finalreport

Project code: **FLOT.125**

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Date published: **June 2006**

ISBN: **9781741917727**

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Rumen inoculum for the efficient use of high grain diets

Abstract

This project identified and isolated starch utilising bacteria present in cattle efficiently utilising grain for inclusion in a probiotic drench, together with the lactic acid utiliser *Megasphaera elsdenii* YE34, to improve the efficiency of grain utilisation and reduce lactic acidosis in lotfed cattle. Using molecular techniques, *Ruminococcus bromii* was identified as a dominant starch utiliser and subsequently isolated. Inclusion of these bacteria in a probiotic drench was evaluated in a feedlot trial with eighty steers. The performance of the steers in the trial was commensurate with high producing commercial feedlot cattle. However, there was little difference between inoculated and Control steers largely due to the rapid acquisition of *M. elsdenii* by the Control animals. It is recommended that the natural rate of increase of *M. elsdenii* in commercial feedlot cattle be determined prior to further development.

Executive Summary

When cattle are fed grain acidotic ruminal conditions and decreased efficiency in starch utilisation can result from the rapid production and accumulation of lactic acid in the rumen. These conditions depress feed intake and the growth rate of young cattle can be constrained by limited protein availability (through decreased microbial protein synthesis). In more extreme cases clinical acidosis may occur, with symptoms such as laminitis, from the rapid production and accumulation of lactic acid in the rumen of grain-fed cattle.

We have investigated the use of probiotic bacteria to reduce acidosis and improve the efficiency of ruminal starch utilisation in lotfed cattle. From earlier work it was identified that *Megasphaera elsdenii* YE34 was an efficient lactic acid utiliser that rapidly established dense populations in the rumen and appeared to be a major and important species in grainfed cattle. Probiotically introduced *M. elsdenii* established a dense and stable population 5 to 7 days earlier than in untreated Control steers. Unfortunately, a superior starch utilising bacterium that would efficiently utilise starch without producing lactic acid was not identified during this early work but would be advantageous in developing a live microbial inoculant to increase the efficiency of ruminal starch utilisation.

The purpose of this project was to identify and isolate the major starch utilising species of bacteria that, in CRC project 2.5.4, were present in the rumen of cattle efficiently utilising grain without signs of acidosis. A probiotic drench using the starch utilising bacteria and *M. elsdenii* YE34, the lactic acid utiliser, was then used to determine whether this would improve the efficiency of grain utilisation and reduce the incidence of lactic acidosis in lotfed cattle.

Specific objectives were to:

1) Examine samples from the CRC pen-trial and identify the dominant starch utilising species of bacteria;

2) Isolate and characterise these species for use in a probiotic drench containing a combination of lactic acid and starch utilising bacteria; and

3) Evaluate in a field trial the benefits from using the probiotic drench in terms of feed intake, liveweight gain and incidence of acidosis.

Samples from the CRC pen trial were examined by denaturing gradient gel electrophoresis (DGGE) and dominant DNA bands from steers fed 75% barley were selected. DNA sequenced from these bands identified *Ruminococcus bromii* as the predominant bacterium present. Four strains of this bacterium were subsequently isolated and characterised. A Real-Time PCR assay was developed to enumerate *R. bromii* and to confirm that in the CRC trial this bacterial species rapidly developed high density populations in all the steers soon after the introduction of grain into the diet. *R. bromii* YE282 was selected for inclusion in a probiotic drench along with *M. elsdenii* YE34.

A feedlot trial was undertaken at the Queensland Department of Primary Industries & Fisheries Brigalow Research Station. The trial utilised 80 steers in 10 pens in a randomised complete block design. An empty-pen-buffer was maintained between treated and Control groups to avoid transfer of inoculant bacteria to Controls. Following allocation into treatment groups all Control replicates were moved to pens. Treatment replicates were then inoculated with *M. elsdenii* YE34 and *R. bromii* YE282 before being moved to pens. These cattle each received 100 ml of each bacterial species by oral inoculation using standard drenching equipment. The cattle were fed for a total of 70 days with commercial, barley-based, feedlot rations.

High growth rates (1.91 kg/d) were achieved throughout the experiment in both the inoculated and Control steers. Liveweight changes generally reflected similar intake and growth rates in inoculated

and Control cattle. With both liveweight change and intake there was less variability between inoculated steers as compared to Control steers. There were no marked differences between treatments in carcass weight, dressing percentage or P8 fat cover. The general lack of differences between the inoculated cattle and Control cattle and the fact that all performed very well is probably due to the rapid spread of one of the inoculant bacteria and the transient dominance of the other. The Control group acquired dense populations of *M. elsdenii* much earlier in the trial than expected or could be anticipated on current knowledge. This meant that the Controls, in terms of the presence of this important bacterium, were similar to the inoculated group before day 14 (and in some steers by day 3). This effectively reduced the period of time over which differences between the groups could have developed. With hindsight, the experimental design did not take into account the possibility of aerosol spread despite considerable effort being made to ensure no direct physical contact between inoculated and Control steers occurred. This highlighted the importance of experimental design where live bacterial inoculants are used and the importance of isolating the Control group from inoculated animals. The presence of high density *M. elsdenii* populations appears to correlate with a stable, even reduced, *Streptococcus bovis* (implicated in producing acidosis) population, higher pH and markedly reduced lactic acid accumulation.

R. bromii was only transiently dominant in the rumen ecosystem. Populations established at high densities within the first two weeks but then declined and were undetectable by 50 days. Coincidentally, the cattle in the CRC trial from which *R. bromii* was identified as the dominant starch utiliser had been on grain for just 14 days. *R. bromii* appears to be replaced as the dominant bacterial species by *Ruminobacter* spp. These species became dominant between 14 and 28 days on grain, in all the steers examined, and persisted as dominant species through to the end of the trial. The role of *R. bromii* in adaptation of the rumen ecosystem to a grain diet is unclear.

The performance of the steers in the trial was commensurate with normal to high producing commercial feedlot cattle. They showed few signs of acidosis (none in inoculated steers). Inoculation with *M. elsdenii* YE34 appears to be a viable alternative to rumen modifiers such as monensin for Controlling acidosis.

We recommend further research to confirm and clarify results, particularly:

- Independently determine the natural rate of increase of *M. elsdenii* in commercial feedlot cattle.
- Repeat the experiment with an improved experimental design to re-determine the possible benefits of *M. elsdenii* inoculation.
- Confirm the uniformity in growth performance of the steers inoculated with *M. elsdenii*.

The longer term benefits from the use of the technology developed in this project are likely to include a reduction in the incidence of acidosis, faster introduction to a grain based diet and improved efficiency of starch utilisation. Although it requires confirmation, a more uniform response to grain feeding resulting in a greater percentage of the mob reaching market specifications within a given time frame may also result.

An additional impact may arise from the ability to safely feed grain to cattle in the field, which will enable opportunity benefits to be gained by a larger section of the industry when cheap grain is available and not impinge on the welfare of these cattle while doing so.

Chemical rumen modifiers may have a limited lifespan through lack of public acceptability, their use as trade barriers and the increase in microbial resistance to their action. Live microbial inoculants may well be able to replace these modifiers and deliver similar benefits more cheaply and be more acceptable in the longer term.

Contents

1 Background

Acidotic ruminal conditions and decreased efficiency in starch utilisation are associated with the rapid production and accumulation of lactic acid in the rumen of grainfed cattle. High concentrations of lactate are found in the rumen of cattle and feed intake is highly variable, particularly during the feedlot introductory period. Specifically:

- When cattle are fed grain the pH of the rumen contents often falls to very low levels decreasing the efficiency with which feed is converted to volatile fatty acids (VFAs) and microbial protein for animal production.
- Feed intake can be depressed when the rumen pH falls below 5.5.
- Microbial protein synthesis decreases as pH declines, and low rumen pH during feedlot induction could result in a critical reduction in microbial protein available for intestinal digestion.
- The growth rate of young inducted cattle is likely to be constrained by limited availability of protein in the intestine.
- In more extreme cases of acidosis, other symptoms such as laminitis may result from the rapid production and accumulation of lactic acid in the rumen of grain-fed cattle.

Within the Cooperative Research Centre for the Cattle and Beef Industry (Meat Quality) the use of probiotic bacteria to reduce acidosis and improve the efficiency of ruminal starch utilisation in lotfed cattle was investigated.

Lactic acid-utilising and competitive starch-utilising bacteria were selected from the rumens of cattle adapted to a high grain diet and inoculated into the rumen of cattle being introduced to a diet of 75% rolled barley.

The major outcomes from this work have been published (Klieve *et al.* 1999; Ouwerkerk *et al.* 2002; Klieve *et al.* 2003). In summary:

- *Megasphaera elsdenii* YE34 (lactic acid utiliser) when used probiotically, rapidly established dense populations in the rumen and appeared to be a major and important species in grainfed cattle. Probiotically introduced *M. elsdenii* established a dense and stable population 5 to 7 days earlier than in untreated Control animals.
- *Streptococcus bovis* did not appear to be a significant starch utilising bacterium under grain feeding conditions.
- *Butyrivibrio fibrisolvens* (the probiotically introduced starch degrader) did not appear to be a significant starch utilising bacterium *in vivo* as it was rapidly eliminated from the ecosystem shortly after grain feeding began.

Unfortunately, a superior starch utilising bacterium that would efficiently utilise starch without producing lactic acid was not selected by growth in pure culture. A major question that arose from this work was – what were the major starch utilising bacteria in these cattle that were efficiently utilising grain without signs of acidosis? The bacteria that had been assumed to be performing this function were found not to be. The identification and isolation of the major starch-digesting microbes operating in these animals would be of considerable value for inclusion in an inoculum for grainfed cattle, together with the lactate-degrading bacterium *M. elsdenii*.

2 Project Objectives

The purpose of the project was to identify and isolate the major starch utilising species of bacteria that were present in the rumen of cattle efficiently utilising grain without signs of acidosis in CRC project 2.5.4. A probiotic drench using these starch utilising bacteria and *M. elsdenii* YE34 was then formulated and was used to demonstrate whether this would improve the efficiency of grain utilisation in the period following feedlot induction and reduce the incidence of lactic acidosis.

Specifically we:

- Examined samples from the CRC pen-trial and identified the dominant starch utilising species of bacteria.
- Isolated and characterised these species for a probiotic drench containing a combination of lactic acid and starch utilising bacteria.
- Evaluated in a field trial the benefits from using the probiotic drench in terms of feed intake, liveweight gain, days to turnoff, feeding costs and incidence of acidosis.

3 Methodology

3.1 Identification and isolation of a dominant starch utilising bacterium

3.1.1 Samples and their origin

Samples of rumen contents were collected in the CRC for the Cattle and Beef Industry (Meat Quality) Project 2.5.4. Briefly, a pen-trial had been undertaken using 10 rumen cannulated steers to determine whether bacterial cultures of *Megasphaera elsdenii* YE34 and *Butyrivibrio fibrisolvens* YE44 would establish in cattle rapidly introduced to a high grain diet (75% rolled barley). *M. elsdenii* established extremely well but *B. fibrisolvens* did not (for full details refer to CRC final report and Klieve *et al.,* 2003). The animals all adjusted rapidly to the diet without adverse reactions indicating that populations of bacteria capable of rapidly and efficiently utilising starch were present in these animals. Rumen fluid samples were collected and stored for analysis of microbial populations throughout the experimental period. These samples (from 8 of the animals) were the basis for the current work.

3.1.2 DNA extraction, PCR amplification and denaturing gradient gel electrophoresis

DNA was extracted from samples by physical disruption using a bead beater as described by Whitford *et al.* (1998).

The hypervariable V2V3 region of the bacterial 16S rRNA gene was amplified from all of the bacteria in each sample. The V2V3 region of the 16S gene of the rumen samples was amplified using primers 341F-GC (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG –3') and 534R (5'- ATT ACC GCG GCT GCT GG –3') (Muyzer *et al.,* 1993).

Denaturing gradient gel electrophoresis (DGGE) was used to separate the PCR products into a profile of the diversity of bacteria present in the sample. DGGE separates DNA fragments on sequence differences as they travel through an increasing gradient of denaturants (urea and formamide) present in the gel matrix. AT bonds in DNA denature at a lower denaturant concentration then GC bonds therefore DNA fragments with a high ratio of AT to GC bonds will appear at the top of the gel while sequences with a low AT to GC ratio will appear at the bottom of the gel. The PCR

products were loaded onto a DGGE gel with a 30 to 60% denaturant range. A V2V3 ladder was also loaded onto the gel to allow comparison between denaturing gradient gels. The gel was then electrophoresed at 100 volts for 18 hours in 0.5X TAE (Tris, acetic acid, EDTA) buffer, at 60 ^{o}C.

Following electrophoresis the gel was silver stained to visualise the DNA. This results in the entire rumen bacterial population in a given sample being profiled as a ladder of light and dark bands in a lane on the gel. In most cases, a single band represents a single species and the more intense the band the greater the density of that specific bacterial population.

3.1.3 Identification of dominant bacterial species

The DNA from selected dominant bands was isolated and sequenced to identify the bacterial species. A number of criteria were used to select the dominant starch utilising bacteria:

- The intensity of the band on the gel. The cattle had been feeding on grain for at least 9 but mostly 14 days.
- The presence of the band in multiple animals.

The DNA sequence from selected bands was determined and used to identify the bacterial species represented by that band. The basic process involved obtaining a sample of the DNA by stabbing through these bands with a needle (or pipette tip). The V2V3 region was then re-amplified by PCR and a clone library of the PCR products produced in *E. coli* cells (TA Cloning Kit (Invitrogen, San Diego, CA, USA)). Approximately 20 clones for each band that had been stabbed were selected, plasmid DNA containing a copy of the original product was extracted from the clones, the V2V3 region amplified and run on a DGGE gel alongside the DNA banding profile from the animal to confirm the clones contained the correct insert, i.e. the V2V3 PCR product migrated to the same position as the band that the stab was taken from. Five clones containing the correct insert had their V2V3 region sequenced (Griffith University sequencing facility) and three identical sequences from different clones was deemed sufficient for assigning the sequence to a specific band. The sequence was subsequently compared with others in the GenBank database to determine species identity and phylogenetic position (Ouwerkerk and Klieve 2001).

3.1.4 Isolation of the dominant starch utilising bacterium

From stored samples of rumen contents from the grain-fed cattle, standard anaerobic microbiological methods (Hungate 1969; Holdeman *et al.* 1977; Ogimoto and Imai 1981) were used to isolate the species identified above. A variety of anaerobic media (rumen-fluid based medium supplemented with either wheat starch, barley, maltose or cellobiose and glucose) was used in roll tubes, agar plates and broths. Once isolated the bacteria were confirmed by PCR amplification of the V2V3 region of the 16S rRNA gene and comparing this, on a DGGE gel, with the position of the band in the original bacterial profile from rumen contents. Bacteria were characterised on morphology, growth habit, colony morphology, 16S rRNA gene sequence and phylogeny (Ouwerkerk and Klieve, 2001). The bacteria were then stored for future use as a probiotic inoculant along with *Megasphaera elsdenii* YE34.

3.1.5 Real-time PCR detection of *Ruminococcus bromii*

To confirm the dominance of *Ruminococcus bromii* YE282 in the grainfed cattle from the CRC trial, a Real-time PCR assay was designed and calibrated in the same manner as previously reported for *M. elsdenii* YE34 and others (Ouwerkerk *et al.* 2002; Klieve *et al.* 2003)

3.2 Pen trial – inoculation of feedlotted steers with *M. elsdenii* and *R. bromii*

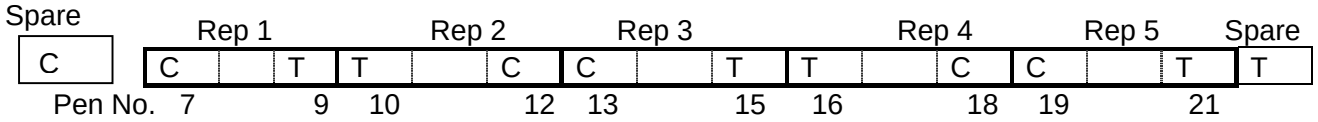
3.2.1 Site and climatic conditions

A feedlot growth study over 70 days was undertaken at DPI&F's Brigalow Research Station, Theodore, Qld (latitude 24°50'S; longitude 149°48'E) between February and May 2006. The climatic conditions over this period were: maximum temperature, average 30.6°C (range 20.2 - 36.5°C); minimum temperature, average 16.5°C (range 4.8 - 24.5°C); relative humidity, average 58.0% (range 17.0 - 100.0%); and temperature humidity index, average 71.1 (range 56.3 - 80.2). Rainfall over the trial period was 80.5 mm, most of which fell on two successive wet days in early April.

3.2.2 Animals and experimental design

The experiment was approved by the DPI&F's Staff Access Animal Ethics Committee (approval number SA 2005/09/49). Two groups of steers were sourced from central Queensland saleyards (i.e. Emerald and Gracemere). The group sourced from Gracemere comprised high-grade (>75%) *Bos indicus* steers; the other steers were Droughtmaster and Droughtmaster x Brahman crossbreds (*ca*. 50% *B. indicus*). The estimated age of both groups was 12 months, with none of the steers having any permanent teeth, and the initial liveweight was 347.1 ± 31.7 (± s.d.) kg. The steers arrived at the property 4 days prior to induction. Upon arrival, steers were branded, vaccinated with 2 ml Ultravac™ 5in1 Vaccine (Pfizer Animal Health), implanted with Compudose®100 (Elanco®) hormonal growth promotant and some steers were dehorned prior to returning to the paddock.

The study utilised 80 steers in 10 pens in a randomised complete block design with five replicates of two treatments (Control and Inoculated). Steers were randomly stratified by property of origin, phenotype description within one property group, and unfasted live weight at induction. Within each pen, three steers from one property of origin from the same blocks were selected at the beginning as rumen sampling animals. Treatment groups were assigned to pens as indicated in the diagram below (C – Control; T – treated) such that an empty-pen-buffer was maintained between treated and Control groups to avoid transfer of inoculant bacteria to Controls.



3.2.3 Induction, inoculation and feeding

Following allocation into treatment groups all Control replicates were moved to pens. Treatment replicates were then inoculated with *Megasphaera elsdenii* YE34 and *Ruminococcus bromii* YE282 before being moved to pens. These cattle each received 100 ml of each bacterial species by oral inoculation using standard drenching equipment.

The cattle were fed for a total of 70 days with the following commercial feedlot rations:

Table 1. Proportional composition and chemical composition of the three rations fed

	Starter (1 – 6 d)	Intermediate (7 – 12 d)	Finisher
Ration composition (g/kg total, as fed)			
Barley	500	610	705
Sorghum silage	60	90	90
Wheat straw	60	60	20
Lucerne hay	100	-	-
Cottonseed – high lint	70	80	90
Molasses	150	80	30
Copra meal	35	35	-
Vegetable oil	-	10	20
Pre-mix	25	35	45
Chemical composition (DM basis)			
OM (g/kg)	939	946	951
CP (g/kg)	139	141	144
Fat (g/kg)	33	48	67
CF (g/kg)	111	94	90
Ca (g/kg)	5.9	5.7	6.0
P (g/kg)	2.6	2.7	3.0
ME (MJ/kg) [A]	12.4	12.9	13.4

[A] Estimated from NRC (1996).

Rations were prepared in a horizontal feed mixer wagon. The barley was coarse rolled so there were no whole grains present and the straw was processed to approximate 50 to 80 mm lengths. The barley contained (/kg DM) 979 g OM, 128 g CP, 22 g fat, 0.5 g Ca and 2.5 g P and had an estimated ME concentration of 13.5 MJ/kg DM. Monensin was not included in the rations. The steers were fed once daily around noon using a "clean bunk at midday" management system (Lawrence 1998). Daily feed allocation was determined based on the amount of feed remaining just prior to feeding but once weekly any residual feed was removed, weighed, sub-sampled for DM content, and returned to the bunk. Feed that became wet was removed, weighed, sub-sampled for DM content and discarded. Samples of the ration fed were taken daily, refrigerated (4^{o}C), bulked weekly, and 2 sub-samples were taken, one for DM determination and the other, which was stored frozen, for chemical analysis. All samples for DM determination were dried at 100^{o}C for 24 h. At the end of the experiment, one bulked sub-sample of the starter and intermediate feeds, and two of the final ration (weeks 3-6, 7-10) were mixed, dried at 60^{o}C and ground to pass through a 1 mm screen prior to analysis.

3.2.4 Sampling and data collection

The health status of animals was checked via a pen walk each morning and any symptoms of acidosis, e.g. lameness, were recorded.

The steers were weighed at the beginning (day 1) and then at days 14, 28, 50 and 70. Hip height and body condition score (US score; (Herd and Sprott 1996)) were recorded at each weighing. Rumen sampling of the selected steers (three steers per pen) was carried out on days 3, 14, 28 and 50. Rumen fluid was collected *per os* using a stomach tube and vacuum pump, as previously reported (Klieve *et al.* 1998), for pH determination and microbial analysis. Rumen fluid (50 - 100 ml)

was collected, strained through nylon gauze into an open-mouthed jar and the pH recorded. From each sample four 1 ml aliquots were placed in 1.5 ml eppendorf tubes and centrifuged at 15,000 rpm for 15 mins. The supernatant fluid was discarded and pellets frozen on dry ice prior to transport and storage at -20^{o}C. A 4 ml aliquot was placed into a container with mercuric chloride (to prevent further fermentation) for determination of lactic acid content and a further 4 ml was placed into 16 ml of formal saline for enumeration of protozoal populations, as previously reported (Klieve *et al.* 1998). The samples for lactic acid determination were frozen on dry ice prior to transport and storage at -20^{o}C.

The steers were slaughtered at a commercial abattoir and data were collected on carcass weight and fat cover at the P8 site.

3.2.5 Sample analysis

Samples to determine lactic acid concentration and to enumerate protozoa were analysed as previously reported by Ouwerkerk and Klieve (2001) and Klieve *et al.* (1998) respectively.

Real-time PCR assays to enumerate populations of *M. elsdenii* and *S. bovis* were those reported by Klieve *et al.* (2003), and for *R. bromii* as reported above (section 3.1.5). PCR, DGGE, band excision, DNA sequencing and sequence analysis were as reported in sections 3.1.2 and 3.1.3 above.

3.2.6 Statistical analysis

Live animal data (weight, body condition score (US) and hip height) and carcase data (carcase weight, dressing percentage and P8 fat) were analysed by standard analysis of variance (ANOVA) with pen as the experimental unit and animal as a sampling term. Further, live weight growth profiles were examined by repeated measures analysis using the method of residual maximum likelihood (REML) and modelling the variance-covariance matrix.

Average pen feed intakes per head per day were calculated for selected periods. Feed conversion efficiency (FCE) over the selected periods was calculated for each pen as the ratio of average DMI per head per day and the average live weight gain per head per day over the respective periods. As feed intakes were collected on a pen basis, average feed intake ('as-fed' and DMI) and FCE over the selected feedlot periods were analysed by ANOVA excluding the sampling term. Weekly DMI profiles were also assessed by repeated measures analysis using REML and modelling the variance-covariance matrix.

Ruminal pH data were calculated from $[H^+]$ ions by

$$pH = \log_{10}(1/[H^+])$$

and pH values on days 3, 14, 28 and 50 were analysed by ANOVA with pen as the experimental unit and animal as a sampling term. Two obvious data values were excluded (Animal 3673 on Day 14 (7.9) and Animal 3654 on Day 50 (5.5)). The treatment profiles were further assessed by repeated measures analysis using REML and an unstructured variance-covariance matrix.

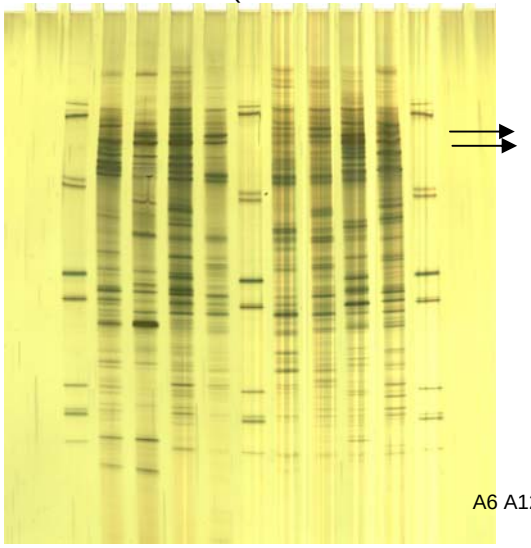
4 Results and Discussion

4.1 Identification and isolation of a dominant starch utilising bacterium

4.1.1 DNA extraction, PCR amplification and denaturing gradient gel electrophoresis

The CRC trial was run for a total of 21 days with grain being introduced into the diet from day 7 onwards. The CRC trial DGGE results are presented in appendix 1. Steers numbered 6, 12, 14 and 18 were uninoculated Controls. Steers numbered 1, 4, 13 and 16 were the probiotically treated animals. Bands arrowed were those that have been selected for DNA excision, sequencing and identification to bacterial species level.

As an example of these results, the DGGE gel of samples after 9 days on grain in the CRC trial is shown below. Many of the dominant species (bright bands) were present in many steers simultaneously suggesting that the dominant bacteria were important in the efficient utilisation of a starch-based diet (in this case 75% rolled barley).



4.1.2 Identification of dominant bacterial species

A summary of the bands selected and stabbed from DGGE gels are presented in Figure 1.

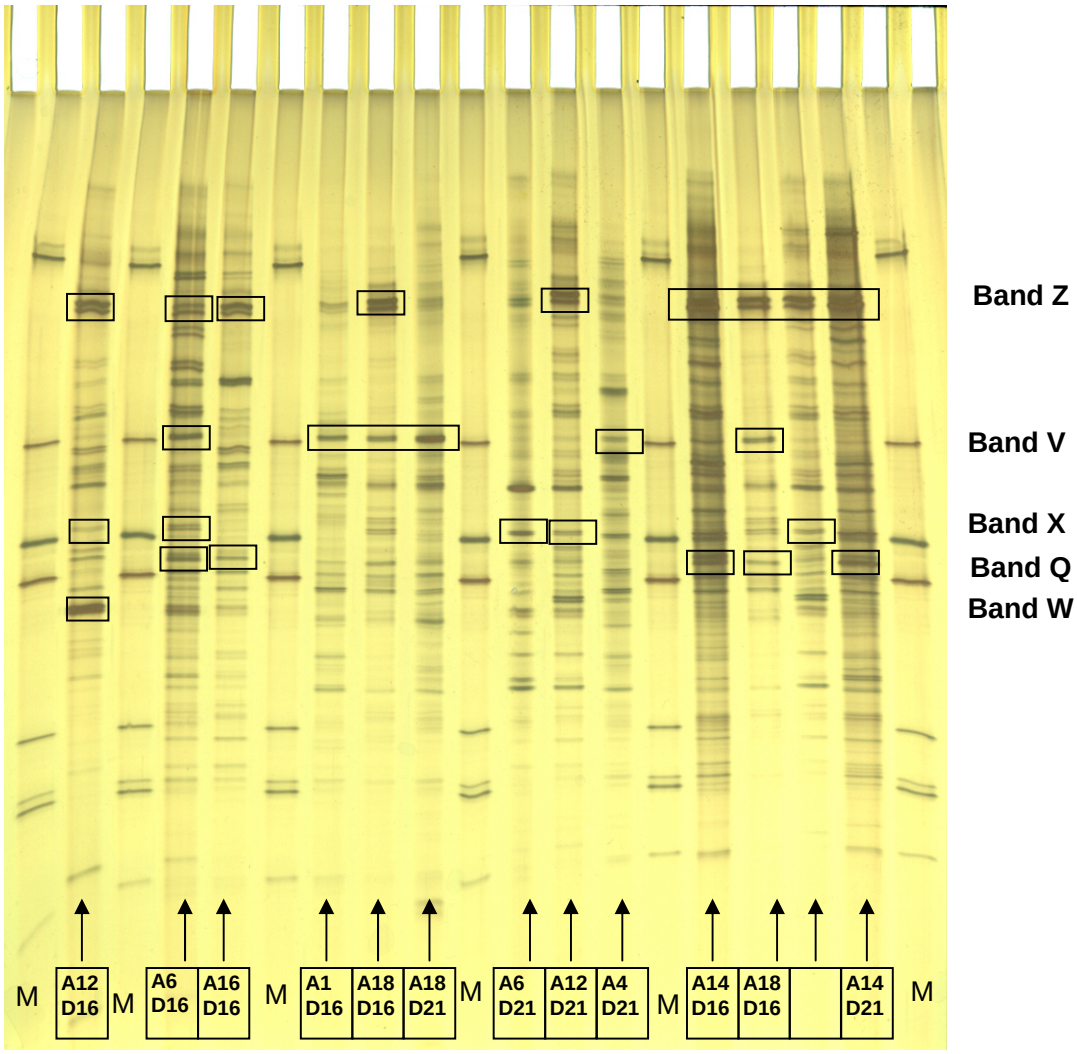


Figure 1. Dominant DNA bands selected for stabbing and DNA sequencing. M – Marker lanes for reference. A – Animal number; D – Day sample taken.

As can be seen band Z, in particular, was very distinct (bacteria present at high density) and present in many animals fed grain for 9 and 14 days. The identities of the bacteria represented by the bands selected in Figure 1, as determined from the DNA sequence of the V2V3 region of the 16S rRNA gene, is presented in Table 2.

Table 2. Nearest match (% similarity) to known bacteria in the GenBank database of bacteria representing dominant bands in DGGE gels.

Band	No. of Animals	No. of Clones sequenced	Closest match by BLAST Identification (% similarity of V2V3 region of 16S rRNA gene)
Z	4	20	*Ruminococcus bromii* (99%)
V	3	15	*Prevotella aff. ruminicola* (96%)
X	2	10	*Prevotella ruminicola* strain 223 (97%)
Q	2	10	*Prevotella dentalis* (94%)
			Anaerovibrio lipolytica DSM 3074(T) (92%)
			Prevotella bryantii (93%)
W	1	5	Butyrate-producing bacterium SM4 (97%)

From DNA sequence comparisons it was apparent that the bacterial species that was extremely dominant and represented by band Z was *Ruminococcus bromii*, and that another dominating group of bacteria were *Prevotella* spp. The latter were present in a number of different dominant bands and were likely to be different species and strains in the different animals and at different times. Also evident from the work was the fact that some species of bacteria produced more than a single band on DGGE gels, e.g. *R. bromii* always produced a close doublet of bright bands, whereas not all single bands represented a single species, e.g. band Q represented three species of bacteria.

From this work it was evident that *R. bromii* was the primary target to isolate for inclusion in the probiotic product with *M. elsdenii.*

4.1.3 Isolation of the dominant starch utilising bacterium

A large number of colonies were selected and screened on cell morphology, 16S rRNA gene restriction digestion pattern and DNA sequence of the 16S rRNA gene (including the V2V3 region) to determine whether they were the correct bacteria. Four bacteria isolated on maltose supplemented medium were cocci with 16S rRNA sequence relating them closely to *R. bromii*. These isolates were designated *R. bromii* YE281, YE282, YE283 and YE284. Isolate YE281 grew as single cells and diplococci and was morphologically distinct from the remaining three strains which grew in extremely long chains (100's of cells in a single chain). The latter strains appeared identical to each other following digestion of the 16S rRNA gene by endonucleases and DNA sequencing, while YE281 was related but distinct from the other isolates. The sequence of the V2V3 region of YE282 was identical (100% match) to the sequence of Band Z from the DGGE results. Further confirmation that isolates YE282 – 284 were responsible for Band Z were obtained by running a DGGE gel with the V2V3 region of the isolates alongside the profiles from the cattle. The results are presented in Figure 2.

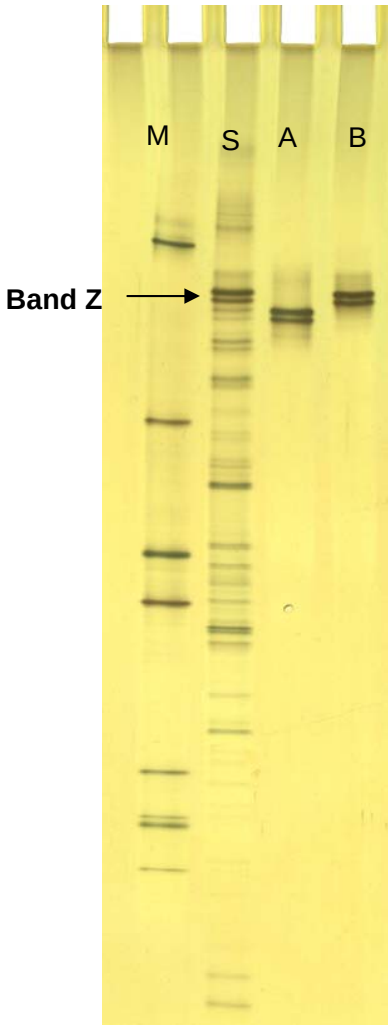


Figure 2. Comparison of migration of V2V3 DNA from a steer (S) showing the dominant band Z and the V2V3 DNA from R. bromii *YE281 (A) and YE282 (B). M – reference marker.*

The V2V3 band from YE281 was present in the steers rumen bacterial profile but it did not appear to be a dominant population and was not developed further. The V2V3 band from YE282 migrated to the same position as band Z confirming that the YE282 group of isolates comprised this dominant band in the profile and was worthy of continued development for use in the probiotic drench.

4.1.4 Confirmation of dominance of *Ruminococcus bromii* by real-time PCR

Primers and probes were designed for a Real-Time PCR assay and the assay has been developed and calibrated to enumerate YE282. This allowed the dominance of the YE282 group of isolates to be confirmed in the samples from CRC Project 2.5.4 (Figure 3) and for future use in the feedlot trial to determine the rate of establishment and colonisation in inoculated animals. The *R. bromii* YE282 group of isolates were below detectable levels in all eight cattle before the introduction of grain into the diet but after 10 days on grain were at populations above 10^9 *R. bromii* cell equivalents (RBCE)/ml in all animals and reached as high as 10^{10} RBCE/ml in some animals after 14 days on grain. This confirms the selection for this species in grain (barley) fed cattle and the dominance of the species in the rumen of cattle on a grain diet.

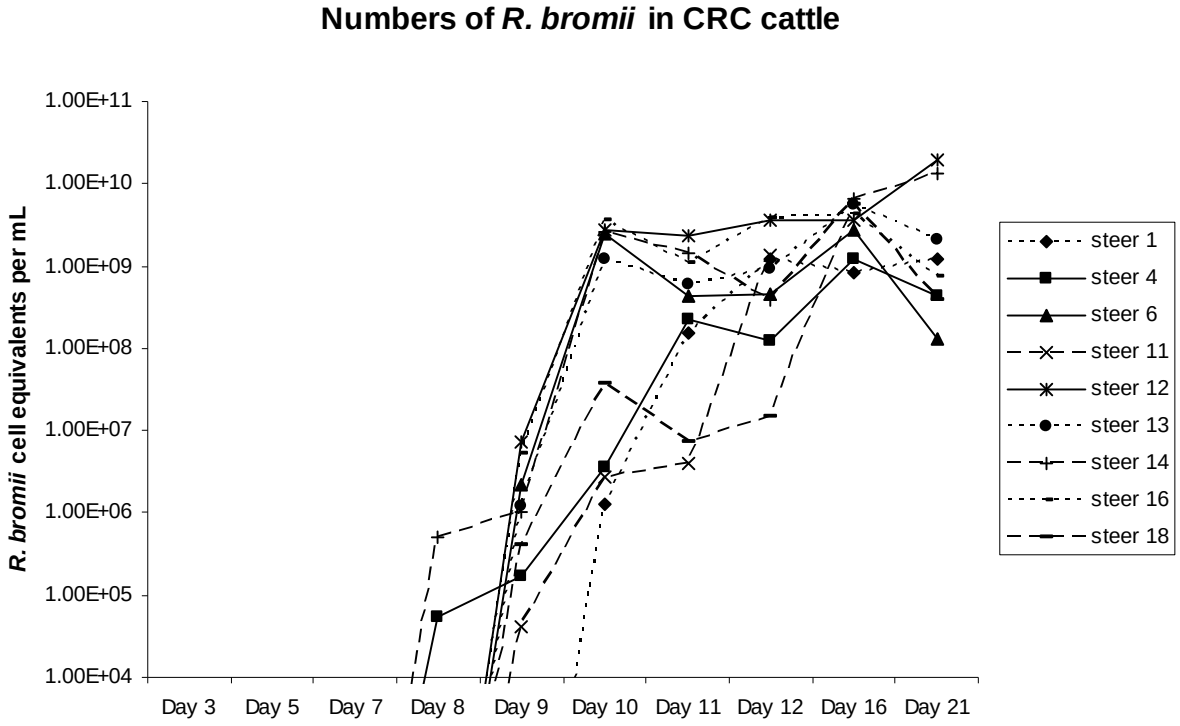


Figure 3. Population densities of the R. bromii *YE282 group of bacteria in cattle before (prior to Day 7) and during grain feeding.*

R. bromii YE282 was included in the probiotic inoculant as the major starch utiliser along with *M. elsdenii* YE34 as the lactic acid utiliser.

Attempts to isolate a dominant *Prevotella* isolate were unsuccessful and none of the *Prevotella* isolates present in our culture collection matched to the dominant *Prevotella* spp. present in the grainfed cattle. Therefore, YE282 and YE34 were used as a bivalent mix in the feedlot trial.

4.2 Pen trial – inoculation of lotfed steers with *M. elsdenii* and *R. bromii*

4.2.1 Cattle growth and welfare

4.2.1.1 Feed intake, liveweight gain and carcase parameters

There were no statistically significant differences between treatments in relation to liveweight, intake, pH or slaughter parameters. The cattle in general ate well throughout the trial and this was reflected in the high rate of liveweight gain. Data relating to intake, growth and body condition pre- and post-slaughter are summarised in Table 3.

Table 3. Feed intake, liveweight gain, body condition and carcass characteristics averaged across cattle in the Control and inoculated treatments.

		Control	Inoculated
DM intake (kg/d)	Weeks 1-4	7.69	7.57
	Weeks 5-10	9.48	9.74
	Weeks 1-10	**8.76**	**8.87**
DM intake (g/kg LW/d)	Weeks 1-4	20.3	20.2
	Weeks 5-10	21.3	22.0
	Weeks 1-10	**21.1**	**21.5**
Initial liveweight (kg)		349.8	345.3
Final liveweight (kg)		480.5	481.4
Liveweight change (kg/d)	Weeks 1-4	2.11	2.08
	Weeks 5-10	1.71	1.85
	Weeks 1-10	**1.87**	**1.94**
Feed conversion ratio (kg/kg) - Total trial		4.70	4.57
Carcass weight (kg)		250.0	251.0
Dressing percentage		52.0	52.1
P8 fat cover (mm)		10.0	10.3
Hip height change (mm)		44.4	52.5
Body condition score change		2.19	2.36
Final body condition score		6.8	6.8

There was little difference in DM intake between treatment groups in the first 4 weeks of the trial (see Table 3). While intakes were marginally higher for the inoculated cattle in the last 6 weeks and overall, these differences were not significant. The pattern of intakes (see Figure 4) indicates a trend toward higher intakes by the inoculated cattle in the last 4 weeks of the trial. However, this coincides with poor performance of one pen in the Control group (Pen 7), and one steer in that pen in particular. This steer lost 2 kg over the last 3 weeks. As illustrated in Figure 4, intakes in the inoculated groups of cattle tended to be less variable than in the Control groups. This finding suggested that introduction of the inoculant bacteria would create a more uniform response to feeding grain due to the fact that all the cattle would have similar high populations of the main bacteria responsible for fermenting the grain starch in the rumen. However, this finding needs to be confirmed as two Control groups performed slightly better than the inoculated groups.

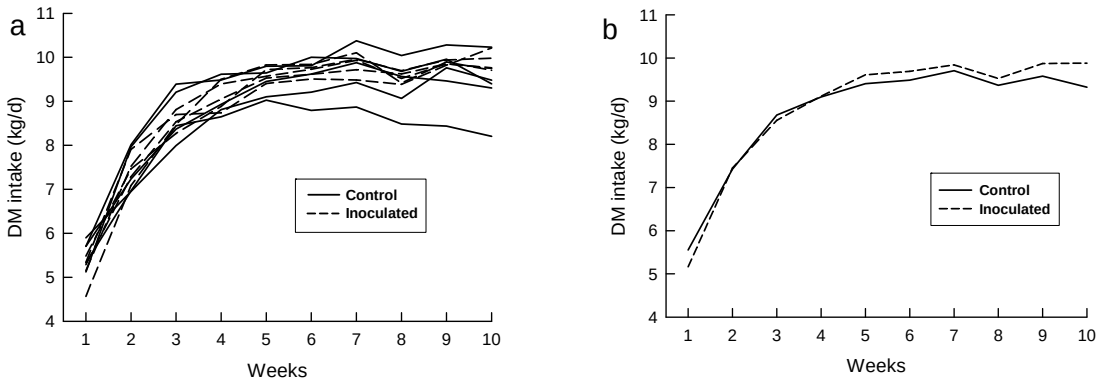


Figure 4. Changes in DM intake by steers over the trial period, for individual pens (a) and the treatment averages (b).

High growth rates were achieved throughout the experiment in both the inoculated and Control steers (Figure 5), but particularly in the first 4 weeks which could have resulted partly from some realimentation after low intakes in the paddock prior to induction. Liveweight changes generally reflect changes in intake and growth rates were slightly higher for inoculated than Control cattle for the final 6 weeks, but again poor performance of a few individual steers in the Control group may have been an influence. As with intakes, there was slightly less variability in liveweight change within inoculated steers as compared with Control steers.

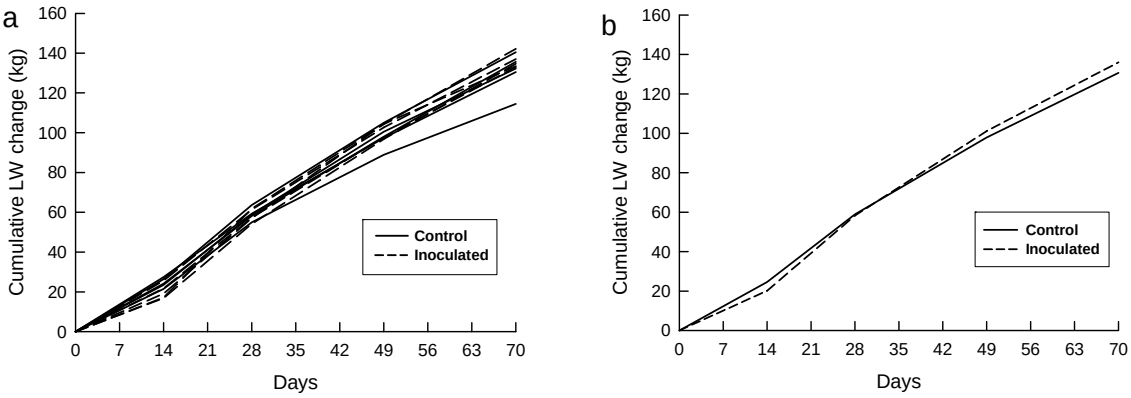


Figure 5. Cumulative liveweight changes of steers over the trial period, for individual pens (a) and the treatment averages (b).

There were no differences between treatments in carcass weight, dressing percentage or P8 fat cover. The slight advantage to the inoculated groups for carcass weight and P8 fat reflect small differences in liveweight.

The general lack of differences between the inoculated cattle and Control cattle and the fact that all performed very well is probably due to the rapid spread of one of the inoculant bacteria and the transient dominance of the other. The data relating to microbial changes will be presented and discussed below (section 4.2.2).

4.2.1.2 Acidosis – physical symptoms, ruminal pH, and lactic acid concentrations

There was only one obvious case of laminitis, a Control steer at 4 weeks, but this steer subsequently recovered to some extent and was not removed from the experiment. One other Control steer

appeared lethargic at week 5 but showed no signs of laminitis and was monitored but not removed. This steer only maintained liveweight during the last 3 weeks of the experiment.

The ruminal pH of sampled steers is presented in Figure 6.

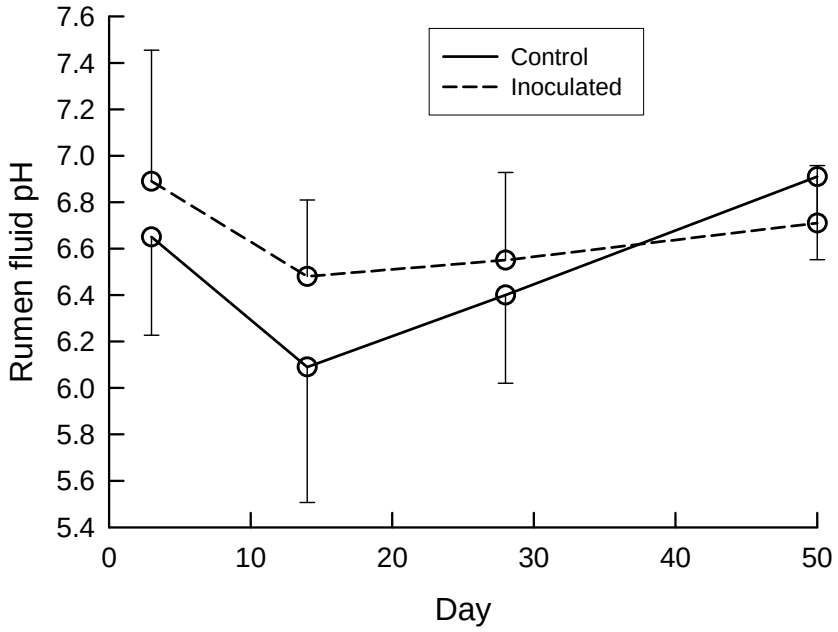


Figure 6. Ruminal pH measured at 3, 14, 28 and 50 days post-inoculation in Inoculated and Control steers. Bars represent the standard error from the mean.

Ruminal pH was not affected (*P*>0.10) by inoculation at any recording time and remained relatively high (non-acidic) commencing on day 3 at an average of 6.8, dropping on day 14 before returning to original levels by day 50. Lactic acid concentrations and pH values tend to indicate that ruminal lactic acidosis was not an issue for animals in this experiment, even without monensin in the diet. Although pH and lactic acid concentrations were only measured once on the day of sampling and early in the morning, when diurnal fluctuation would suggest that pH in particular would be at a high point (least acidic), the pH values across all animals remained above 5.5 throughout the trial and except for three Control group steers lactic acid remained at trace levels (below 1 mM). Even in these three Control group cattle the maximum lactic acid concentration was 11.45 mM, well below concentrations exceeding 100 mM reported in acidotic cattle (Klieve *et al.* 2003). No treated animals that were sampled showed any accumulation of lactic acid in the rumen.

4.2.2 Rumen microbial observations

4.2.2.1 Bacterial population changes

Real-Time PCR was used to enumerate populations of the inoculant bacteria, *M. elsdenii* and *R. bromii,* and also *S. bovis*, a lactic acid producing bacterium often implicated in lactic acidosis (Mackie *et al.* 2002). Numbers of these bacterial species in ruminal contents of the sampled cattle at days 3, 14, 28 and 50 are presented in Figures 7, 8 and 9. Data are presented as frequency graphs with the percentage of sampled animals in each treatment group with populations within each log value range (e.g. numbers of bacteria between 10^5 and 10^6 per ml) being plotted.

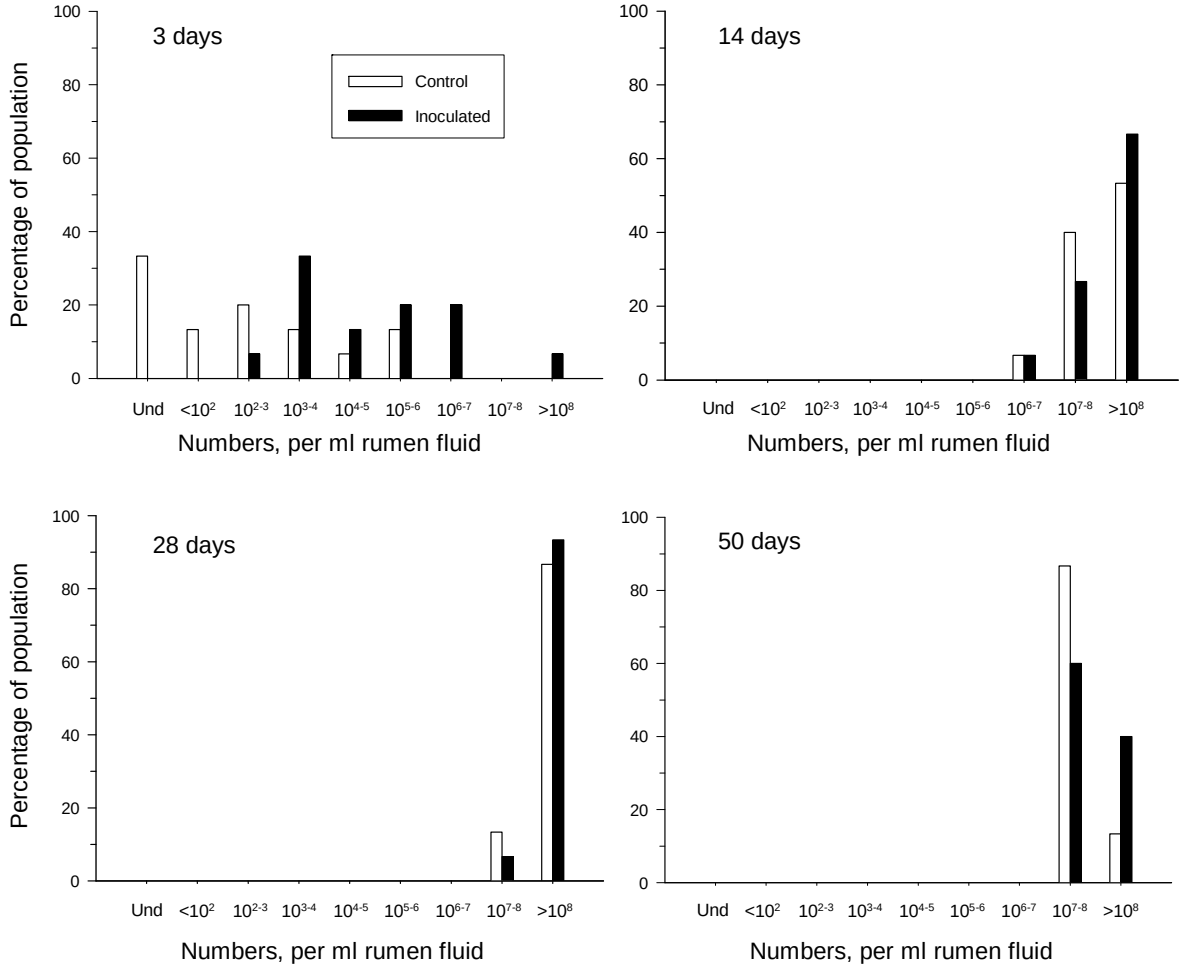


Figure 7. Frequency of occurrence of M. elsdenii *at given population densities in sampled cattle from the Control and Inoculated groups. Scale is based on grouping on a logarithmic population density basis; for example, 10^{5-6} represents a population density between 10^5 and 10^6* M. elsdenii *cell equivalents per ml of rumen fluid. Und – population density was below detectable limits.*

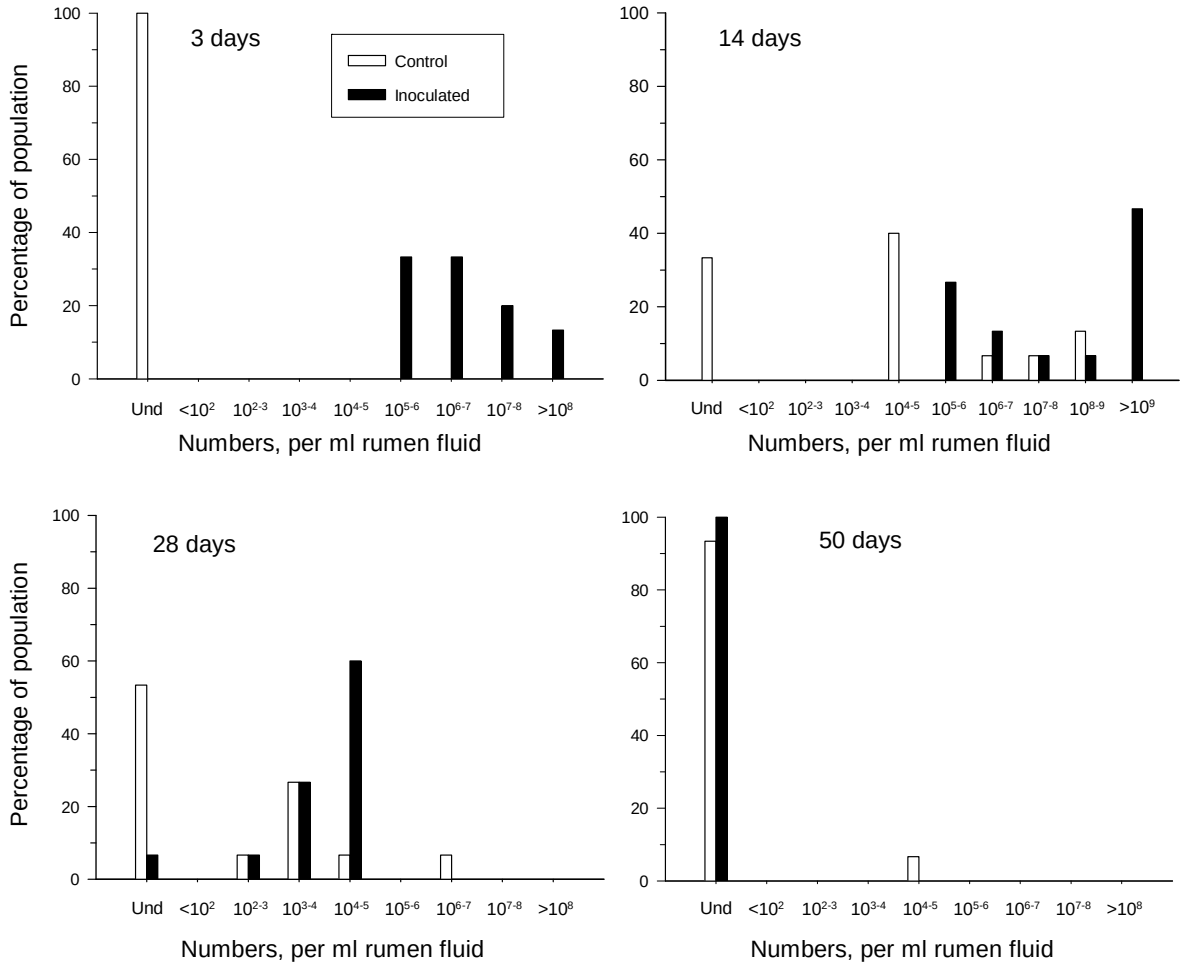


Figure 8. Frequency of occurrence of R. bromii *at given population densities in sampled cattle from the Control and Inoculated groups. Scale is based on grouping on a logarithmic population density basis; for example, 10^{5-6} represents a population density between 10^5 and 10^6* R. bromii *cell equivalents per ml of rumen fluid. Und – population density was below detectable limits.*

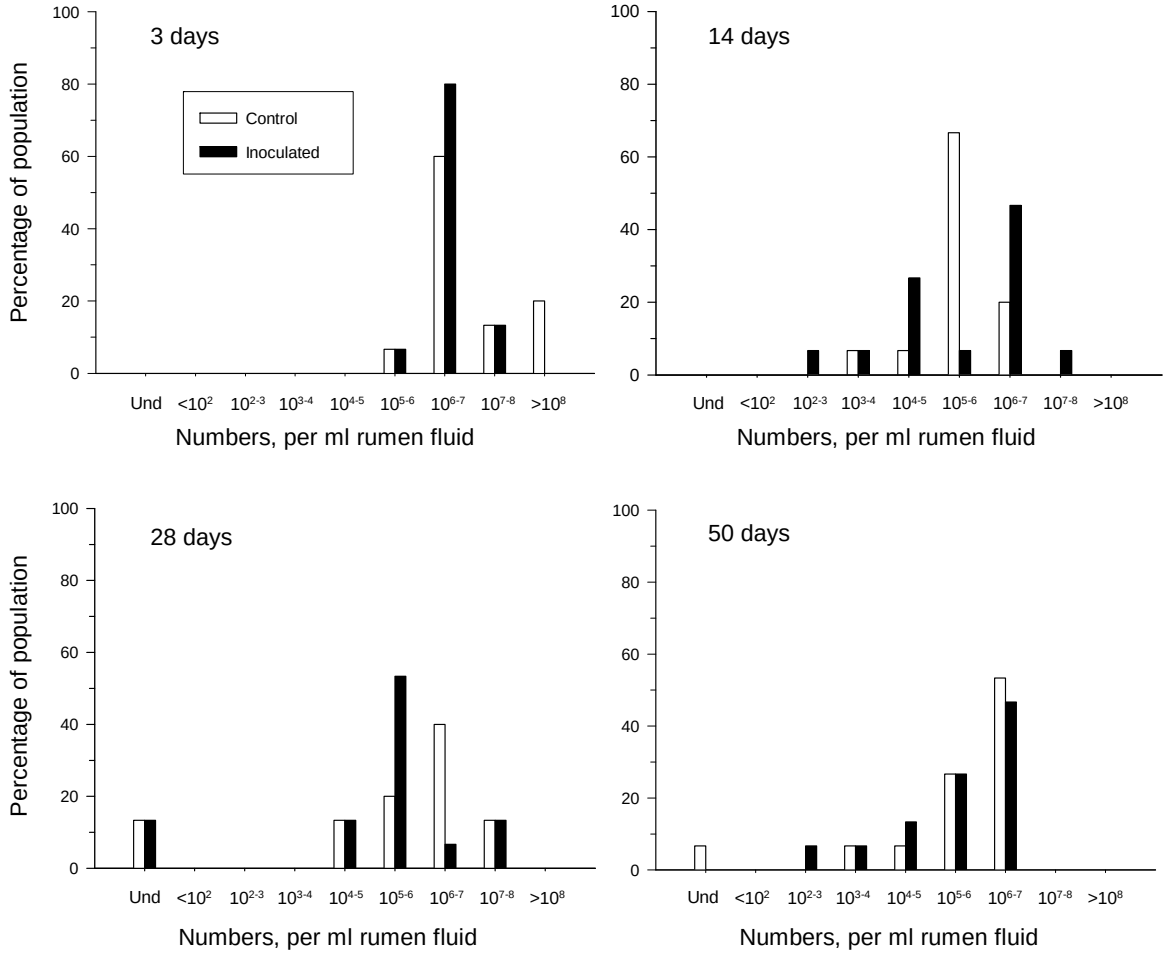


Figure 9. Frequency of occurrence of S. bovis *at given population densities in sampled cattle from the Control and Inoculated groups. Scale is based on grouping on a logarithmic population density basis; for example, 10^{5-6} represents a population density between 10^5 and 10^6* S. bovis *cell equivalents per ml of rumen fluid. Und – population density was below detectable limits.*

Populations of *M. elsdenii* were present in all inoculated cattle generally at densities between 10^3 and 10^7 cells per ml by day 3. Unfortunately, by day 3 *M. elsdenii* was also detected in 70% of the Control animals as well, generally at a lower density but as high 10^6 per ml. The acquisition of detectable populations of *M. elsdenii* was much more rapid than expected and more rapid than previously observed. Klieve *et al.* (2003) inoculated *M. elsdenii* directly into the rumen and did not detect *M. elsdenii* in any Control animals (all were sampled) for five days. While it is possible that the Control group had *M. elsdenii* naturally present at higher levels than previously observed, it is also possible that the Controls were compromised by spread of inoculum to them at an early stage. The observation that the Control steers at the end of the pen complex from which the wind prevailed had the highest population density of *M. elsdenii* could suggest greater exposure to prevailing wind (and thus to wind borne transfer of inoculant) than for other Control groups further along the row of pens and thus buffered more from the wind by the pens of animals upwind. On the day of inoculation, treated animals reached their pens late in the afternoon when it was overcast with a cool wind blowing and the humidity was high with some drizzly rain. Figure 10 shows the position of the pens and *M. elsdenii* population densities in sampled Control group animals relative to wind direction.

M. elsdenii density.

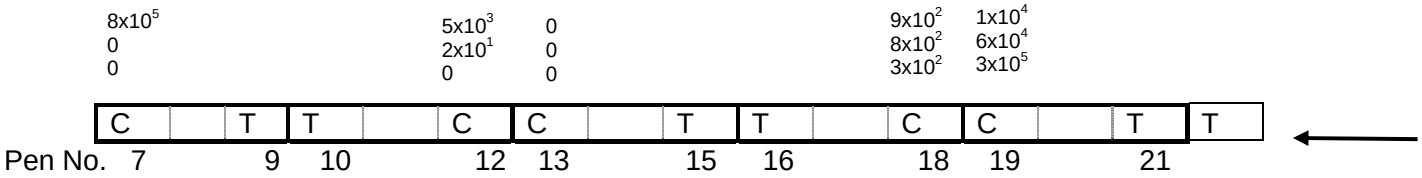


Figure 10. M. elsdenii *population densities (cells per ml of RF) in sampled Control group animals on day 3. The arrow indicates wind direction.*

While not conclusive, there does appear to be a clustering of Control cattle with higher population densities nearest to treated animals at the end of the row where the prevailing wind effect would have been the strongest. While we had been very careful to eliminate direct physical contact between animals we had not been aware that aerosol drift could be a problem. Since this time we have been informed that work with *Synergistes jonesii* and leucaena feeding (Ray Jones, personal communication) had shown that it was not possible to maintain a Control group as aerosol transmission rapidly spread this bacterium to animals that had not been inoculated. Moreover, Keith Gregg and colleagues in Western Australia have posted a draft paper on their website monitoring the aerobic transmission of three ruminal obligate anaerobic bacteria. They measured spread of up to 30 metres under the right conditions, which included high humidity. Unfortunately neither of these reports were published and only came to our attention recently. However, this meant that many of the Controls had appreciable populations of *M. elsdenii* very early in the experiment which would have at least protected them from acidosis (including subclinical).

M. elsdenii populations rapidly increased in all animals and by 14 days were present at relatively high densities (10^7 to 10^8 cells per ml of rumen contents), where they appeared to stabilise for the remainder of the experiment.

The changes in *R. bromii* populations were unexpected. At three days, *R. bromii* had established in all inoculated cattle at reasonable densities between 10^5 and 10^9 cells per ml and was below detection limits in all the Controls, suggesting that it did not spread (or was not able to colonise as quickly) to the Controls in the manner that *M. elsdenii* may have done. By 14 days, the *R. bromii* population was still behaving as expected for a dominant starch utiliser with the population density in inoculated animals increasing markedly with numbers above 10^9 cells per ml in 50% of inoculated

cattle and increasing in the Controls with a few animals (~ 20%) with high density populations (10^6 to 10^9 cells per ml), 40% around 10^4 to 10^5 cells per ml and still undetectable in ~ 30% of the Control cattle. Unexpectedly, by day 28 populations had declined markedly in both groups of cattle with all the inoculated cattle having populations of < 10^5 cells per ml and *R. bromii* was not detectable in 55% of the Control cattle. Given this unexpected turn of events we approached the animal ethics committee and had approved an additional sampling of these cattle at 50 days. At this time the *R. bromii* population had declined to the extent that it was below the detectable threshold in all but one of the Control animals that was sampled. It appears that *R. bromii* is only transiently dominant during the early weeks of grain feeding and was not detected at any time in 20% of the Control animals and the presence or absence of this bacterium does not appear to have had an influence on intake or liveweight gain. Unfortunately, we noted in hindsight that the cattle that were examined to determine the dominant starch utilising bacterium (from the Beef CRC experiment) had been on grain for 14 days, the time at which the *R. bromii* populations were maximal in the current experiment. Given the transience of *R. bromii* it was important to find out what bacterial species had replaced *R. bromii* and were dominant in these steers. This has been undertaken and is reported below (section 4.2.2.3).

S. bovis is often implicated in lactic acidosis but recent work suggests that the population density rarely increases unless the ruminal ecosystem is compromised (Mackie *et al.* 2002; Klieve *et al.* 2003). This conclusion is supported in this work and there is the possibility that *M. elsdenii* limits the growth of *S. bovis. S. bovis* populations were generally 10^6 to 10^7 cells per ml throughout the entire trial, which is very much the same situation as was reported previously (Mackie *et al.* 2002; Klieve *et al.* 2003). In the current trial a few Control animals had higher populations at day 3 (above 10^8 cells per ml) but these did not persist. As the trial progressed the population density of *S. bovis* decreased in many of the sampled animals and even became undetectable in a few animals at 28 and 50 days into the trial.

4.2.2.2 Protozoal population changes.

Populations of ciliate protozoa rapidly disappeared from the animals in the trial. At three days all animals had healthy populations (averaging 4 x 10^5 per ml in inoculated cattle and 4.2 x 10^5 per ml in Controls) dominated by *Entodinia* spp. (70 – 80%) and *Diplodinia* spp. (~ 20%). By day 14 protozoans were present in most animals but at low densities (~10^4 per ml and below). By day 28 protozoa were sporadically present in a few animals in each group and at day 50 were absent from all animals, with the exception of one steer in the inoculated group which had quite high numbers (1.7 x 10^5 per ml).

4.2.2.3 Identification of starch utilisers displacing *R. bromii*

It was important to determine the identity of the dominant starch utilising bacteria that replaced the transiently dominant *R. bromii,* as these bacteria may further improve starch digestibility if used in the future to replace *R. bromii* in the probiotic mix along with *M. elsdenii.*

To determine what changes had occurred in the rumen ecosystem of these animals over the time period involved and whether the bacteria replacing *R. bromii* were always the same or not, DGGE gels were run on total DNA from ruminal contents from eight of the steers (six inoculated and two Control) on days 3, 14, 28 and 50. The steers selected were those in which *R. bromii* had initially established and then disappeared. Results are presented in Appendix 2 with an example presented below as Figure 11.

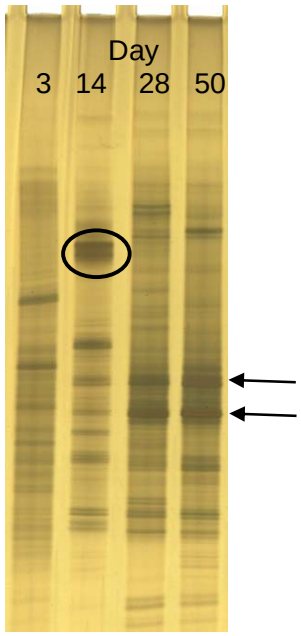


Figure 11. DGGE profiles of bacterial diversity and abundance in ruminal contents of steer 3620 (an inoculated group animal) on days, 3, 14, 28 and 50 post-inoculation. Banding typical of R. bromii *is circled and bands that became extremely prominent after day 28 are arrowed.*

Figure 11 shows that *R. bromii* appeared dominant at day 14 in steer 3620 but dropped away after that and two other bands, in particular, became very dominant indeed and remained that way through to at least day 50. While *R. bromii* is not as evident in all the DGGE profiles (Appendix 2), the two dominant bands that arose after day 14 in 3620 also became dominant in all the other steers (and in some were dominant from day 14 onwards) and remained so. A comparison of DGGE patterns for all eight steers at day 50 is presented in Figure 12. This clearly shows the similarity in the bacterial population profiles of the cattle by day 50 and the dominance of these two bands (designated A and B) and also of two slightly less dominant bands that were still very strongly represented and present in all the steers. DNA from these bands (marked on Fig. 12) was excised, cloned and sequenced to determine the identity of the bacteria represented by these bands.

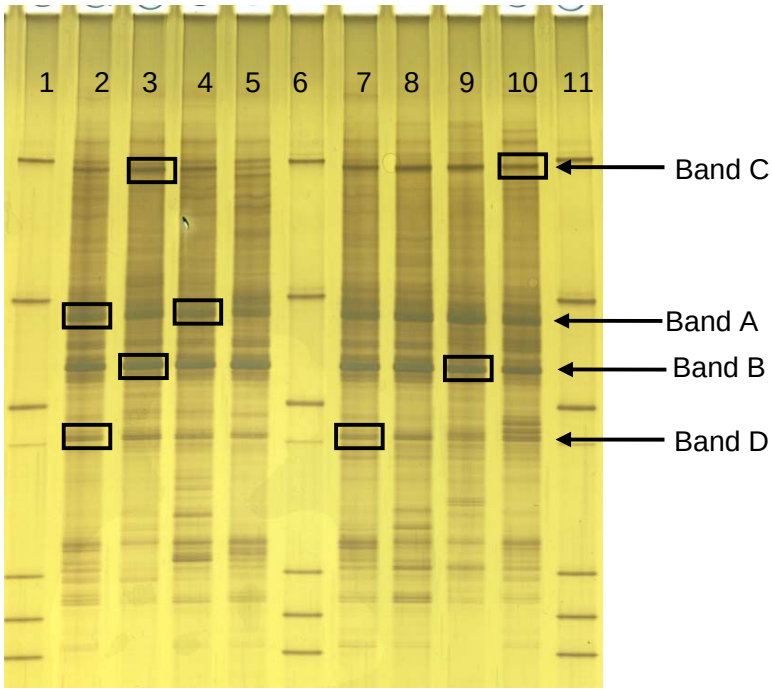


Figure 12. DGGE profiles from rumen contents of eight steers. Lanes 1, 6 and 11 are reference markers; Lanes 2, 3, 4, 5, 7, 8, 9, and 10 were from steers 3647, 3654, 3625, 3613, 3620, 3651,3614 and 3632 respectively. Bands from which DNA was excised, cloned and sequenced are boxed.

The sequences from bands A and B are closely related and both are from bacteria that belong to the genus *Ruminobacter*. However, they are sufficiently different from known culturable bacteria within this genus to be regarded as different species.

5 Success in Achieving Objectives

5.1 Overall

In general the project achieved the majority of the objectives set but in doing so has generated some further questions that will require clarification in the future.

5.2 Specific objectives

Objective 1. Examine samples from the CRC pen-trial and identify the dominant starch utilising species of bacteria.

Objective one was fully achieved. Samples from the CRC pen trial were examined by DGGE and five DNA bands that appeared dominant and common when steers had been fed 75% barley for 14 days were selected. DNA was isolated, cloned and sequenced from these bands and the dominant bacteria were identified as *Ruminococcus bromii* and a range of *Prevotella* spp. Both groups represented known starch fermenting bacteria and the most dominant species in terms of abundance in the population (band density) and frequency of occurrence in steers appeared to be *R. bromii.*

Objective 2. Isolate and characterise identified species for inclusion in a probiotic drench containing a combination of lactic acid and starch utilising bacteria.

This objective was achieved with the isolation of four strains of *R. bromii*, three of which matched 100% to the dominant DGGE bands in the steer profiles. Development of a real-time PCR assay to enumerate *R. bromii* confirmed that in the CRC trial this bacterial species rapidly developed high density populations in all the steers soon after the introduction of grain into the diet. *R. bromii* YE 282 was selected for inclusion in a probiotic drench along with the lactic acid degrading bacterium *M. elsdenii* YE34. Unfortunately, attempts to isolate a dominant *Prevotella sp.* were unsuccessful and none of the *Prevotella* isolates present in our culture collection matched to the dominant *Prevotella* spp. present in the grainfed cattle.

Objective 3. Evaluate in a field trial the benefits from using the probiotic drench in terms of feed intake, liveweight gain, days to turnoff, feeding costs and incidence of acidosis.

Objective three was only partially achieved with the results showing that there was no benefits to inoculation in comparison with a Control group under the trial conditions. It appears that the Control group was compromised by the acquisition of dense populations of *M. elsdenii* much earlier in the trial than expected. This meant that the Controls, in terms of the presence of this important bacterium, were similar to the inoculated group. With hindsight, the experimental design did not take into account the possibility of aerosol spread. While considerable effort was put into ensuring no direct physical contact between inoculated and Control steers occurred, it was only after the fact that the possibility of aerosol spread arose and this was from personal communication and unpublished literature. Despite this the cattle performed very well growing at almost 2 kg per day throughout the trial with few signs of acidosis (none in inoculated steers) even though monensin had been omitted from the ration.

6 Impact on Meat and Livestock Industry – now & in five years time

6.1 Impact on Meat and Livestock Industry – now

As the results of this project require confirmation and clarification the impacts will not be immediate.

6.2 Impact on Meat and Livestock Industry – in five years time

The potential benefits of this technology have already been espoused, viz., reduced acidosis, faster introduction to grain rations and improved utilisation of starch. Such effects would likely be translated into improved animal welfare, increased growth rate of grain-fed cattle, reduced time on feed, increased feed conversion ratio, or a combination of these. The impact on a rapidly expanding feedlot industry could be immense. However, the present experiment failed to show a response in terms of animal production, perhaps due to design inadequacies, although there were indications of a small reduction in incidence of acidosis related symptoms. Thus the impacts on the meat livestock industry at this stage have not established or are equivocal at best.

The main impact of the current research at this stage has been in providing world-first in-depth description of the changes in microbial populations in the rumen of cattle fed high-grain rations over the medium term. This new information will underpin future research and innovations in feedlot nutrition and management. The impact of this is difficult to quantify but should not be dismissed.

An additional impact may arise from the ability to safely feed grain to cattle in the field, which will enable opportunity benefits to be gained by a larger section of the industry when cheap grain is available and not impinge on the welfare of these cattle while doing so.

Chemical rumen modifiers may have a limited lifespan through lack of public acceptability, their use as trade barriers and the increase in microbial resistance to their action. Live microbial inoculants may well be able to replace these modifiers and deliver similar benefits more cheaply and be more acceptable in the longer term.

7 Conclusions and Recommendations

7.1 Conclusions

1. The molecular biological techniques that were used to identify, target for isolation and monitor changes in bacterial populations over time, primarily DGGE and real-time PCR, were very successful.
2. The inoculation procedure worked very effectively and the simple drenching procedure could be readily implemented under normal commercial induction of cattle into the feedlot.
3. *Megasphaera elsdenii* established high density populations in the rumen of grainfed cattle very rapidly, even while the percentage of grain in the diet was relatively low, and persisted at these levels. The presence of high density *M. elsdenii* populations appears to correlate with a stable, even reduced, *Streptococcus bovis* population, higher pH and markedly reduced lactic acid accumulation.
4. *Ruminococcus bromii* is only transiently dominant in the rumen ecosystem of steers consuming a barley based diet. Populations established at high densities within the first two weeks but then fell away and were undetectable by 50 days. Coincidentally, the cattle in the CRC trial from which *R. bromii* was identified as the dominant starch utiliser had been on grain for just 14 days. *R. bromii* appears to be replaced as the dominant bacterial species by *Ruminobacter* spp. These species became dominant between 14 and 28 days on grain, apparently in all the steers, and persisted as dominant species through to the end of the trial. The role of *R. bromii* in adaptation of the rumen ecosystem to a grain diet is unclear.
5. The lack of difference between inoculated and Control steers may have been related to the rapid acquisition of high population densities of *M. elsdenii* in the Control group which effectively reduced the period of time over which differences between the groups could have developed. This highlights the importance of experimental design where live bacterial inoculants are used and the importance of isolating the Control group from inoculated animals.
6. The performance of the steers in the trial was commensurate with normal to high producing commercial feedlot cattle.

7.2 Recommendations

1. Further research be instigated to confirm or clarify results to date:-
    - Independently determine the natural rate of increase of *M. elsdenii* in commercial feedlot cattle where there is no possible source of contamination with inoculant bacteria.
    - Repeat the experiment with an improved experimental design to confirm the benefits of *M. elsdenii* inoculation. Maintaining the Control and inoculated groups at differing sites, e.g. Brigalow and Brian Pastures Research Stations, would prevent any contact, including possible air-borne spread, between treated groups.
    - Investigate whether *R. bromii* and *Ruminobacter* spp. provide any additional advantage to inoculation with *M. elsdenii* alone.

- Confirm the improved uniformity in growth performance of the steers. The limitation of undertaking a single experiment is that trends towards traits such as uniformity in feedlot performance need to be repeated to show that the response is reproducible.
- Undertake experiment to determine whether inoculated cattle can be introduced to full grain feeding faster than the Controls.

2. Once the value of *M. elsdenii* YE34 has been confirmed, investigate the commercial development of the *M. elsdenii* inoculum in line with the Commercial Development Plan (Appendix 3).

3. Further research be instigated to:-
    - Expand the research to determine the efficacy of inoculation when other grain types, e.g. wheat or maize, are used.
    - Investigate a possible role for *M. elsdenii* in improving the safety of feeding grain to cattle in the paddock.
    - Investigate the benefits of inoculating with *M. elsdenii* with and without monensin added to the diet as a possible future replacement for monensin.

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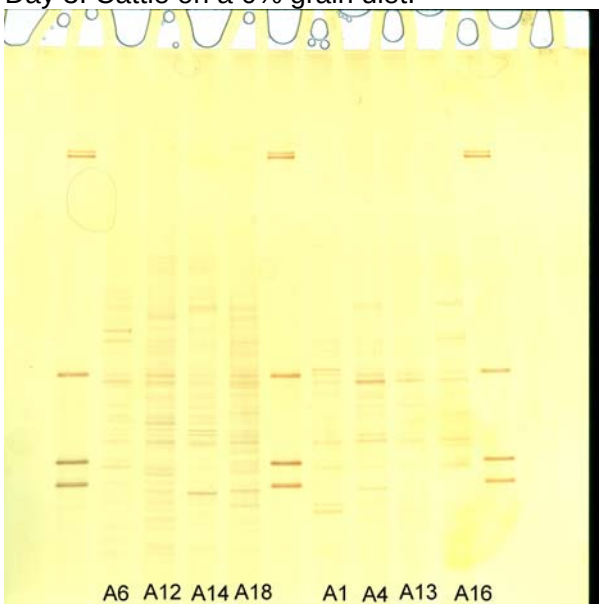
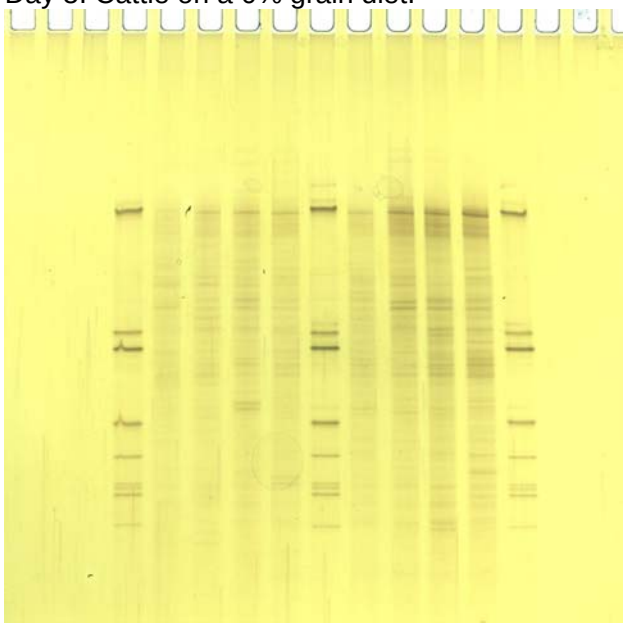
9 Appendices

9.1 Appendix 1. Identification of dominant species from CRC trial - DGGE results

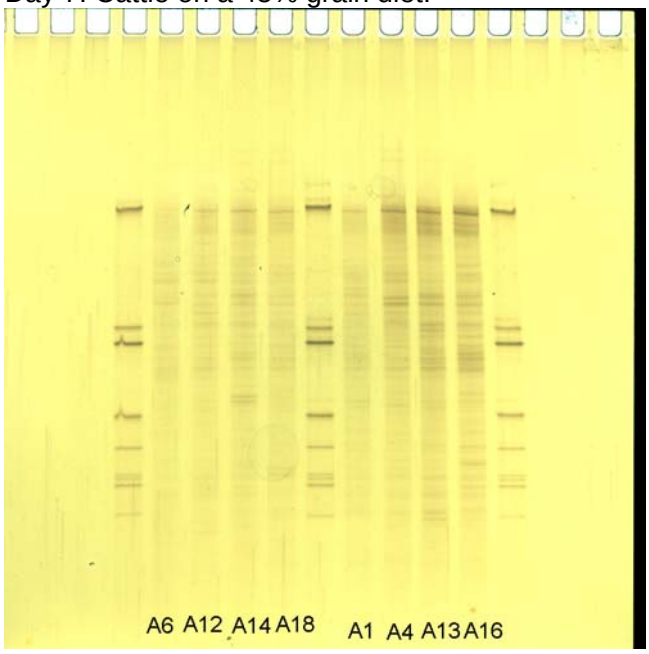
Day 3. Cattle on a 0% grain diet.



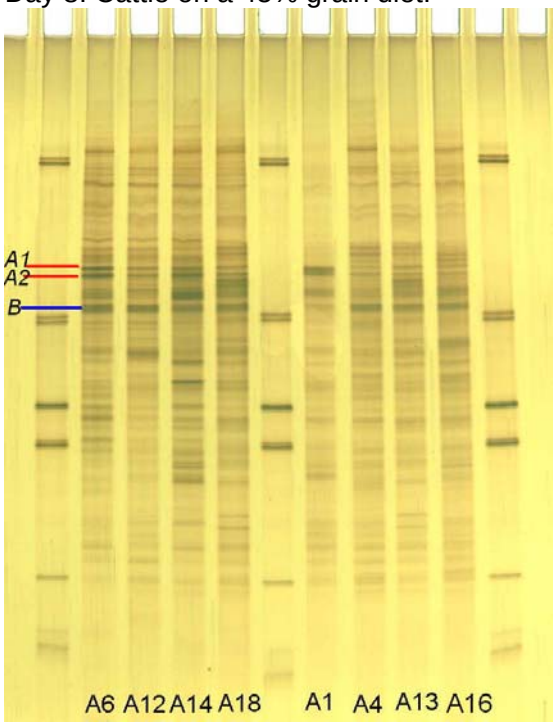
Day 5. Cattle on a 0% grain diet.



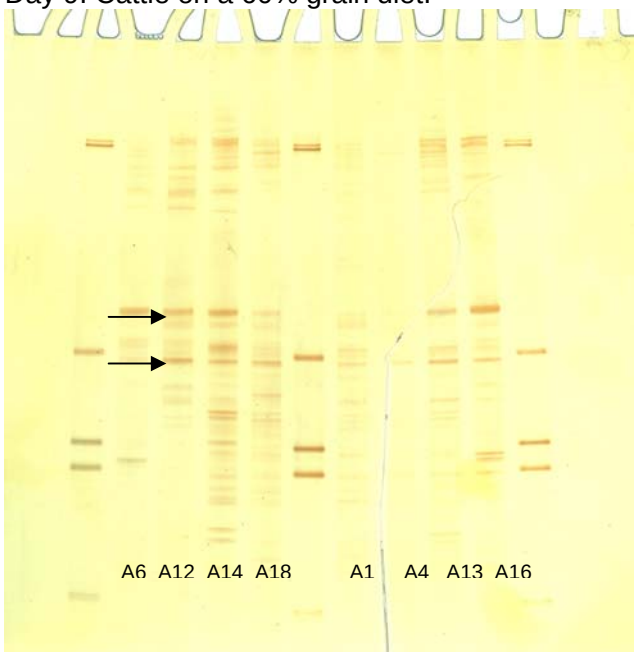
Day 7. Cattle on a 45% grain diet.



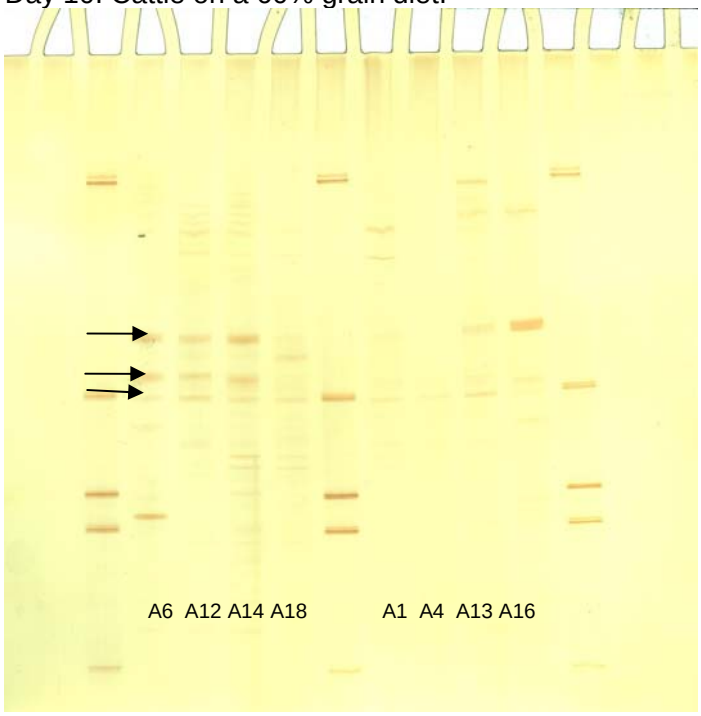
Day 8. Cattle on a 45% grain diet.



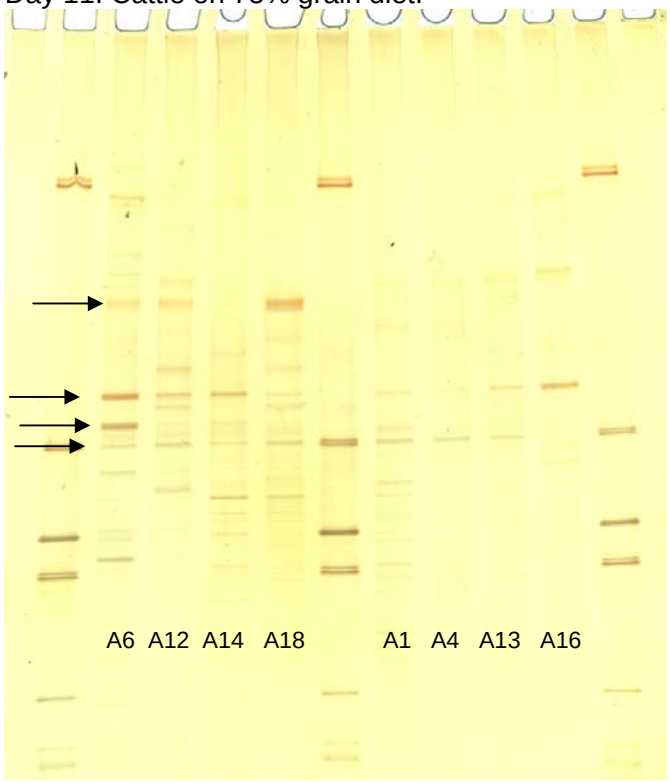
Day 9. Cattle on a 60% grain diet.



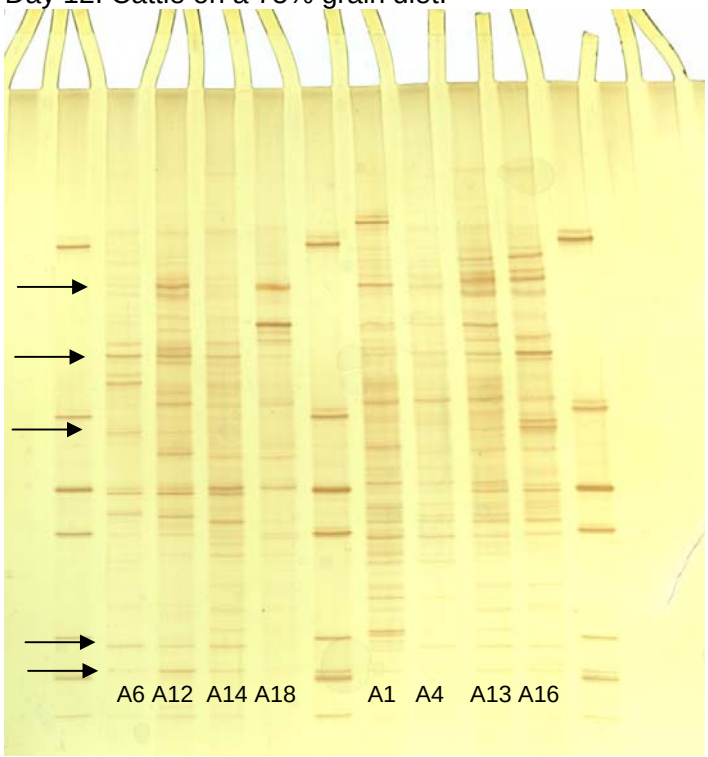
Day 10. Cattle on a 60% grain diet.



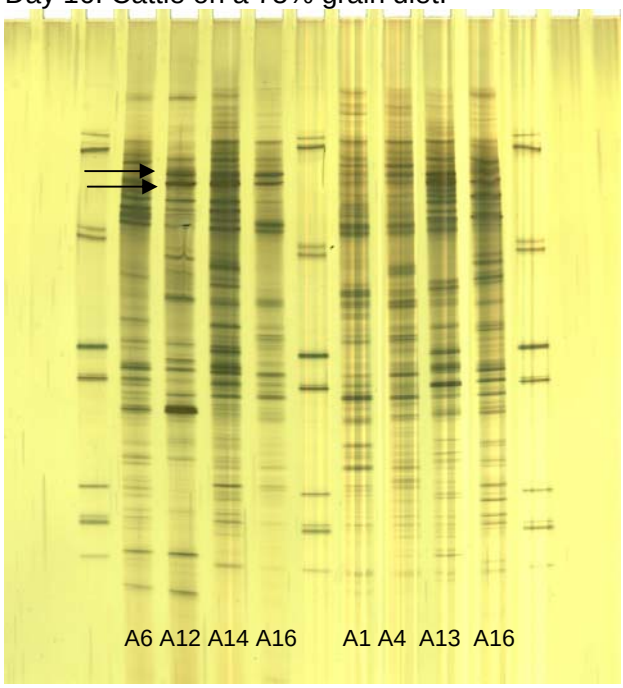
Day 11. Cattle on 75% grain diet.



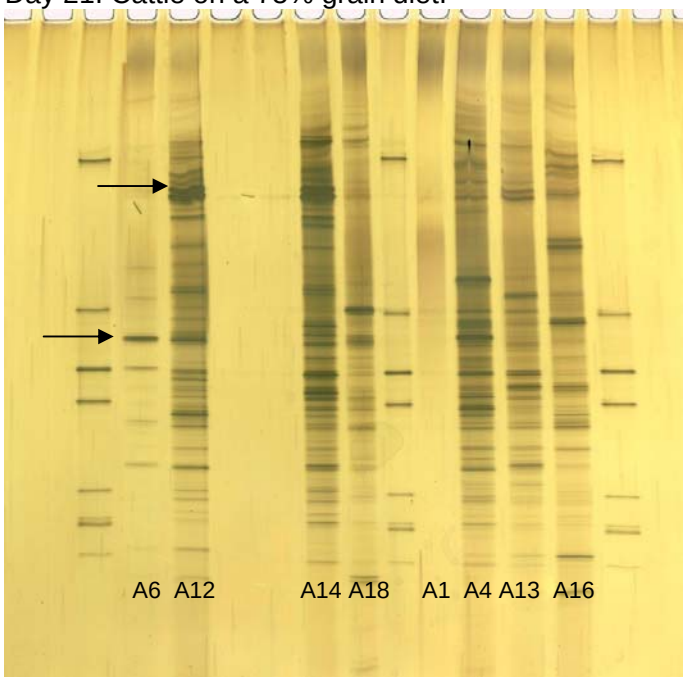
Day 12. Cattle on a 75% grain diet.



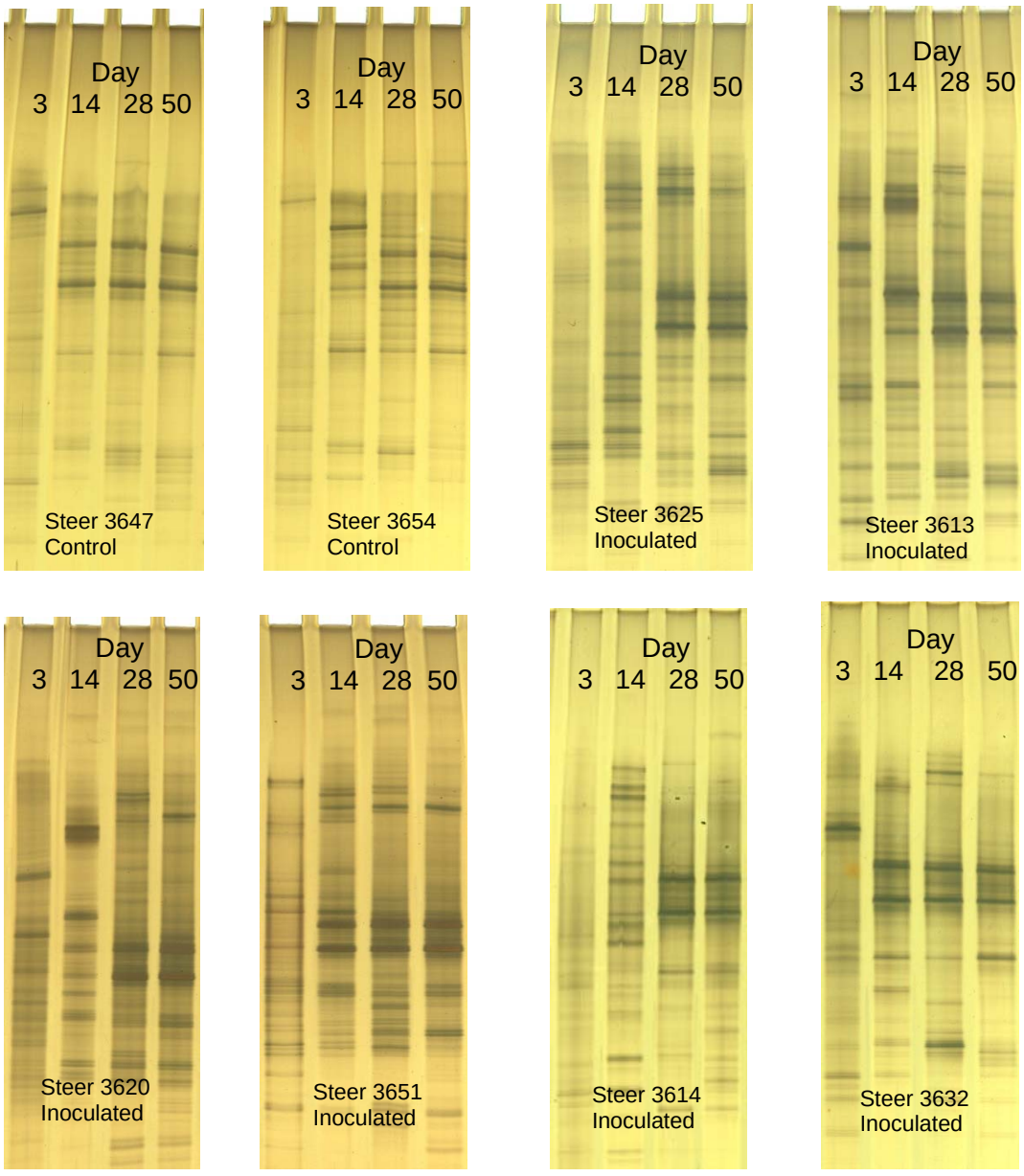
Day 16. Cattle on a 75% grain diet.



Day 21. Cattle on a 75% grain diet.



9.2 Appendix 2. Changes in dominant bacterial species in six steers - DGGE results.



9.3 Appendix 3. Commercial Development Plan

Rumen inoculum for the efficient use of high grain diets

Plan for Commercial Development

The purpose of project FLOT.125 is to identify and isolate the major starch utilising species of bacteria that were present in the rumen of cattle efficiently utilising grain without signs of acidosis in CRC project 2.5.4. A probiotic drench using these starch utilising bacteria and *Megasphaera elsdenii* YE34 (a lactic acid utilising bacteria) will be formulated and demonstrated to improve the efficiency of grain utilisation and reduce the incidence of lactic acidosis in the period following feedlot induction.

Given that this objective is successfully achieved by 30 June 2006, it will then be necessary to engage a partner in developing the inoculum into a commercial product available to feedlot owners and managers for use in feedlot cattle. The steps and options in commercialising the product are outlined below. A flow diagram summarising these steps is presented over page.

- To be able to administer the inoculum to commercial cattle it is necessary to first obtain a permit/registration of the product through the Australian Pesticides and Veterinary Medicines Authority (APVMA). A permit has been obtained (No. PER8343) for the experimental work encompassed within FLOT.125 but further development and commercialisation of the product will require additional permits and registration by this authority. Furthermore, proven efficacy of the product, obtained through the feedlot trial, will be required by the APVMA before the product can be registered.
- An economic evaluation of the value of the product, it's value to the market and benefits to producers will be required to enable decisions to be made as to which is the best way to proceed with engaging a commercial partner. This will also dictate the most successful way of getting the product to market and adopted by our primary clients, beef cattle lotfeeders. A number of possible directions have been foreseen that would follow the economic evaluation.
    - Direct publication of results and placing all details in the public domain. No specific commercialisation partner would be identified and it would be up to market forces and competitive advantage as to further development of the product. In my opinion, this is the least likely manner in which to get an efficacious product to market and widely adopted.
    - Patenting or otherwise protecting the product (i.e. trade secret) and proactively seeking a commercial partner, either exclusively or non-exclusively. Publication would still be extremely important for both registration of the product (to achieve peer review of efficacy claims) and to inform the scientific community (who might in the future improve on the original idea) and producer groups, to increase awareness of the product and its benefits.
    - A non-exclusive approach would involve putting the product specifications out to tender to attract a commercial partner. The partner would therefore need to compete with other commercial companies for the right to negotiate a license and production agreement.
    - An exclusive approach would entail approaching a specific company to become a partner in commercialisation. Such a partner would need to be engaged earlier in the development process and would most likely have involvement in economic evaluation of the product alternatives and with commercial protection of the product. It would be

envisaged that a partner in these circumstances would also financially contribute to this process.

There are both benefits and drawbacks when comparing the exclusive/non-exclusive approaches to engaging a commercialisation partner and these will need to be determined in relation to the economic considerations and achieving maximal market penetration and adoption by lotfeeders. These scenarios and a possible hybrid of these approaches could be considered in the economic evaluation.

- Once selected the commercial partner will have to negotiate a licence to produce and market the product. Production of the product could well involve further research to ensure that the product is economically produced and viable in storage.
- The product will then need to be marketed and supplied to lotfeeders.
- A communication strategy through MLA, CRC and DPI&F may need to be devised in association with commercial marketing to create an awareness of the product and the benefits from using it amongst the lotfeeding community.

Commercial Development Flow Diagram