate of 1^{o}C per 30 s from 60^{o}C to 95^{o}C.

5.3 Objective 3

To develop an oral fungal inoculum for increased voluntary intake and digestion of poor quality feed (crop residues) by cattle and buffaloes

This objective was addressed by:

5.3.1 The transfer of elite strains of anaerobic fungi to domesticated large ruminants

In Australia, the persistence of selected elite strains of anaerobic fungi was tested in the rumen of cattle held in pens and fed diets based on crop residues. The animals were initially adapted to the diet for 18 days in pens during which samples were taken and ARISA performed to determine baseline fungal populations. Treated animals received 500 ml of 48 hr culture of a Piromyces spp isolate and Orpinomyces spp. isolate F11 via a stomach tube. The control animals were given an equivalent amount of un-inoculated culture media. Rumen samples were collected via stomach tubing before dosing and every subsequent week for a period of three weeks.

In India, fifteen crossbred heifers of 18-24 months age were divided into three groups of five each -control, treatment 1 and treatment 2. All the heifers were fed on limited concentrate mixture (1kg on DM basis/day) and greens (1kg fresh para grass/day) and ad lib finger millet straw and their body weights were recorded at weekly intervals and the dry matter intake was recorded daily. The treatment groups - T1 were drenched with two elite fungal cultures obtained from large ruminants (@ 100ml per culture) while T2 consisted of two elite fungal cultures isolated from the small ruminants and wild ruminants. The control group received a placebo (media devoid of any fungal culture). Dosing of the fungal inoculm was carried out at weekly intervals. Composition of the concentrate mixture used in the growth trial Maize-30%, Soybean meal –30%, Wheat bran-37%, Mineral mix –2%, Salt –1%

5.4 Objective 4

To improve beef/milk production from poor quality feed by use of cost effective fungal treatments in India

This objective was addressed by:

5.4.1 Feeding trials with cross-bred cows in India

Eighteen crossbred milking animals were divided into three groups based on the milk yield from day of calving, days of lactation, parity and daily average milk yield. The dietary treatment consisted of control, treatment (T1) with inorganic sulphur supplement –sodium sulphate, 6.6g daily per animal and treatment (T2) with organic sulphur supplement-sodium salt of mercaptopropanesulfonate, 9.1g daily per animal. Three fistulated animals were used to assess the effect of dietary treatment on rumen fermentation by assigning them to each treatment for a period of one month.

The animals were fed on a concentrate mixture @ 40-50% of milk yield, limited silage and ad lib chaffed sorghum straw for a period of four months and the daily dry matter intake, milk yield and weekly milk composition were recorded. A digestibility trial was carried out at the end of the feeding trial and the nutrient digestibility was calculated. The rumen fermentation pattern was also studied in fistulated animals.

5.4.2 Feeding trials with lactating buffaloes in India

A feeding trial was conducted at NIANP India to assess the influence of the fungal treatments on feed intake and milk production by lactating animals held in pens. The design of the trials was the same as those described for cross-bred cows.

6 Achievements against activities and outputs/milestones

Objective 1: To obtain superior fibre-degrading strains of anaerobic fungi in India and Australia that have the potential to persist in the rumen of cattle and buffaloes.

No.	Activity	Outputs/ Milestones	Completion date	Comments
1.1	Isolation and purification of new strains of both monocentric and polycentric anaerobic fungi from rumen fluid and/or faeces of indigenous (wild) and domesticated ruminants in India and Australia	Preserved culture collection of anaerobic fungi from Indian herbivores Indian scientists trained in anaerobic microbiology techniques Contribution to information on fungal biodiversity in India	12/2005	All milestones achieved Superior fibre-degrading strains have been isolated from a wide range of domestic and wild, large and small ruminants. Most extensive database of ruminal fungal isolates in the world established in India a) Results b) Exchange/collaboration c) Capacity building/infrastructure d) Future Australia: a) 30 isolates characterised at molecular level, chose 5 to pursue, all genera represented, wild ruminants, persistency unknown b) Information on fungal isolates c) Four Indian scientists trained in anaerobic microbiology PC: a) Isolation from large and small ruminants. 1) Large ruminants: in vitro gas production technique, 2000 samples from faecal samples from cattle and buffalo; also some collections from rumen fluid in cattle. All 5 genera obtained and characterised morphologically and molecular. Cyllamyces – novel and only second recorded; first report in Buffalo; 161 isolates with information on gas production and NDF% - 15 isolates considered elite. 2) Small ruminants: isolates from sheep, goats and variety of wild animals. Morphological and molecular characterisation. Found 2 isolates that ranked 1 and 2 for all measures of efficiency that were used. Also found isolates that could not be classified morphologically b) No exchange of isolates but exchange of information about culture conditions and storage and scientists trained training

No.	Activity	Outputs/ Milestones	Completion date	Comments
				c) Now nominated and recognised as the site of central fungal culture collection/database and expertise in India; fully equipped and functional microbial ecology facility; skilled workforce; most diverse ruminal fungal collection in the world, anaerobic hood, real time PCR machine d) Preservation of fungal isolates needs to be addressed and made a priority. Maybe need to use DMSO. Expand fungal culture collection but targeted towards enzymatic discovery and potential industrial uses or fodder pre-treatment technology
1.2	In vitro assessment of the fibre degrading abilities of the fungal isolates	Fibre-degrading abilities of new strains compared with fungal isolates in the CSIRO collection Ranking of all fungal strains determined to reveal potential superior (elite) fungi and responsiveness to fungus-specific nutrients	12/2003	All milestones achieved Australia: Screened MTB, MPA, MPS in vitro on bacterial species, all responded so not fungal specific, cellulolytic bacterial species responsive. 30 isolates characterised for fibre degrading ability and chose 5 to pursue for animal trials PC: 161 isolates from large ruminants with information on gas production and NDF% - 15 isolates considered elite. Small ruminants: Found 2 isolates that ranked 1 and 2 for all measures of efficiency that were used.
1.3	Development and use of modern molecular biology techniques to detect the presence of specific anaerobic fungi and fungal diversity in treated ruminant animals	Screening of new fungal specific sulfur nutrients for safe cost effective S compounds DNA probes specific for anaerobic fungi with a demonstrated high capacity for degrading the dietary fibre component in poor-quality fodder Indian scientists trained in molecular biology techniques Dynamics of fungal populations in the rumen over time and under different dietary influences	12/2004 12/2004 12/2008 12/2005	All milestones achieved Australia: Molecular-based method developed for tracking fungi (ARISA), increased fungal database, database of molecular information of variety of fungal isolates Characterisation or better description of the molecular ecology of fungal populations in the rumen and answered questions of persistence, species differences and dietary responses. Industrial applications of fungal enzymes? Four Indian scientists trained in molecular ecology techniques for gut ecosystems

PC = partner country, A = Australia

Objective 2: To evaluate the fungal-specific nutrient developed as a supplement for sheep in cattle and buffaloes under conditions where they receive poor-quality feeds

no.	Activity	outputs/ milestones	completion date	comments
2.1	In vitro and in vivo assessment of the fungal-specific nutrient for Indian large ruminants and Australian cattle	Measurements of in-vitro digestibility responses due to supplementation with fungal-specific nutrients Most effective daily amount determined for stimulation of rumen fungal populations in cattle and buffaloes Measurements of production responses in animals due to supplementation with fungal-specific nutrient	12/2003 12/2004 12/2005	The persistency of these strains was not determined and there was no work undertaken in buffalo. The original plan to include buffaloes in time available was probably over ambitious A range of fungal-specific nutrients were evaluated in cattle eating poor quality diets. There was some influence on feed intake but greatest effect on nitrogen metabolism. Effect seems to be more general in nature, not fungal specific. MPS more practical than MPA. a) Results b) Exchange/collaboration c) Capacity building/infrastructure d) Future Australia: a) In vivo experiments, 12 cattle with control of inorganic S, increase FI linked to S, no change digestibility, rumen microbial protein significantly higher, qPCR of cellulolytic bacterial species and fungi increased but S-linked. MPA no better than inorganic S supplement and also toxicity issues. Same expt. design but used MPS. FI higher, much better microbial protein synthesis. No obvious change in microbial populations. Not fungal specific, stimulates other microbial populations. VFA data from the experiment with MPS (but not MPA) indicate sig. difference in A:P ratio with increased propionate (only a trend in this work), no production data because animals were mature b) Information about focus on MPS and experimental design (e.g. increase level of S), chemicals. c) Microbial ecology, developed S-free medium d) Pursue MPS and alternatives rather than MPA, cost effectiveness needs work

no.	Activity	outputs/ milestones	completion date	comments
				PC: a) 2 experiments (18 xbred cattle); Experiment : MPA examined along with Na sulphide and rice straw, urea treated and diets formulated to same amount of S; MPA and S didn't influence feed intake; ammonia N much lower; didn't look at microbial protein. Exp2, MPS: paddy straw (better quality), urea adjusted to keep N:S ratio 10:1, DMI didn't change nor did digestibility; low ammonia N and high VFA in MPS but not inorganic S (v. different to expt 1). No explanation for difference between expt 1 and 2 for inorg S results. No buffalo work. b) Planning experimental design c) Much of the infrastructure and expertise in nutritional methodology already existed. d) Evaluate dose response to compound and also effect of purity to help establish cost effective practise

PC = partner country, A = Australia

Objective 3. To evaluate an oral fungal inoculum for increased voluntary intake and digestion of poor quality feed (crop residues) by cattle and buffaloes.

no.	Activity	outputs/ milestones	completion date	Comments
3.1	Transfer of elite strains of anaerobic fungi to domesticated large ruminants in India	Qualitative measurements of colonisation and persistence of specific fungi in dosed cattle and buffaloes Measurements of feed intake and digestibility responses in dosed versus un-dosed cattle fed on crop residues	12/2004 12/2006	Oral fungal inoculums based on elite fungal isolates from milestone 1 were evaluated in cattle in Australia and India. Some effects on feed intake in Australian, but not Indian, experiments. a) Results b) Exchange/collaboration c) Capacity building/infrastructure d) Future Australia: a) 2 experiments: Geelong (8 Steers); Rockhampton (12 Steers). Geelong; dosed twice, significant response to dosing in feed intake and liveweight gains, difficult to determine if dosed strains were taking hold, but minor proportion of population. Both experiments showed increases in feed intake but no liveweight data in Rocky because mature animals were used. In Rocky had transfer of rumen contents from a donor animal, complicated and confounded design, only clear result was that fungal profiles differed for different diets. Background organisms make it difficult to separate and therefore conclude anything on persistency. b) Training in microbial techniques and ongoing communication for troubleshooting and experimental design c) NA d) Complicated and not cost effective, commercial value? Initial interest of commercial partner mentioned in the original proposal did not eventuate into anything. PC: a) 1 experiment (xbred cattle); (slightly different diet similar S levels) over 10 weeks with weekly dose; two elite fungal isolates from large and small ruminants each, inoculum two strains; trend towards increased body weight but probably no effect; no effect on rumen parameters. Different strains in inocula but the consistent story from across sites is that there is very little value in using fungal inocula.

no.	Activity	outputs/ milestones	completion date	Comments
				b) Information about strains and isolates but no exchange of isolates or DNA was possible due to restrictions on transfer of biological material between countries. c) Experience in culturing, preparing fungal inocula and dosing cattle with prepared mixes. d) Focus on the fungal collection for its value in industrial enzymology and the value of the molecular characterisation of the isolates to better describing and studying fungal ecology in the rumen, rather than as a source of elite fungal species for improving productivity.

Objective 4. To improve milk production from poor quality feed by use of cost effective fungal treatments (fungal specific nutrient and/or fungal inoculum) in India.

no.	Activity	outputs/ milestones	completion date	Comments
4.1	Feeding fungal-specific nutrient and /or dosing of elite strains of anaerobic fungi in dairy cows in India and beef cattle in Australia	Measurements of milk production/ beef productivity responses in treated versus untreated animals Interaction and efficacy of fungal dose and fungal-specific nutrient for milk production Pilot study of fungal-specific nutrient and/or fungal inoculants in large groups under pen conditions	03/2007 12/2006 12/2008	Evidence that milk production from cattle fed poor quality feed was improved by adding fungal specific nutrient. a) Results b) Exchange/collaboration c) Capacity building/infrastructure d) Future Australia: b) Offered advice in the planning of the experimental design. Participation in this type of experiment. c) Not applicable d) Involved in the planning and support for two more experiments that will be undertaken in India to confirm the effects observed on milk yield, to tease out the mechanism and examine dose response for MPS or alternative. PC: a) 1 experiment with cattle (18 xbred); standard dairy diet (practical as possible); started at peak of lactation curve; 40% increase in milk yield for MPS and also effect of inorg S. Response in milk production and lactation curve was maintained for longer in MPS>inorg S>control; fat remains the same; no impact on VFI; ammonia-N lower and VFA higher. Milk yield not statistically significant ($p<0.06$) but if outliers removed from analysis then it is highly significant.

7 Key results and discussion

7.1 Objective 1

To obtain superior fibre-degrading strains of anaerobic fungi in India and Australia that have the potential to persist in the rumen of cattle and buffaloes.

7.1.1 Summary

Two thousand faecal and rumen samples were taken from large and small, domestic and wild, ruminants. Approximately 200 fungal isolates were obtained from these samples and characterised morphologically and at the molecular level. All 5 genera normally found in the rumen were represented as well the genus Cyllamyces. This was only the second report of Cyllamyces being identified in cattle and the first in Buffalo. There were others that could not be classified morphologically. The NIANP has been nominated by ICAR as the site of a central fungal culture collection/database and expertise in India and arguably holds the most diverse ruminal fungal collection in the world. The fibre degrading capability of all of these isolates has been determined and approximately 20 were found to be elite and could be used as a fungal inoculum. Molecular-based methods (ARISA) for tracking fungi were developed by the Australian team, which were useful for describing fungal populations in the rumen but not for determining the persistency of fungal isolates.

Isolation and purification of new strains of both monocentric and polycentric anaerobic fungi from rumen fluid and/or faeces of indigenous (wild) and domesticated ruminants in India and Australia

INDIA

Both polycentric and monocetric fungi were isolated and purified from buffalo and cow dung samples procured form Anand, Chennai and Dharwad. All the five genera were obtained and among these Orpinomyces and Anaeromyces genera were predominant. Under the Orpinomyces genera's *O. joyonii* and *O. intercalaris* were the two species isolated. *Anaeromyces elegans* species with pointed or acuminate apex developing on solitary sporangiophores predominated. Of the six species of Piromyces only two species were isolated of which *Piromyces communis* was predominant in all the samples studied till date. *Caecomyces communis* was isolated in large numbers from the faecal samples of cattle and buffaloes with very active zoospores. The isolation of the recently described fifth genera of *Cyllamyces spp* in Indian cattle and buffalo was a novel finding and represents one of the few isolates of this organism held in culture collection.

Studies were also conducted to isolate anaerobic fungi from the faecal material of zoo animals from Bannerghatta National Park, Bangalore. The faecal material was collected from zebra (*Zebra equi*), hippopotamus (*Hippopotamus amphibus*), mithun (*Bos frontalis*), elephant (*Elephus maximus*),sblack buck (*Antilope cervicapra*) and spotted deer (*Chital axis*), horse and camel. Anaeromyces and Orpinomyces genera were found to predominate.

In vitro assessment of the fibre degrading abilities of the fungal isolates

AUSTRALIA

Thirty new strains of both monocentric and polycentric anaerobic fungi from rumen fluid and/or faeces of indigenous (wild) and domesticated ruminants in Australia have been isolated, identified and preserved as viable cultures. The fibre-degrading abilities of new strains were compared with fungal isolates in the CSIRO collection. These isolates were obtained from large ruminants, which were adapted to tropical fibrous diets. The isolates were compared with fungi in the CSIRO culture collection, which contains representatives

from many regions of the world. Several strains of fungi have been identified as having elite fibre degrading capacity (Table 1).

Table 1. Extent and rate of digestion of Rhodes and Spear grass by rumen fungi isolated from large ruminants

		NDF Digestibility (%) 96 h		NDF digestion rate (%/h)	
Isolate	**Genus**	**Spear grass**	**Rhodes grass**	**Spear grass**	**Rhodes grass**
TAP F6	Neocallimastix		43.9		
TAP F7	*Neocallimastix*		42.3		
TAP F8	*Orpinomyces*	59.8	46.2	1.34	1.61
TAP F9	*Orpinomyces*	61.6	62	1.74	2.24
TAP F10	*Orpinomyces*	40.7	61.2	0.97	1.47
TAP F11	*Orpinomyces*	49.1	44.3	1.34	1.80
TAX.1	*Piromyces*		30.9		
TAX.2	*Piromyces*		29		
TBT.1	*Orpinomyces*		15.7		
TBT.2	*Neocallimastix*	37.8	49	0.91	1.26
TBT.3	*Neocallimastix*	30.8	40.2	0.93	1.49
TED.1	*Piromyces*		46.9		
TGB.1	*Neocallimastix*	44.5	61.7	1.27	1.83
TGB.2	*Piromyces*		49.3		
TGB.3	*Neocallimastix*		49.5		
TNL.1	*Piromyces*	36.2	51.2	1.49	2.19
TNL.2	*Piromyces*	36.2	48.3	1.39	2.14
TOX.1	*Piromyces*	35.4	38.6	0.72	1.11
TSB.1	*Neocallimastix*	33.4	40.5	0.97	1.42
TZB.1	*Piromyces*	48.3	51	1.50	1.74
TZB.2	*Piromyces*		49.8		
TBB.1	*Piromyces*		3.3		
KS13	*Piromyces*	50.5	49.8	1.17	1.77

INDIA

Several fungal isolates which had superior rates and extent of fibre digestion were chosen for *in-vivo* studies as shown in table 2.

Table 2: Some of the elite strains of anaerobic rumen fungi isolated from large ruminants

Strain. No.	Place	Animal species	Genus	In vitro gas production(ml)	Total gas production (ml)	NDF digestibility (%)
1.	ELU	Cow	Anaeromyces	29.0 (24)	63.0	89
2.	Chennai	Buffalo Native Breeds	Cyllamyces	39.42 (24)	59.0	80
3.	Devegowda Layout	Cattle	Cyllamyces	31.00 (48)	50.0	71
4.	Chennai	Buffalo Native Breeds	Cyllamyces	31.00 (48)	50.0	79
5.	ELU	Rumen Liquor	Orpinomyces	32.00 (48)	51.0	77

6.	Chennai	Buffalo Native Breeds	Cyllamyces	32.00 (48)	56.0	79
7.	ELU	Rumen Liquor	Orpinomyces	32.00 (43)	48.3	74
8.	Shimoga	Buffalo	Orpinomyces	27.0 (24)	48.0	83
9.	Chennai	Jersey cross	Cyllamyces	32.00 (48)	48.0	75
10.	Devegowda Layout	Cattle	Anaeromyces	28.00 (48)	48.0	64
11.	ELU	Rumen Liquor	Orpinomyces	31.22 (48)	47.5	73
12.	Devegowda Layout	Cattle	Anaeromyces	31.00(48)	43.0	77
13.	ELU	Rumen Liquor	Anaeromyces	29.24 (48)	42.0	79
14.	ELU	Rumen Liquor	Anaeromyces	30.00 (48)	41.7	72
15.	ELU	Rumen Liquor	Anaeromyces	30.6 (48)	41.1	61

Numbers in parenthesis represent hours of fermentation

Development and use of modern molecular biology techniques to detect the presence of specific anaerobic fungi and fungal diversity in treated ruminant animals

AUSTRALIA

The goal of this work was to develop robust quantitative methods for measuring cellulolytic microbial populations in the rumen so that microbial responses to the sulfur and fungal treatments can be measured. Traditional methods for enumerating and identifying microbial populations in the rumen can be time consuming and cumbersome. Methods that involve culturing and microscopy can also be inconclusive when studying anaerobic rumen fungi, due to difficulties in discriminating between species, especially the polycentric anaerobic rumen fungi. DNA-based molecular methods do not depend on the culturability of micro-organisms, and therefore offer an attractive alternative for the study of complex fungal community structures. Other advantages include accelerating identification and increasing the accuracy of discrimination.

For this project we have used the internal transcribed spacer region 1 (ITS1) of the rRNA genes for studying genetic diversity of the fungi. PCR amplification of the length polymorphic region of ITS 1 using anaerobic fungal specific primers, followed by separation of the PCR products on high-resolution gels has provided a useful tool for quickly identifying fungal members from rumen samples.

Fungal population fingerprinting utilising length polymorphism of the anaerobic fungi ITS1 region on DNA extracted from the faecal samples of cattle fed different diets is shown in figure 1.

Quantitative real time PCR methods were also developed to measure the total fungal population in the rumen. Several primer pairs were designed for the amplification of total rumen anaerobic fungi, total rumen bacteria and several specific species of fibre degrading rumen bacteria including *Ruminococcus albus, Ruminococcus flavefaciens* and *Fibrobacter succinogens.*

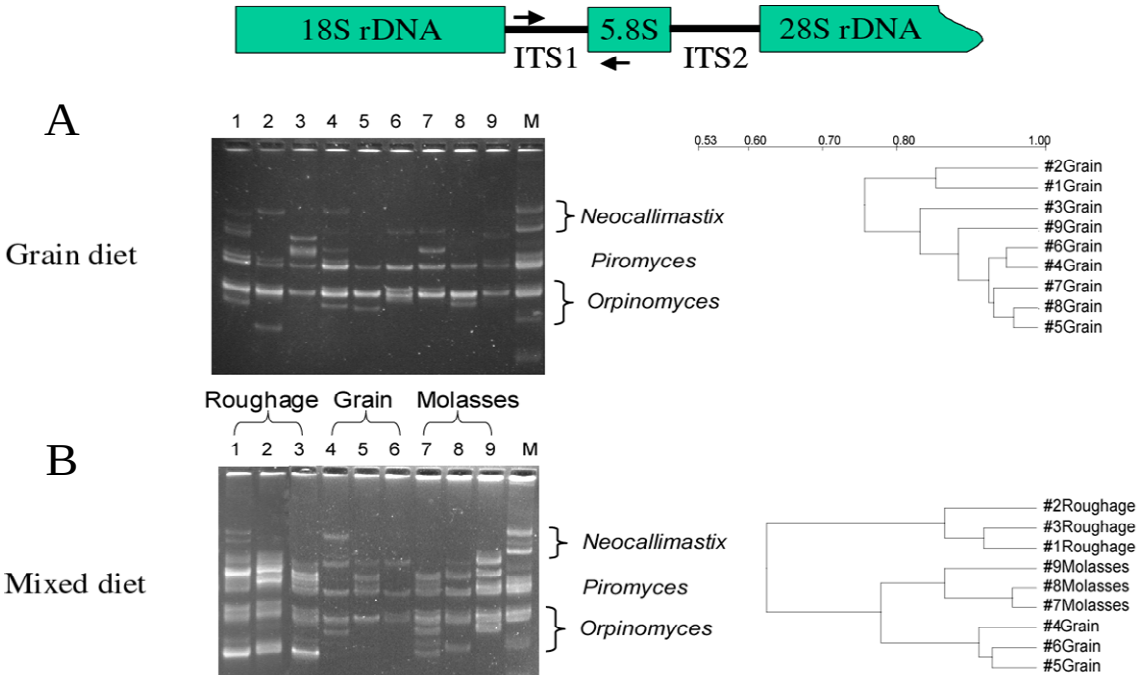


Figure 1. ITS1 anaerobic fungal specific amplicons were separated on a spreadex EL 600 gel for 4.5 hrs at 55 °C. A) Lanes 1-9 animals on a grain diet, M anaerobic fungal marker. B) Lanes 1-3 Roughage, 4-6 grain and 7-9 molasses diet, M anaerobic fungal marker. Phylogenetic trees are drawn based on population similarities of bands present for each animal.

7.2 Objective 2

To evaluate the fungal-specific nutrient developed as a supplement for sheep in cattle and buffaloes under conditions where they receive poor-quality feeds

Summary

A variety of fungal-specific nutrients were examined both in vitro and in vivo for their effects on microbial populations in the rumen and production parameters. The 'fungal-specific' nutrients mercapto-1-propionic acid (MPA) and 3-mercapto-1-propanesulfonic acid (MPS) were tested in vivo in three different cattle experiments and compared to an inorganic S control. The effects on feed intake in the three experiments were variable and there was no effect on digestibility, but there was consistent improvement in nitrogen utilisation and microbial protein production. The range of organic compounds that was evaluated do not appear to be fungal specific. Cellulolytic bacterial species also used these compounds as a sulfur source and the results from quantitative real time PCR analysis of rumen fluid indicated that there is a more general effect of these compounds on microbial populations in the rumen. The effects in cattle appear to be due to a more

general improvement in the efficiency of microbial fermentation of lignocellulose (better N metabolism and some evidence for volatile fatty acid production) not from specific stimulation of fungi. The benefits observed in animals supplemented with organic compounds were over and above inorganic S and the mechanism behind this improvement warrants further investigation. The decision to replace MPA with MPS for all other in vivo experiments was made because of toxicity concerns about MPA. At this stage none of the compounds screened could be used cost effectively but the effects are significant enough to pursue the development of cost effective practices.

In vitro and in vivo assessment of the fungal-specific nutrient for Indian large ruminants and Australian cattle

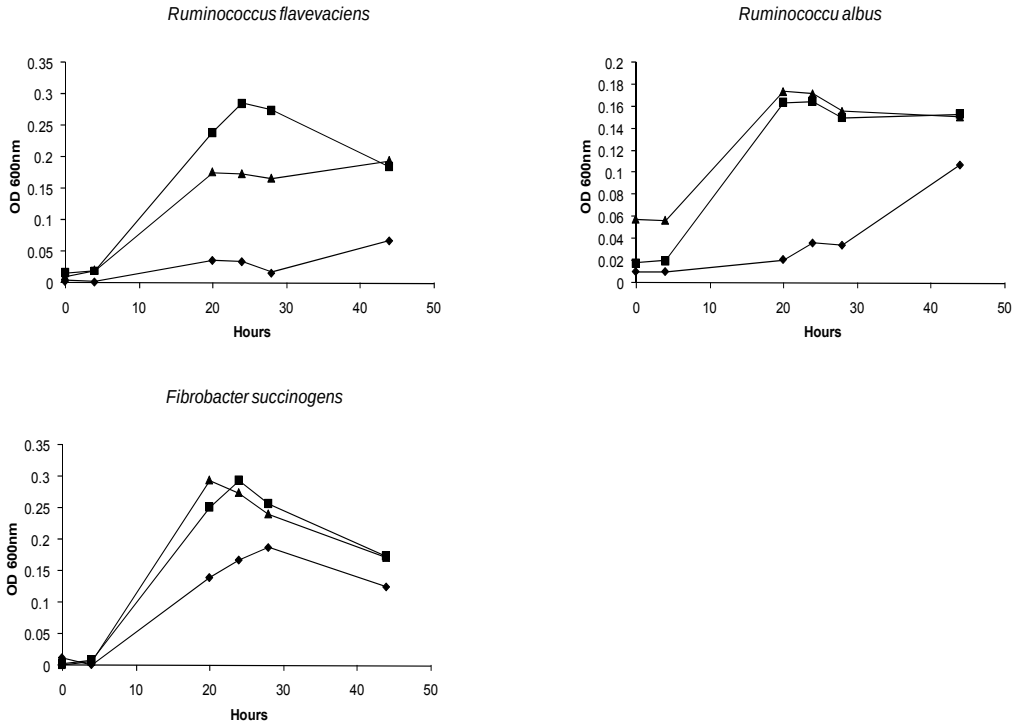
AUSTRALIA

In vitro assessment

In vitro studies of the effect of MPA and other sulfur compounds on fungi and rumen bacteria were performed initially by tracking the growth of the bacteria through an increase in optical density in the culture tubes. These studies were done to demonstrate whether thiol type sulfur compounds could be used by cellulolytic microrganisms in the rumen and thus potentially stimulate fibre degradation. Primary experiments focused on the development of a defined sulfur free media that could be used to test the addition of specific compounds. Inoculation of the sulfur free media with fibrolytic bacteria and specific sulfur compounds indicated that the fibrolytic rumen bacteria can use MPA as a sulfur source (Figure 2). *Ruminococcus flavefaciens* was observed to have a faster growth rate on MPS (3-Mercapto-1-propanesulfonic acid) than on MPA. All organisms tested grew slowest on the sulfur compound MTB (S-Methylthiobutyrate). Although *Fibrobacter succinogens* was least inhibited by this compound.

Several other sulfur-containing compounds were also tested, but were found to be inhibitory to the bacterial and fungal growth.

Figure 2. Growth rates of three rumen bacteria (*Ruminococcus albus, Ruminococcus flavefaciens* and *Fibrobacter succinogens*) were monitored for their response to three different sulfur compounds. MTB: (♦), MPS (■) and MPA (▲).



Due to the growth characteristics of filamentous fungi it is impractical to use this method to determine fungal growth curves, instead a method that examined sulfur supplement utilization was developed.

In vivo assessment

Cattle trial 1

A cattle trial was conducted to examine the effect of different sulfur supplements on feed intake, digestibility and rumen function. Twelve steers, 212 ± 4.2 kg liveweight (mean±sem) were fed a basal diet of Angelton grass which was deficient in S, N and P. Urea/phosphoric acid supplement solution (urea, 447.4g/l; 85% w/w orthophosphoric acid, 98.5g/l) was sprinkled on the feeds (34.4ml/kg) to increase the N and P content to 1.2% and 0.273% respectively on a dry weight basis. The S/N ratio in the feed for control animals was 0.055. The animals were initially adapted to the Angleton grass for three weeks in pens during which dry matter intake (DMI) was measured before transferring to metabolism cages for a nine-day digestion trial. The animals were then allocated equally on feed intake to three treatment groups as follows: (1) Angelton grass, (control); (2) Angelton grass plus 6.64 g/d anhydrous sodium sulphate (Sulphate); Angelton grass plus 6.0 g/d anhydrous sodium 3-mercaptopropionate, (Na-MPA). The sulfur supplements (equivalent to 1.5 g S/d) were mixed with 40 g sucrose to ensure complete consumption and placed in a separate container beside the Angleton/urea/P feed. The controls were given an equivalent amount of sugar. After adaptation to the diets for two weeks the animals were again placed in metabolism crates for digestion studies.

Feed and rumen chemical analysis

Feed intake, faecal output and urine production were determined for a six-day period during the digestion trials and then rumen samples collected for three days at approximately 3,7,11 and 23 hours after the start of feeding.

Measurements taken for the six days of the digestion trial for dry matter intake (DMI), % digestibility and digestible dry matter intake (DDMI) were analysed. A significant increase in DMI for those animals being offered the sulphate supplement was observed when compared with those on the control diet (Table 3). Similarly an increase in DDMI was also observed on the sulphate-supplemented diet with no change to the % digestibility. There was a marked increase in microbial N flow from the rumen for both sulfur supplements.

Table 3. Effect of Sulfur supplement on intake and digesibility of roughage.

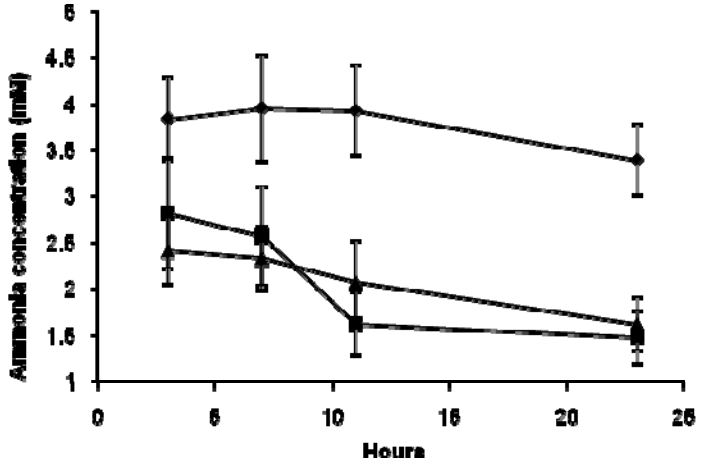
	Control	Sulphate	MPA
DMI (kg)	2.94 ±0.23^{a*}	3.81 ±0.43^{b}	3.28 ±0.20^{a,b}
% Digestibility	47.9 ±1.5^{a}	42.5 ±2.5^{a}	46.0 ±1.3^{a}
DDMI (kg)	1.41 ±0.12^{a}	1.62 ±0.09^{b}	1.50 ±0.13^{a,b}

** Values in rows with an uncommon superscript are significantly different (P<0.05); DMI: Dry matter intake, DDMI: Digestible dry matter intake.*

The mean theoretical N/S ratios, based on intakes for the six-day digestion period were 18.2 for controls, 11.4 for sulphate steers and 10.7 for the MPA supplemented steers.

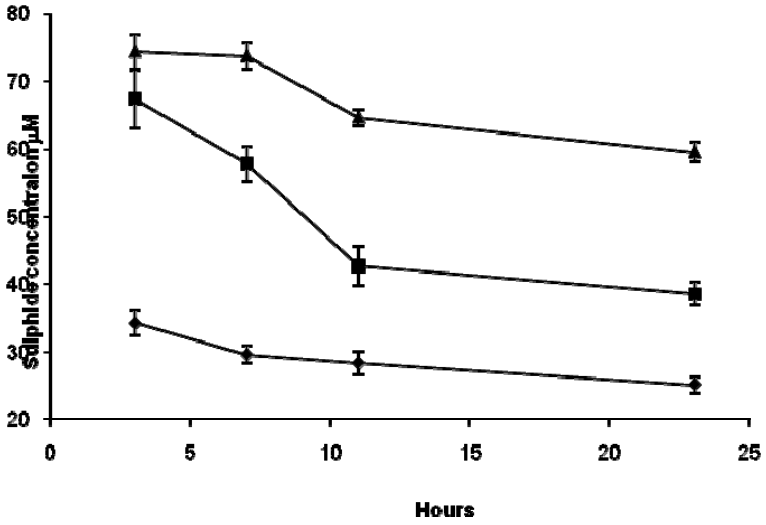
Ammonia levels at 3.5 hrs after feeding reflected this difference in N/S ratios, where the animals on the sulphate and MPA diets did not have as high a level of ammonia compared with that of the control group. The control group revealed only a slight decrease in ammonia levels over a 24 hour feeding cycle. The sulphate group of animals produced a traditional ammonia response curve over the 24 hours. Although the MPA animals were able to reduce the amount of ammonia in the rumen to the equivalent of that by the sulphate animals the animals did not show a large decrease in ammonia seven hours after feeding (Figure 3). However both sulfur supplementation groups were at sub-optimal N levels by 3.5 h after feeding commenced.

Figure 3. Ammonia levels in rumen fluid expressed as mM concentration. Points are the mean (± SEM) of four animals over three sampling periods, (n=12). Control animals, (♦); Sulphate animals, (■); MPA animals,(▲)



Sulphide measurements from rumen samples indicate a significant differences between all the feed treatments offered. The highest rumen sulphide levels were found in the MPA supplemented animals followed by the sulphate and then control animals (Figure 4). The higher levels of sulphide found in the MPA animals indicates that there was probably insufficient N available for efficient use of the available sulphide. Furthermore it is apparent that MPA can be converted to sulphide by rumen microorganisms.

Figure 4. Sulphide fluid levels expressed as μM concentration. Points are the mean (± SEM) of four animals over three sampling periods, (n=12). Control animals, (♦); Sulphate animals, (■); MPA animals, (▲).



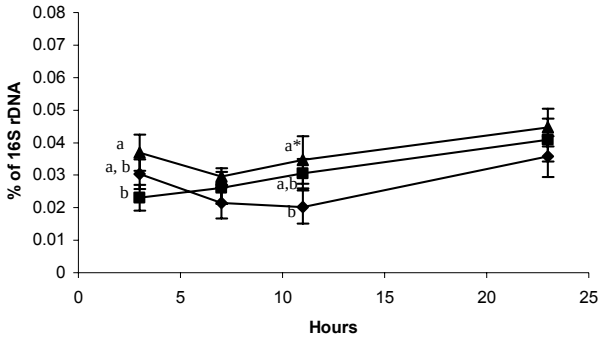
Molecular biological techniques for microbial population analysis:

qPCR results for all bacterial and fungal primers revealed distinct growth patterns relating to microbial flux in relations to times sampled after feeding regardless of the supplement added to the feed ($p<0.05$; Figure 5). Population analysis indicates that MPA stimulated the fungal populations while sulphate stimulated the Fibrobacter populations which are

cellulolytic. The analysis to date indicates that in the case of the total rumen fungal populations that they reach their highest numbers some hours later at around eleven hours after feeding before declining in numbers again. The fibrolytic rumen bacteria *Ruminococcus albus* and *Ruminococcus flavefaciens* showed a similar growth pattern in the rumen to that of the rumen fungi, in that they do not reach their peak in the rumen until the latter part of the 24 hour feeding cycle. *Fibrobacter succinogenes* also did not reach peak population numbers until later in the feeding cycle. It is most probable that the fibrolytic species of the rumen would follow this sort of growth pattern and not like that portrayed by the total bacteria population, as these organisms have the ability to attack plant material unlike some other microbes in the rumen which mainly survive on the soluble components of the feed. The soluble components of the plant material would be easily accessible at the start of feeding and exhausted quite rapidly, hence the drop of in bacterial numbers.

Figure 5. qPCR data representing specific bacterial targets with respect to percentage of total 16S rDNA. A) Ruminococcus flavefaciens, B) Fibrobacter succinogenes and C) total anaerobic fungi presented as μg of fungal biomass ml -1. Time points represent the mean (± SEM) of four animal samples over three consecutive days (n=12). Control animals, (♦); Sulphate animals, (■); MPA animals, (▲). Values that do not share common letters indicate significant differences (P < 0.05) within a given time point only. *Significantly different with a P < 0.06. † Significantly different with a P < 0.01.

A.



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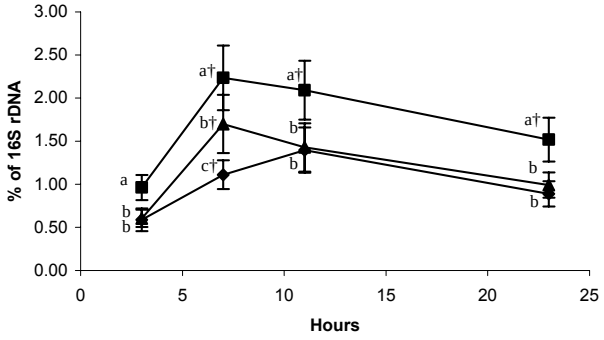
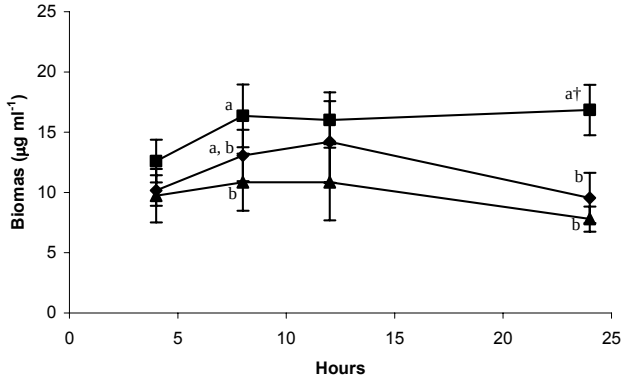


C.



Cattle trial 2

A cattle trial was conducted to examine the effect of different sulfur supplements on feed intake, digestibility and rumen function. Twelve steers, 333 ± 9.5 kg liveweight (mean±sem) were fed a basal diet of Angleton grass (Dicanthium aristatum) (mg/kg; Al 42, B 14, Ca 3242, Cu 0.5, Fe 71, K 4083, Mg 848, Mn 47, N 2670, Na 107, P 1043, S

997, Zn 14) which was deficient in S, N and P. Urea/phosphoric acid supplement solution (urea, 447.4 g/l; 85% w/w orthophosphoric acid, 98.5g/l) was sprinkled on the feeds (48.8 ml/kg for controls and 68.3 ml/kg for sulfur supplemented) to increase the N and P content to 1.4% and 0.345% respectively for control animals and 1.8% and 0.441% respectively for sulfur supplemented animals on a dry weight basis. The S/N ratio in the feed for the animals was 0.073. The animals were initially adapted to the Angleton grass for three weeks in pens during which dry matter intake (DMI) was measured before transferring to metabolism cages for a nine-day digestion trial. The animals were then allocated equally on feed intake to three treatment groups as follows: (1) Angleton grass, (control); (2) Angleton grass plus 9.56 g/d anhydrous sodium sulphate (Sulphate); (3) Angleton grass plus 6.0 g/d anhydrous sodium 3-mercapto-1-propane sulfonic acid, (Na-MPS). The sulfur supplements (equivalent to 1.5 g S/d) were mixed with 40 g sucrose to ensure complete consumption and placed in a separate container beside the Angleton/urea/P feed. The controls were given an equivalent amount of sugar. After adaptation to the diets for two weeks the animals were again placed in metabolism crates for digestion studies.

Feed and rumen chemical analysis

Feed intake, faecal output and urine production were determined for a six-day period during the digestion trials and then rumen samples collected for three days at approximately 4,8,12 and 24 hours after the start of feeding.

Measurements taken for the six days of the digestion trial for dry matter intake (DMI), % digestibility and digestible dry matter intake (DDMI) were analysed. A significant increase in DMI for those animals being offered the sulphate supplement was observed when compared with those on the control diet (Table 4). Similarly an increase in DDMI ($P < 0.06$) was also observed on the sulphate-supplemented diet with no change to the % digestibility. There was a marked increase in microbial N flow ($P < 0.06$) and a significant increase in purine levels ($P < 0.05$) from the rumen for sulphate supplementation. Similarly there was a marked increase in microbial N flow ($P < 0.06$) and a marked increase in purine levels ($P < 0.07$) from the rumen for MPS supplementation.

Table 4. Effect of sulfur supplement on intake and digesibility of roughage.

	Control	Sodium sulphate	MPS
DMI (kg)	$5.76 \pm 0.43^{a*}$	6.44 ± 0.26^{a}	6.11 ± 0.38^{a}
% Digestibility	48.1 ± 1.2^{a}	48.0 ± 1.4^{a}	47.9 ± 1.4^{a}
Purines (mmol/d)	52.5 ± 3.1^{a}	71.9 ± 3.6^{b}	69.7 ± 3.6^{b}
Intestinal flow N (g/d)	35.6 ± 2.6^{a}	51.4 ± 3.0^{b}	50.1 ± 3.0^{b}

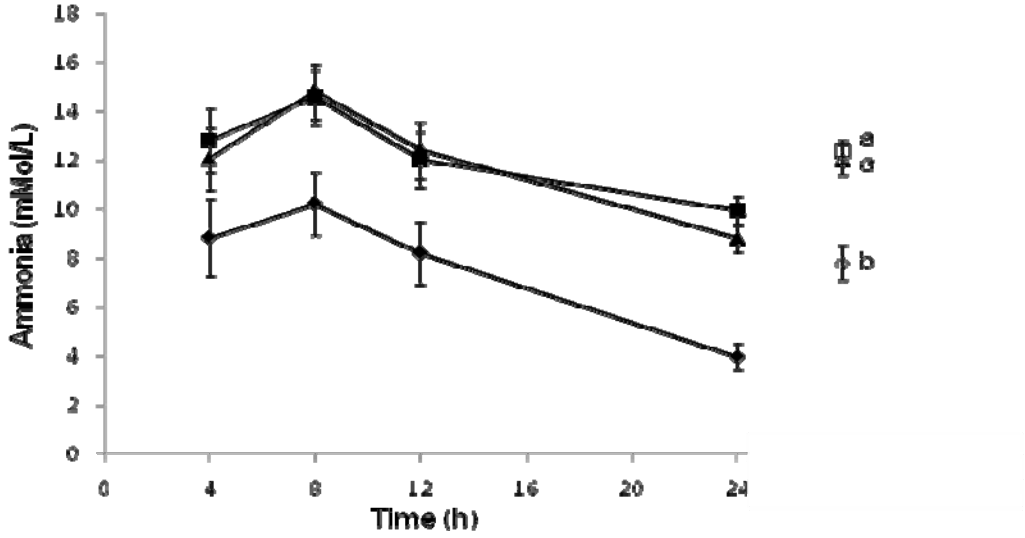
** Values in rows with an uncommon superscript are significantly different ($P \leq 0.05$). DMI: Dry matter intake*

The mean theoretical N/S ratios, based on intakes for the six-day digestion period were 13.9 for controls, 13.7 for sulphate steers and 13.5 for the MPS supplemented steers.

All groups of animals produced a traditional ammonia response curve over the 24 hours. Although the levels in the control animals were approximately two thirds that of the sulfur treated groups which reflected the differences in urea supplementation (Figure 6). However in all groups ammonia nitrogen levels were above minimal requirement for microbial protein synthesis throughout the entire day.

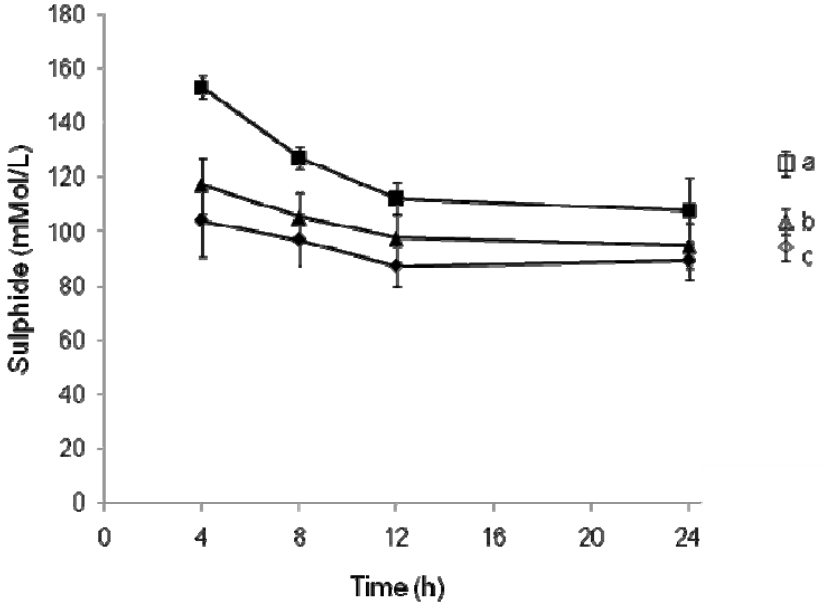
Figure 6. Ruminal ammonia levels in response to S supplementation. Data points are the mean (± SEM) of three or four animals over three sampling periods. Control, 3 animals (♦); Sulfate, 4 animals (■); MPS, 4 animals (▲).Pooled means (open symbols) and standard error

bars for each treatment are presented and values that do not share common letters indicate significant differences (P < 0.001).



Sulphide measurements from rumen samples indicated a significant difference between the sulphate treatment and those of MPS and control treatments ($p < 0.05$). The highest rumen sulphide levels were found in the sulphate supplemented animals followed by the MPS which tended to produce an intermediate response and then control animals (Figure 7). MPS contains two sulfur residues, although one of these may not be available for microbial sulphide production and this may reflect the difference in rumen sulphide levels between MPS and sulphate.

Figure 7. Ruminal sulphide levels in response to S supplementation. Data points are the mean (± SEM) of three or four animals over three sampling periods. Control, 3 animals (♦); Sulfate, 4 animals (■); MPS, 4 animals (▲).Pooled means (open symbols) and standard error bars for each treatment are presented and values that do not share common letters indicate significant differences (*P* < 0.05).



Molecular analysis of microbial populations.

Analysis of total rumen bacterial, fungal and two fibrolytic bacteria revealed significant differences in population numbers with respect to diet offered. Total rumen bacterial populations were observed to be approximately 1.5 fold higher and significantly different ($p<0.05$) for animals supplemented with sulphate compared to the control and those animals supplemented with MPS. Total fungal populations were observed to be significantly higher ($p<0.05$) in animals that were supplemented with respect to the control animal group, however no significant difference was noticed between the two types of supplements offered. MPS supplementation produced opposite responses for the two fibrolytic bacteria monitored. *Fibrobacter succinogenes* was observed to have a significant ($P<0.05$) reduction in numbers on MPS supplementation compared to both the sulphate supplement and control animals. While *Ruminococcus flavefaciens* was not affected by MPS and was shown to possess significantly ($p=0.05$) higher numbers then those animals supplemented with sulphate. Previous in vitro analysis of MPA and MPS supplementation on growth conditions of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* also correlates with data collected here.

INDIA

In vivo assessment

An experiment was carried out to assess the effect of supplementing organic (mercapto propionosulfonic acid sodium salt -MPS) and inorganic (sodium sulphate) sulphur sources on dry matter intake and digestibility of nutrients in heifers fed on low sulphur diets. Eighteen crossbred heifers of 24-30 months age were selected and divided into three groups of six each in a randomized block design and were fed on paddy straw ad lib for a period of six weeks. At the end of five weeks of supplement feeding a digestibility trial was carried out with seven days collection period for assessing the effect of sulphur supplements on dry matter intake and nutrient digestibility. Rumen samples were collected through stomach tube at the end of the trial at different hours to study the rumen fermentation pattern. Spot urine samples were collected consecutively for 2 days for estimating purine derivatives and thereby assessing the microbial protein synthesis. No signifficant changes in the dry matter intake was observed between the treatment groups (Table 5).

Table 5. Dry matter intake during the trial period

	Control	Sodium sulphate	MPS
Body wt (kg)	276 ±10.	275 ± 10	274 ± 9
DMI (kg/d)	6.30 ± 0.50	6.15 ± 0.33	6.08 ± 0.28
Apparent DMD (%)	43.3 ± 0.6	44.4 ± 0.3	45.1 ± 1.6
ADF digestibility (%)	40.9 ± 0.4	42.9 ± 0.7	43.2 2.6

DMD: dry matter digestibility, ADF: acid detergent fibre

Total volatile fatty acid analysis for the animals revealed a significant increase in values for the animals supplemented with MPS six hours after feeding, indicating a significant increase in microbial fermentation activity within these animals (Table 6).

Table 6. Rumen fermentation pattern

		Control	Sodium sulphate	MPS
pH	0 hr	7.15± 0.06	7.26 ± 0.07	7.20 ± 0.09
	6 hr	7.20 ± 0.10	7.29 ± 0.06	7.45 ± 0.15
TVFA (meq/dl)	0 hr	4.16 ± 0.54	4.27 ± 0.29	3.29 ± 0.37
	6 hr*	6.96 ± 0.55[a]	6.54 ± 0.36[a]	8.94 ± 0.29[b]

**Values with different superscripts vary significantly (P<0.05).*

Significantly low ammonia–N in the MPS supplemented group indicates better utilization of the nitrogen, probably by increased microbial protein synthesis for this group (Table 7). This is being ascertained further through the analysis of micirobial protein from collected spot urine samples which is currently underway.

Table 7. Rumen fermentation pattern

		Control	Sodium sulphate	MPS
NH_3N (mg/dl)	0 hr* 6 hr**	3.82 ± 0.51^{a} 11.46 ± 1.24^{c}	4.30 ± 0.29^{a} 11.28 ± 1.49^{c}	2.78 ± 0.35^{b} 7.56 ± 0.55^{d}

**a,b Values with different superscripts vary significantly (P<0.05)*

***c,d values with different superscripts vary significantly (P<0.01)*

The fungal counts undertaken from the rumen liquor of the trial animals clearly showed an increase in fungal numbers within both sulfur supplemented groups with the highest numbers being found in animals supplemented with MPS (Table 8).

Table 8. Rumen fungal counts of animals under trial

	Control	Sodium sulphate	MPS
Fungal counts (cfu/ml)	300	63	663

The results of this trial correlate well with those observations from the feeding trials in Australia.

7.3 Objective 3

To evaluate an oral fungal inoculum for increased voluntary intake and digestion of poor quality feed (crop residues) by cattle and buffaloes.

7.3.1 Summary

Different fungal inoculums containing elite fungal isolates were developed in Australia and India and tested in vivo in 3 different experiments with cattle fed low quality feed. The results from these experiments were inconsistent and this may be related to complementarity of fungal strains in the inoculum with those already present in the rumen. However, this is speculative because it was difficult to differentiate the elite isolates in the dosed animals from the background strains already present in the animals. What was clear from the work in both India and Australia was that this approach for improving productivity is not practical or cost effective at this time because of the time and effort involved in the process of isolating, characterising and culturing enough of the fungal cocktail for inoculation and the complicated nature (and our lack of understanding) of fungal ecology in the rumen.

AUSTRALIA

In vivo assessment

Trial 1

A cattle trial was conducted to examine the effect of dosing cattle with elite fibre degrading fungi. Eight steers, 231 ± 4.9 kg liveweight (mean±sem) were fed a diet of oaten chaff (mg/kg; Al 22, B 37, Ca 988, Cu 4, Fe 55, K 14195, Mg 899, Mn 62, N 8340, Na 2811, P 1360, S 797, Zn 21) supplemented with urea 40-60 g/d. The animals were initially adapted to the diet for 18 days in pens during which dry matter intake (DMI) was measured. The animals were then allocated equally on feed intake to two treatment groups as follows: (1) no fungal dose, (control); (2) fungal dose (treatment). Each animal to be inoculated with fungi received 2000 ml of 48 hr culture, comprising of 500ml each of the four individual fungal strains (F9, F11, TNL1 and TZB1). The controls were given an equivalent amount of un-inoculated culture media.

Feed intake, and animal weights were recorded throughout the trial period, rumen samples were collected via a stomach tube at weekly intervals both prior to and after dosing with fungal inoculum.

Measurements taken during the trial for dry matter intake (DMI) and animal weights were analysed. A significant increase in DMI for those animals being dosed with fungi ($P<0.05$) was observed along with a significant weight gain ($P< 0.05$) when compared to those animals that were not dosed with elite fungi (Table 9).

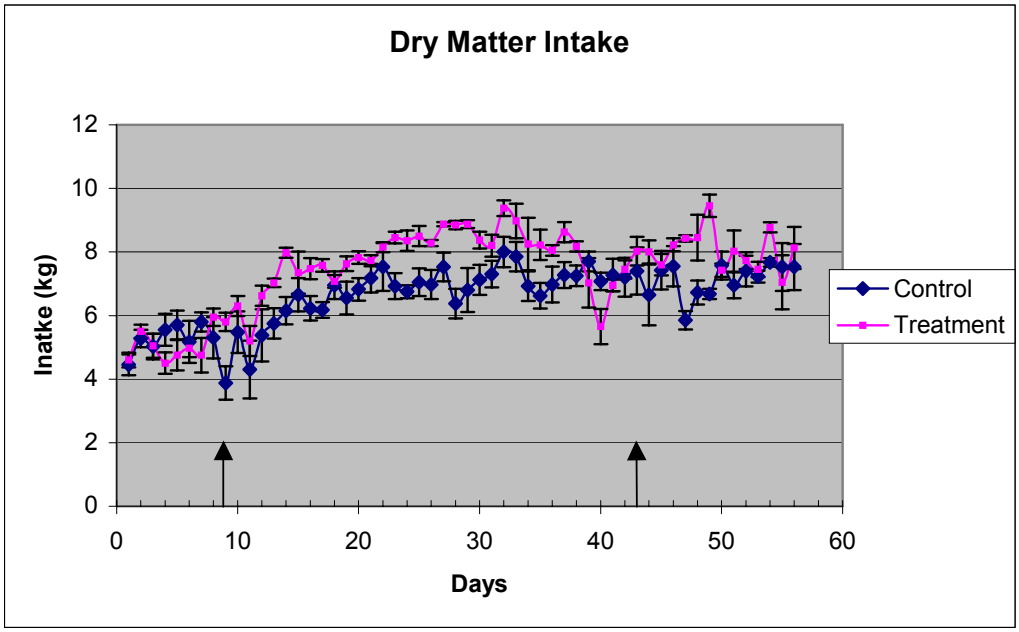
Table 9. Effect of fungal dosing on intake and animal weight gain.

	Control	Treatment
DMI (kg)	$6.83 \pm 0.09^{a*}$	7.83 ± 0.08^{b}
Weight gain (kg)	82 ± 2.9^{a}	98 ± 4.0^{b}

** Values in rows with an uncommon superscript are significantly different ($P \leq 0.05$). DMI: Dry matter intake.*

Analysis of the feed intake data across the entire trial revealed that on both occasions some three to four days after inoculating the treatment animals with fungi their DMI was observed to increase significantly compared to the control animals (Figure 8).

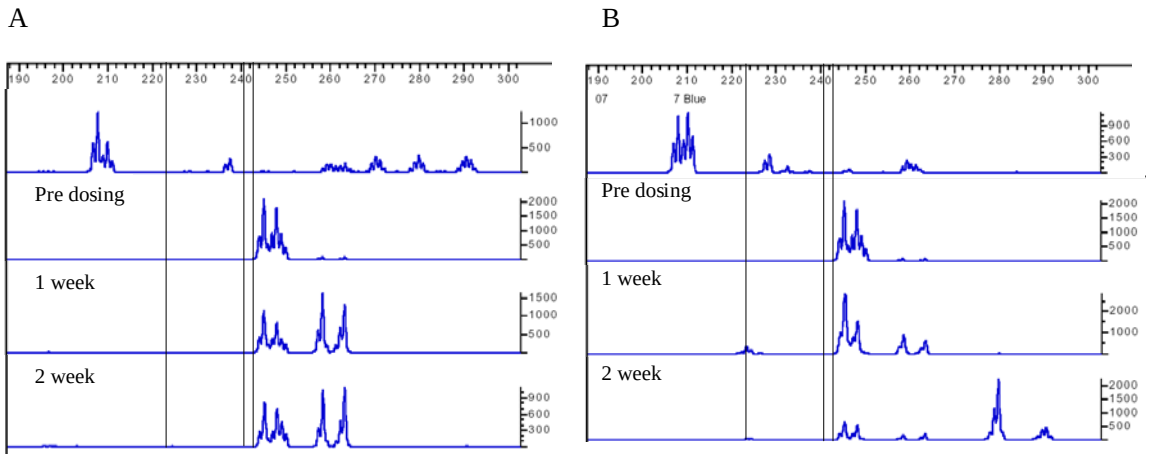
Figure 8. Dry matter intake values collected over the trial period. Arrows indicate fungal dosing days at nine and 43 days after trial start day. Points are the mean of four animals error bars are standard errors (n=4). Control animals: blue line, fungal dosed animals: magenta line.



ARISA analysis was used to track the presence of introduced fungi within the rumen of the treated animals. Peaks were expected to be observed at specific positions based of fragment size for the four individual fungal strains (F9 and F11 at 223bp, TNL1 at 241bp and TZB1 at 243bp). No peaks representing any of the introduced fungal species were observed in the control animals (Figure 9A). A peak representing the fungi F9 or F11 was observed in a single animal from the treatment group but no peak was observed for TNL1 or TZB1 (Figure 9B). It is possible that because fungal strains F9 and F11 were originally isolated from Bantang cattle while TNL1 and TZB1 were isolated from Nilgi and Zebra respectively they may be expected to have less difficulty in colonising within the rumen of cattle. Observations of introduced fungi in only one of the treated animals may reflect that

the current method is limited by detection threshold limits rather than the absence of these fungi from the other treated animals.

Figure 9. ARISA electropheragrams of anaerobic fungal populations from a [A] control animal and [B] a treated animal. Scale across the top represents peak migration in base pairs, vertical lines indicate where peaks are expected to be observed for introduced fungi.



As samples could only be collected once a week via stomach tube it is not possible to draw any conclusions about wether the fungal inoculum was detectable in the first few days after dosing and then failed to colonise.

Trial 2

A cattle trial was conducted to examine the effect of dosing cattle with elite fibre degrading fungi. Twelve steers, 449 ± 10.9 kg liveweight (mean±sem) were fed a diet of Rhodes grass (Chloris gayana) supplemented with urea 70 g/d. The animals were initially adapted to the diet for 18 days in pens during which dry matter intake (DMI) was measured. The animals were then allocated into two treatment groups as follows: (1) no fungal dose (control); (2) fungal dose (treatment). Each animal to be inoculated with fungi received 2500 ml of 48 hr culture, comprising of 500ml each of the five individual fungal strains (F9, F11, TGB1, TNL1 and TZB1). The controls were given an equivalent amount of un-inoculated culture media. Feed intake, and rumen samples were collected at weekly intervals both prior to and after dosing with fungal inoculum.

Measurements taken during the trial for dry matter intake (DMI) revealed a significant increase in DMI for those animals being dosed with fungi ($P < 0.05$) when compared to those animals that were not dosed with elite fungi (Table 10).

Table 10. Effect of fungal dosing on dry matter intake (kg/d).

	Period 1 (undosed)	Period 2 (Fungal dosed)
Control group	8.68 ± 0.13^{a*}	8.87 ± 0.10^{a}
Treatment group	9.26 ± 0.07^{a}	9.71 ± 0.08^{b}

** Values in rows with an uncommon superscript are significantly different ($P \leq 0.01$). DMI: Dry matter intake.*

These results are in agreement with the fungal dosing from trial 1 involving animals on a temperate diet and field conditions; thus showing that in two independent trials an increase in dietary feed intake was observed when animals were dosed with these elite rumen fungi.

The second stage of the trial was designed to investigate the effect diets may have on recipient animals when being inoculated with rumen contents from a donor animal. Animals were separated into three groups and inoculated with rumen contents from an animal grazing on Rhodes grass pasture. Prior to inoculation the previously fungal dosed

animals were adapted to an Angleton diet (*Dicanthium aristatum*), no significance in DMI was observed at this point between all animals (Table 11). From the fungal dosed group four animals were chosen for dosing with rumen contents while the other four remained as a control. In addition the control group for the first trial that had remained on a Rhodes grass diet were also inoculated with rumen contents from the grazing animal.

Table 11. Effect of rumen dosing on dry matter intake (kg/d)

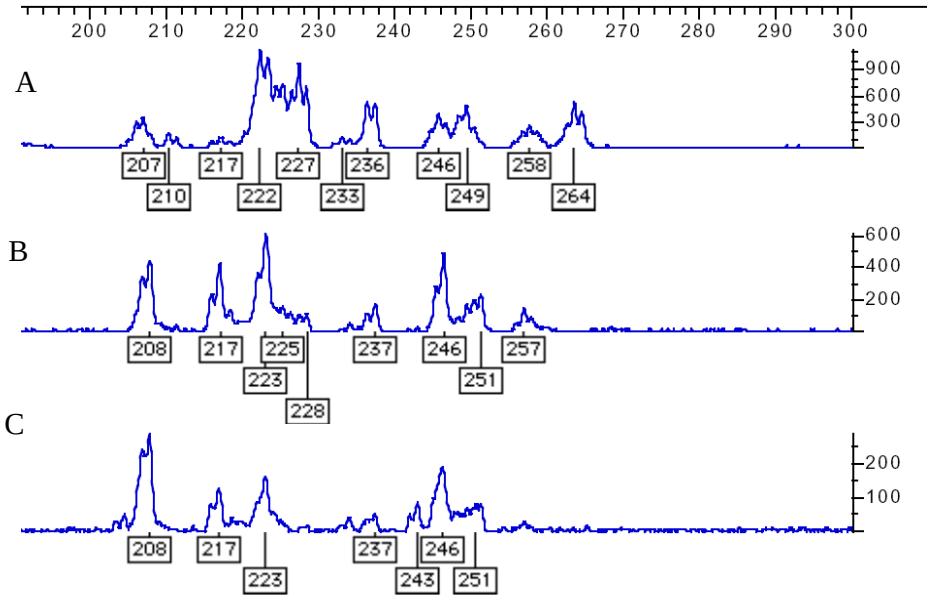
Group	Control	Treatment
A	9.35 ± 0.16[a*]	9.37 ± 0.13[a]
B	9.49 ± 0.10[a]	9.64 ± 0.09[a]
C	9.52 ± 0.16 [a]	8.96 ± 0.14 [b]

** Values in rows with an uncommon superscript are significantly different (P≤ 0.01). Group A: Not previously dosed with elite fungi, dosed with rumen contents, B: previously dosed with elite fungi, not dosed with rumen contents, C: previously dosed with elite fungi, dosed with rumen contents.*

Results indicate that there was no significant change in DMI for the animals that had remained on the Rhodes grass diet and were dosed with rumen contents. However, there was a significant decrease in DMI for those dosed animals which were being fed Angleton grass. There was however no significant change in DMI for the animals that were being fed Angleton grass and not dosed with the Rhodes grass rumen contents. This data would suggest that diet contributes significantly to the colonisation and persistence of microbial populations within the rumen.

During the first stage of the trial ARISA analysis could not be used to track the presence of the introduced fungi within the rumen of the treated animals due to endogenous rumen fungal populations that produced similar peak positions to that of the introduced fungi (Figure 10). Peaks were expected to be observed at specific positions based of fragment size for the five individual fungal strains (F9 and F11 at 223bp, TNL1 at 241bp and TGB1 and TZB1 at 243bp). ARISA could be used within the second stage of the trial to successfully show population changes within the rumen. A Neocallimastix species (peak 264 bp, Figure 10) was consistently present in animals fed a Rhodes grass diet but did not persist in animals on an Angleton diet when dosed with rumen contents containing this species (Figure 10).

Figure 10. ARISA electropheragrams of anaerobic fungal populations from a [A] Rhodes grass diet, [B] Angleton grass diet and [C] Anleton grass diet plus rumen dosing. Scale across the top represents peak migration in base pairs.



7.4 Objective 4

To improve milk production from poor quality feed by use of cost effective fungal treatments (fungal specific nutrient and/or fungal inoculum) in India.

7.4.1 Summary

A major cattle experiment was undertaken in India to address objective 4. Cattle were fed a standard diet used for dairy cattle in India and supplemented with MPS, inorganic S or given no supplement (control). The response in milk production, fat content and rumen fermentation parameters were measured after peak lactation. MPS improved milk production more than the inorganic supplement, which was also higher than the control. The level of significance for the difference was not high, as there were only 6 animals per treatment and the variation within treatments was high. There was no difference in voluntary food intake but N metabolism and VFA were both improved significantly in the MPS animals. There appears to be a real effect on milk production that is most likely due to the improved patterns of rumen fermentation, lower rumen ammonia and increased VFA, but it needs to be examined in animals over an entire lactation and confirmed in a larger experiment to separate effects.

Dairy cattle and buffaloes in India are largely fed on crop residues that are energy deficient. Improving the utilization of crop residues has the potential to improve the nutrient utilization and production in milking animals, particularly under small livestock holding systems. This has been demonstrated and well documented through urea treatment of crop residues which has resulted in increased milk production by 0.5 to 1.0 kg per day (Prasad & Sampath, 1992). The earlier studies using fungal specific nutrients

in growing heifers fed on crop residues has shown positive trends in the rumen fermentation pattern and microbial protein synthesis. In order to confirm the results an experiment was carried out in crossbred milking cattle using fungal specific supplements to see the production response.

Eighteen crossbred milking animals were divided into three groups based on the milk yield from day of calving, days of lactation, parity and daily average milk yield. The dietary treatment consisted of control, treatment (T1) with inorganic sulphur supplement (sodium sulphate @ 6.6g daily per animal) and treatment (T2) with organic sulphur supplement sodium salt of mercaptopropanesulfonate (SMPS, 9.1g daily per animal). The quantity of SMPS was increased from 8.0g in the earlier experiment to 9.1 g based on the sulfur content and the assay of the compound. Three fistulated animals were used to assess the effect of dietary treatment on rumen fermentation by assigning them to each treatment for a period of one month on a switch over design.

The animals were fed on concentrate mixture @ 40-50% of milk yield, limited silage and ad lib chaffed sorghum straw for a period of four months and the daily dry matter intake, milk yield and weekly milk composition were recorded. A digestibility trial was carried out at the end of the feeding trial and the nutrient digestibility was calculated. The rumen fermentation pattern was also studied in fistulated animals.

The details of animals assigned to different treatment groups are presented in table 12. The animals under the experiment were in the post peak lactation stage and the average parity of the groups were 3-4. Further, the total milk yield from the calving date to the beginning of experiment was similar in all the groups. The dry matter intake and nutrient digestibilities did not differ between the groups.

Table 12. Details of animals in different treatment groups

	Parity	Days in milk	Initial Milk yield (kg)	Body weight (kg)	Daily milk yield (kg)
Control	3.7±1.3	77.7±16.9	589.4±192.0	321.2±14.6	7.1±1.0
Na_2SO_4	3.8±1.4	78.2±19.0	585.53±135.3	389.0±33.1	7.9±0.9
SMPS	3.00±1.2	78.0±16.6	582.42±85.0	352.0±9.9	8.8±1.4

The milk yield in sodium sulphate and SMPS supplemented group were higher by 18 and 39% respectively as compared to the control group (Table 12 & 13). Although the milk yield in sulphur supplemented groups showed a positive effect over the control, the increase in the milk yield was not statistically significant. Excluding the extreme values in each group (one animal each) the milk yield was found to be statistically significant ($P<0.06$) between SMPS and control group.

Table 13. Monthly average milk yield (4% FCM) in different groups

	First month	Second month	Third month	Fourth month	Total yield
Control	158.8	126.5	96.7	91.7	473.7
Na2SO4	179.1	148.5	113.4	117.1	558.1
SMPS	221.6	181.2	130.1	124.1	657.0

No significant difference was noted between the groups for the various milk constituents (totals solids, SNF, fat, protein, lactose, ash).

The rumen fermentation pattern did not differ with regard to the pH. The ammonia nitrogen values tended to be lower than the control in sulfur supplemented groups during different hours of collection though it was not statistically significant. However the total volatile fatty acids tended to be significantly ($p<0.05$) higher in SMPS supplemented group as compared to other two groups Table 14.

Table 14. Rumen fermentation pattern

ammonia N (mg/dl)					
	0 hr	**2hr**	**4hr**	**6hr**	**8hr**
control	11.9±1.9	17.8±0.6	23.7±0.9	25.3±0.9	17.4±0.4
Na_2SO_4	10.4±1.6	16.0±2.1	19.6±2.5	21.2±2.5	16.0±2.0
SMPS	9.9±2.3	15.8±2.3	20.6±3.9	21.9±4.8	15.5±2.2

TVFA (meq/dl)					
Control	4.4±0.1[a]	5.1±0.1[a]	6.1± 0.1[a]	6.6 ±0.1[a]	5.5 ±0.1[a]
Na_2SO_4	4.2 ±0.2[a]	4.5 ±0.2[a]	5.2 ±0.2[a]	6.4 ±0.2[a]	5.7 ±0.2[a]
SMPS	6.9 ±0.5[b]	8.1±0.8[b]	8.5 ±0.5[b]	9.5±0.9[b]	7.8 ±0.6[b]

** Values in columns with an uncommon superscript are significantly different ($P \leq 0.01$).*

8 Impacts

8.1 Scientific impacts – now and in 5 years

There have been 4 key scientific outputs from this project that have the potential to contribute to other scientific projects and activities in the collaborating institutes; particularly in India. The 4 main outputs are:

1. The largest and most diverse collection of ruminal fungal isolates from domestic and wild animals in the world, some of which have been characterised at the molecular level.
2. New molecular technology developed for investigating fungal ecology.
3. New insights into nutritional requirements for optimising utilisation of lignocellulose.
4. The demonstration that the 'fungal specific nutrient', at least in cattle, is probably not fungal specific but more general in its positive effects on the ruminal bacteria as a whole.

The collaborating institutes are in a position to use these outputs to pursue a number of different avenues of research. The collection of fungal isolates puts these institutes in a powerful position to screen for novel fungal enzymes for both industrial and agricultural applications. It also creates opportunities for broadening the collection to non-ruminal fungal isolates and a more fundamental comparison of enzymatic capabilities of fungi. Further research in this area may uncover enzymes or processes that are patentable.

Demonstrating that the effects of the 'fungal specific nutrient' are more general than expected based on the earlier work in sheep creates opportunities for the Indian team to investigate the underlying mechanisms behind the action more closely. They now have the skills in molecular biology to complement their existing skills in nutrition to undertake this properly. This information will improve the chances of identifying suitable (alternative) compounds that increase the efficiency of lignocellulose utilisation and are also cost effective. The organic S compound (mercaptol propanesulfonic acid) identified in this project will be investigated further to verify its potential to improve milk production. More generally, this group is in a position to expand the focus of their gastrointestinal microbiology work and could quite feasibly contribute to scientific projects related to gut health in any livestock species and humans.

In terms of future directions, a project proposal was submitted to the Department of Environment, Science and training (DEST) under the Australia-India Science and Technology Program to undertake collaborative research with two Indian institutes (NIANP and the Indian Veterinary Research Institute, IVRI) into the metagenomics of rumen microorganisms which could reduce methane emissions. A second collaboration has also developed between CSIRO and IVRI through a collaborative project with the International Atomic Energy Agency (Animal Division) on reducing methane emissions from ruminants with the use of molecular microbial ecology analysis. Expertise in rumen microbial ecology established through this project has provided the foundation for a project recently awarded to NIANP by the World Bank to reduce methane emissions from ruminants.

8.2 Capacity impacts – now and in 5 years

A major impact of this project has been on capacity building. The laboratory and team in India had no facilities or expertise to undertake rumen microbiology or molecular microbial ecology. The group had a strong background in traditional nutrition and expertise in the techniques for nutritional analyses, but they were not skilled in microbiology or molecular

biology. They now have a fully equipped (anaerobic hoods, real time PCR machine, electrophoresis equipment) and functional laboratory for molecular microbial ecology studies of, but not limited to, anaerobic microbes in gastrointestinal systems. There are now four research scientists who have all been well trained in anaerobic microbiology and molecular biology. A tour of the laboratories at the time of the review of the project made it clear that the laboratory is fully operational and that the research scientists are very competent and confident in their new area of expertise. They are managing and supervising 5 research associates working in the new laboratory.

In recognition of their capacity in this area, the NIANP has been nominated by ICAR as the central fungal culture collection/database in India; an equivalent centre for the rumen bacterial collection is situated in the north of India. As a direct result of the work undertaken in this project, the project team at NIANP probably has the most diverse ruminal fungal collection in the world. The team in Australia has increased their capacity to monitor and identify fungal populations in the rumen through the development of a molecular-based method for tracking fungi (ITSR and ARISA) and the accumulation of a database of molecular information about a wide variety of ruminal fungal isolates.

With the establishment of a high quality laboratory with very competent research scientists at the NIANP, it is entirely feasible for the collaborating institutes to continue their collaboration. Indeed, they have already developed a research plan for work they intend to continue in collaboration with Australia. Having been nominated as the Fungal Database Centre for India, the team in India is in a good position to attract funding for ongoing research in this area. In addition the NIANP has just been awarded a research project on the microbiology of rumen archaea as a basis for reducing methane emissions from ruminants. Therefore this group is demonstrating an independence and competence to apply the skills developed in the ACIAR project to other areas of livestock science.

8.3 Community impacts – now and in 5 years

The project activities were primarily focussed on basic science and capacity building so that new strategies in ruminant nutrition could be developed for the future. As such it is premature to comment on impacts at the community, farmer or policy level.

8.3.1 Economic impacts

Not applicable

8.3.2 Social impacts

Not applicable

8.3.3 Environmental impacts

Not applicable

8.4 Communication and dissemination activities

The primary mechanism for communication of the project outcomes was via the scientific literature (list of publications from the project is presented in section 10.1) and presentations at the following scientific meetings

- MIE Bioforum 2003 ‘Biotechnology of Lignocellulose Degradation and Biomass Utilisation.
- The Australian Society of Animal Production - "What's New In Animal Science?"
- Proceedings 10th meeting of International Society for Microbial Ecology, Microbial planet: sub-surface to space.

- Proceedings 5th Animal Nutrition Association Biennial Conference, NIANP Bangalore, Izatnagar, India, eds Sharma, K., Pattanaik, A.K., Narayan Dutta and Das, A. pp135-143.
- Proceedings 5th Joint Symposium of Japan-Korea-China Rumen Metabolism and Physiology. Improvement of rumen function for efficient animal production', Huhhot, China.
- The British Society of Animal Science and the Animal Husbandry Association of Thailand, Khon Kaen, Thailand
- XII Animal Nutrition Conference held at Anand, Gujarat from 7-9th January 2006.
- Proceedings of VIth Biennial Conference of Animal Nutrition Association on "Strengthening Animal Nutrition Research for Food security, Environment Protection and Poverty alleviation" held at Sher- E- Kashmir University of Agricultural Sciences and Technology, Jammu
- Proceedings of International Tropical Animal Nutrition Conference (Tropnutricon – 2007) held at National Dairy Research Institute, Karnal , 2007.
- Proceeding ANA world Conference held at NASC complex, New Delhi, 13-17 February, 2009.

9 Conclusions and recommendations

9.1 Conclusions

NIANP is very encouraged by the milk production responses to supplementation with MPS as this could prove to be a cost-effective strategy to optimise the use of lignocellulosic feedstuffs in dairy animals. Further animal experiments are therefore planned to determine the levels of MPS supplementation required to optimise microbial fermentation and protein synthesis in the rumen and to quantify milk production responses over the course of an entire lactation. These experiments will provide the definitive evidence to drive uptake of this new supplementation technology at the farmer level. The research will also be designed to determine the microbial mechanisms underpinning the responses to MPS supplementation, thus providing new insights in nutritional physiology of sulphur utilisation in the rumen and provide the basis for establishing the S requirements for lactation and growth in ruminants fed tropical roughage diets. This work has the potential to improve productivity from lignocellulosic forages while reducing the dependency on expensive protein supplements and alternative energy dense concentrates.

NIANP requested continued support from ACIAR to undertake these studies in collaboration with the project group from CSIRO. While NIANP is committed to providing the resources for these studies, they have identified that continued involvement of CSIRO in the research could best be achieved through the support of ACIAR. CSIRO has provided further advice, exchange of scientific personnel and training in the molecular microbiology planned for these studies that is invaluable in extending the capability of NIANP in this area. ACIAR support enabled the exchange of scientific staff between the institutions for further training in specific techniques planned for these experiments.

This ongoing interest and commitment by NIANP to further research in this area will provide the greatest opportunity for uptake of the technology in India.

9.2 Recommendations

The following 4 recommendations were made as part of the final review of the project. All these recommendations have been acted upon as suggested. Specifically the fungal collection has been extended and further characterised at the molecular level with the intention of using cellulolytic enzymes as feed additives. Work on MPS has been extended by conducting another lactation trial but the outcome of this project was negative probably because the basal feed use was not low enough in sulfur content. In conclusion, supplementation of a S deficient low quality roughage diet with organic S compounds improved rumen function through an increase microbial protein synthesis compared with inorganic S. Thus, the metabolism of organic S in the rumen needs to be investigated further because it may prove to be more effective in stimulating fermentation of roughage diets. Furthermore, the cost and availability of organic S compounds that could be used as animal supplements needs to be addressed before these additives would be adopted by farmers. Inexpensive industrial production of these compounds in India may be possible.

Recommendation 1: The collection and storage of fungal isolates should be ongoing, but the focus of the resource should be for industrial applications of fungal enzymes not for improved inocula (see objective 3) for improving lignocellulose utilisation in ruminants. The molecular characterisation of the collection should be completed to enable a better description of the molecular ecology of fungal populations in the rumen and to answer questions of persistence, species differences and dietary responses.

Recommendation 2: Focus work on developing MPS (or alternative compound) into cost effective supplement for improving productivity. The mechanism behind the improvements

in rumen fermentation in cattle fed these compounds needs to be addressed in an experiment that targets nitrogen and sulfur metabolism in the rumen.

Recommendation 3: discontinue the search for new fungal strains specifically for use as oral inoculums to improve ruminant productivity, but continue the collection for industrial uses (enzymology) and the molecular characterisation of the isolates in the collection to help improve our ability to study fungal ecology in the rumen.

Recommendation 4: Perform an experiment using cattle at the start of their lactation curve (rather than the peak) to get an accurate estimate of the size of the total response in milk production (using more animals per treatment). Collect production data as well as rumen samples for measuring microbial profiles and rumen parameters (including N and S metabolism) to identify the underlying mechanism responsible for the effect.

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