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Enhancing digestibility of native pastures by cattle in northern Australia using kangaroo fibrolytic bacteria

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Abstract

This project investigated whether plant fibrolytic bacteria from the kangaroo gut could establish stable populations in cattle and improve digestion of native pastures. Four fibrolytic bacterial isolates were evaluated. All except one species failed to persist when introduced to a rumen-based fermentation *in vitro* and unfortunately all rapidly disappeared when inoculated into the rumen of rumen cannulated cattle. Analysis of kangaroo forestomach contents from 42 kangaroos indicated that the fibrolytic bacteria that had been evaluated were unlikely to be the dominant fibrolytic species *in vivo*, suggesting a culture-based bias towards these fibrolytic species. Culture-independent, DNA-based techniques have now provided a more accurate picture of the dominant fibrolytic community in the kangaroo gut. However, additional research would be required to isolate, characterise and assess the ability of these fibrolytic bacteria to colonise the rumen and improve the digestion of native pastures.

Executive Summary

A large proportion of Australia's sheep meat and beef industries are reliant upon the use of native pastures as the primary feed source. Ruminants feeding on C_4 pastures, including native pastures, do not achieve their genetic potential for growth performance largely due to their inability to intake sufficient feed to supply the nutrition that they require. The primary constraint on intake is the high fibre content (cellulose, hemicellulose and lignin) of the grasses and the slow rate of degradation via the fermentative processes of the rumen, which physically limits the size of particles that can flow out of the rumen and consequently reduces intake. Very few rumen bacteria are capable of breaking down plant fibre, particularly cellulose, and the rate that plant fibre can be degraded is therefore dependant on the intrinsic metabolic activity and population density that these bacteria can achieve.

The geographic isolation of Australia has meant that large domestic herbivores were not present on the continent when Europeans arrived about 200 years ago. Marsupials had evolved to fill the niche occupied predominantly by sheep and cattle (ruminants) elsewhere, and like the ruminants, the macropodid marsupials (kangaroos) developed an enlarged complex forestomach for fermentation of cellulosic and other complex plant materials prior to further digestion. Like ruminants a complex microbial ecosystem exists in the forestomach of macropodid marsupials. Bacteria, fungi, protozoa and viruses are known to be present but have not been characterised or studied in very much detail. These microbes have evolved to utilise Australian native pasture plants and it is likely that the macropodid marsupials possess gut microorganisms more highly evolved to utilise these plants, particularly the more refractile fibre fractions of these plants.

The purpose of this project was to investigate if fibrolytic bacteria isolated from kangaroo forestomach contents could establish stable populations in cattle, and improve key digestion parameters for native pastures. Specific objectives were 1) to evaluate the ability of fibrolytic bacteria to colonise a model rumen-based microbial ecosystem, and any impact on key digestion parameters; 2) to evaluate the ability of these bacteria to colonise the bovine rumen in a pen trial environment; and 3) to identify the key bacteria responsible for fibre digestion in Wallaroos, Eastern Grey and Red Kangaroos.

Four highly fibrolytic bacterial isolates (three cellulolytic Ruminococci, and one xylanolytic *Butyrivibrio*) were isolated from kangaroo forestomach contents, and isolate-specific quantitative real time PCR assays were developed to enable detection and quantitation of these bacteria in complex microbial ecosystems. The ability of the bacteria to colonise a fermentation based on the bovine rumen microbial population was evaluated, and key digestion parameters monitored to detect improvements in the rate of digestion of the poor quality native pasture provided as feed. Three of the isolates did not persist for more than 5 days at detectable levels, although one isolate did persist throughout the course of the fermentation, establishing a stable population. Each bacterial species increased the rate of dry matter digestion while present in the fermentation, indicating improvement in the fermenter population's efficiency of utilisation of the native pasture.

A pen trial was conducted over 36 days with 12 cannulated steers, inoculated with one of three combinations of the fibrolytic bacteria. The cattle were fed a supplemented low quality native pasture diet, and feed intake and liveweight monitored throughout. Rumen samples were collected at timepoints throughout the trial, and levels of inoculated bacteria quantitated. All inoculated bacteria were reduced to below detectable levels extremely rapidly, through as-yet unknown mechanisms.

Analysis of kangaroo forestomach contents from an additional 30 animals by quantitative real time PCR assay and Denaturing Gradient Gel Electrophoresis indicated that the three cellulolytic *Ruminococci* and one xylanolytic *Butyrivibrio* isolated in the preliminary stage of the project were not dominant or conserved in all species or across the geographical range.

This data supports growing evidence that conventional bacterial isolation biases towards certain bacterial groups, which are not necessarily representative of dominant *in vivo* fibrolytic species. Culture-independent, DNA-based techniques provide a significantly more accurate representation of the diversity and composition of complex microbial ecosystems.

Denaturing Gradient Gel Electrophoresis was used to identify a total of eight bands conserved at significant levels throughout 30 samples of solid-associated bacteria from kangaroo forestomach contents. Four of these bacterial species have been confidently identified, and preliminary data on the remaining four has been gathered, although the current data is not of sufficient rigour for confident identification of the dominant species that contribute to the band. The identified bacteria include cellulolytic clostridia, a hemicellulolytic rumen bacterium, and carbohydrate utilising bacteria. It is suggested that these identified bacteria are involved with fibre utilisation, either directly or as helper bacteria in a "functional core" of fibre utilisation. The identification of these dominant, fibre-associated bands will guide isolation of these bacteria, allowing evaluation of their ability to colonise and improve native pasture digestion in ruminants.

Additional basic research is required to isolate, characterise and assess the impact of the fibrolytic "functional core" bacteria on native pasture digestion in model systems and cattle before potential commercial outcomes are able to be appraised.

Contents

1 Background

A large proportion of Australia's sheep meat and beef industries are reliant upon the use of native pastures as their primary feed source. Ruminants feeding on C_4 pastures, including native pastures, do not achieve their genetic potential for growth performance largely due to their inability to intake sufficient feed to supply the nutrition that they require (Weston, 2002). The primary constraint on intake is the high fibre content (cellulose, hemicellulose and lignin) of the grasses and the slow rate of degradation via the fermentative processes of the rumen (Mackie *et al.,* 2002), which physically limits the size of particles that can flow out of the rumen and consequently reduces intake. Very few rumen bacteria are capable of breaking down plant fibre, particularly cellulose, and the rate that plant fibre can be degraded is therefore dependant on the intrinsic metabolic activity and population density that these bacteria can achieve (Stewart and Bryant, 1988).

The geographic isolation of Australia has meant that large domestic herbivores were not present on the continent when Europeans arrived about 200 years ago. Marsupials had evolved to fill the niche occupied predominantly by sheep and cattle (ruminants) elsewhere, and like the ruminants, the macropodid marsupials (kangaroos) developed an enlarged complex forestomach for fermentation of cellulosic and other complex plant materials prior to further digestion (Hume, 1982). Like ruminants a complex microbial ecosystem exists in the forestomach of macropodid marsupials. Bacteria, fungi, protozoa and viruses are known to be present but have not been characterised or studied in very much detail. These microbes have evolved to utilise Australian native pasture plants and it is likely that the macropodid marsupials possess gut microorganisms more highly evolved to utilise these plants, particularly the more refractile fibre fractions of these plants.

Fibrolytic bacteria often form micro-communities protected by biofilms on the surface of the feed. Other bacterial species are present in these consortia, and stimulate cellulose digestion through substrate exchange. It is unknown if the kangaroo fibrolytic bacteria require specific complementary species to efficiently degrade plant material, or if any suitable complementary species are present in the bovine rumen.

The importance to primary industry and the sustainability of agricultural practices, of investigating Australian native plants and animals has been recognised. The ASTEC Final Report on Research and Technology in Tropical Australia (1993) states … "For many land managers and scientists there is concern that sustainable land management cannot be achieved through improved pastures and that the impact of pasture species on the conservation value of northern Australia is unknown. Researchers are recognising that some native grasses are more resilient than introduced grasses. Another option for reducing grassland degradation involves the better management of native grasses". Obviously, it will be important to optimise the utilisation of those grasses by ruminants to maximise the synergy of ecologically and economically sustainable development.

2 Project Objectives

The purpose of this project was to identify kangaroo-derived highly fibrolytic bacterial species, and evaluate their ability to colonise and persist during *in vitro* and subsequent *in vivo* experiments, demonstrating improved key digestion and fermentation parameters.

Specifically, we:

- Evaluated and compared the ability of fibrolytic bacteria isolated from kangaroos to persist and colonise an *in vitro* fermentation apparatus and increase key digestion and fermentation parameters.

- Evaluated and compared the ability of selected fibrolytic bacteria isolated from kangaroos to persist and colonise in the rumen of cattle and increase key digestion and fermentation parameters.
- Identified a number of key conserved, solid-associated, bacteria in kangaroo forestomach contents.

3 Methodology

3.1 Evaluation of *in vitro* persistence of fibrolytic bacteria from kangaroos

3.1.1 Fibrolytic bacteria – origin and identity

Four fibrolytic bacterial strains had been previously isolated from eastern grey kangaroos harvested near Charleville (Table 1).

Table 1: Fibrolytic kangaroo foregut bacteria isolated prior to the start of B.NBP.0351

Isolate	Source	Closest identified species
YE125	Grey Buck	*R. flavefaciens* strain JM1 (97% over 1496 bases)
YE137	Red Doe	*R. flavefaciens* strain 4 (96% over 1341 bases)
YE161	Red Doe	*B. fibrisolvens* strain 49 (94% over 1354 bases)
YE166	Grey Doe	*R. flavefaciens* strain 17 (93% over 959 bases)

YE125, YE137 and YE166 were highly cellulolytic, while YE161 displayed high xylanolytic activity. YE125 and YE137 were virtually identical (98.7% identity) at the level of 16S rRNA gene sequence, but displayed different rates of cellulose digestion.

3.1.2 Quantitative real time PCR assays

To enable the enumeration and therefore ability to colonise in a rumen-like fermentation system, specific 5' *Taq* nuclease assays (real time PCR assays) were developed for the kangaroo bacteria, using the 16S rRNA gene as a specific target. Primers and fluorescent 3' DNA probes were developed to target unique regions within the 16S rRNA gene of the target bacterium using Primer Express ver. 2 (Applied Biosystems, Inc.), and specificity evaluated *in silico* using BLASTn and Ribosomal Database Project (RDP) searches against GenBank and the RDP database. The 5' *Taq* nuclease assays were conducted in 25 µl volumes, using RealMasterMix probe mix (Eppendorf) with each primer at a final concentration of 900 nM, and probe between 250 and 50 nM. The probe concentration for each assay was optimised to provide the most desirable ΔF and C_T values. The initial default thermal profile was 94ºC for 1 minute, followed by 40 cycles of denaturation at 94ºC for 10 s and annealing/extension at 64ºC for 30 s. Assay specificity for all four assays was evaluated against 0.1 ng of genomic DNA per assay from pure cultures of the bacterial isolates (predominantly rumen) presented in Table 2. These isolates included the kangaroo isolates used in this study.

Table 2: Strains and isolates used to confirm specificity of the assay primers and probes

Species	Strain/ID
Bacteroides fragilis	683
Butyrivibrio fibrisolvens	AR12
Butyrivibrio fibrisolvens	AR27
Butyrivibrio fibrisolvens	AR73
Butyrivibrio fibrisolvens	ATCC 19171[1]
Butyrivibrio fibrisolvens	YE44
Clostrididiae-like isolate	YE257
Clostrididiae-like isolate	YE266
Clostridium butyricum	YE12
Clostridium butyricum	YE15
Clostridium glycolicum	YE255
Eschericia coli	ATCC 15766[1]
Eschericia coli	YE254
Eubacterium ruminantium	AR2
Eubacterium rectale	YE131
Fusobacterium necrophorum	AR4
Lactobacillus sp.	YE07
Lactobacillus sp	YE08
Lactobacillus sp	YE16
Megasphaera elsdenii	YE34
Prevotella ruminicola ss *brevis*	AR20
Prevotella ruminicola	AR29
Prevotella sp.	YE139
Ruminococcus flavefaciens	AR45
R. productus/hansenii	YE168
Ruminococcus albus	AR67
Selenomonas ruminantium	AR55
Shigella sp.	YE261
Streptococcus bovis	AR25
Streptococcus bovis	SB15
Streptococcus bovis	YE01
Streptococcus bovis	2B
Streptococcus intermedius	AR36
Whole bovine rumen contents	"66"

[1] *strain obtained from the American Type Culture Collection.*

Pure cultures of each strain were enumerated by direct count in a Petroff-Hauser chamber. Quantitative standards were prepared for each assay by inoculating resuspended bovine rumen fluid cell pellets with directly enumerated pure cultures of the target isolate to give target cell densities in a logarithmic series from 10^{10} to 10^{3} cells/mL. Total genomic DNA was prepared from 1 mL of the cell suspensions by physical disruption using a bead beater as described by Whitford *et al* (1998) or the modified procedure described by Yu and Forster (2005), and included in the 5' *Taq* nuclease assays. The extraction technique used to prepare the quantitative standards matched that used to prepare the genomic DNA from the assayed samples. Log (target species cells/mL) vs C_T was plotted, and used to estimate the concentration of the target bacterial species in the assayed samples.

3.1.3 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), or as modified by Yu and Forster (2005).

The hypervariable V2/V3 region of the bacterial 16S rRNA gene was amplified from all of the bacteria in each sample. The V2/V3 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). Almost full length 16S rRNA genes were amplified using primers 27F (5'- AGA GTT TGA TCM TGG CTC AG -3') and 1525R (5'- AAG GAG GTG WTC CAR CC -3') (Lane 1991).

Denaturing gradient gel electrophoresis (DGGE) was used to separate PCR products into a profile showing the diversity of bacteria present in the sample. The denaturant gradient used in the gels ranged from 30 to 60% urea/formamide denaturant. A customised ladder consisting of V2/V3 PCR products with defined migrations in the gel gradient was also loaded onto the gel to allow comparison between denaturing gradient gels. The sample V2/V3 products were loaded and the gel 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 using the method of Kocherginskaya *et al* (2005) to visualise the DNA. Images of gels were obtained using a flat bed scanner and GelCompar software (Bio-Rad Laboratories, Hercules, CA).

3.1.4 Fermenter methodology

The methodology associated with using the fermenter is well established and the basic operation was similar to that published in relation to the production of a live inoculum for cattle grazing *Leucaena* (Klieve *et al* 2002). A large set of starter cultures were created from a fermentation inoculated with rumen contents from a rumen cannulated steer grazing pasture at the Animal Research Institute, Yeerongpilly. Fermenter liquor (50 mL aliquots) was combined with an equal volume of rumen fluid/glycerol medium and stored frozen at -20^{o}C. Each fermenter run in this project was initiated with a starter culture from this set to ensure that each experiment started with a nearly identical population of rumen microbes.

Fermentation was commenced with a 100 mL starter culture being added to 2 L of a rumen-fluid-based (RF) medium. At commencement of the fermentation 30 g of finely ground spear grass was added as substrate for the fermenter. On the second day of fermentation the amount of ground hay added was reduced to 10 g and maintained at this level thereafter. Every 24 hours, half of the fermenter liquid was removed and replaced with an anaerobic balanced salts solution. The fermenter vessel was maintained at 39^{o}C and continuously bubbled with a mixture of CO_2:H_2 (95:5 v/v) to maintain anaerobic conditions. On the second day of each experimental run either a pure culture of one of the fibrolytic kangaroo isolates or pure cultures of two isolates were inoculated into the fermenter, following replacement of fermenter liquor with balanced salts solution, to give a final density of 1×10^7 cells/mL of each bacterium. The experiment schedule is given in Table 3.

Table 3: Schedule of kangaroo fibrolytic strains inoculated into fermenter

Experiment ID	Inoculated strain/s
Control	No isolate inoculated
YE137	*Ruminococcus* sp. isolate YE137
YE125/YE161	*Ruminococcus flavefaciens* isolate YE125 and *Butyrivibrio* sp. isolate YE161
YE166	*Ruminococcus*-like isolate YE166

To determine effects of plant fibre digestion in the ruminal environment, 12 nylon bags attached to a metal support were inserted into the fermenter. Each bag contained a pre-weighed amount of ground spear grass (approx 1 g). At zero time and at two day intervals, two bags were removed to determine dry matter disappearance. Total fermentation time per experiment was 11 days.

Each day, at the time of replacement of fermenter liquor with fresh salts solution, samples of fermenter liquor were collected for determination of volatile fatty acid (VFA) production (Ouwerkerk and Klieve, 2001) and to enumerate the fibrolytic kangaroo isolates by real time PCR.

3.2 Pen trial – inoculation of cannulated steers with kangaroo-derived highly fibrolytic bacteria

3.2.1 Trial overview

Twelve cannulated steers were individually penned for 36 days. The steers were allocated into four treatment groups of three steers, following an initial seven day adaptation period to the diet of chaffed spear grass hay, supplemented with cotton seed meal, nitrogen (in the form of urea) and a commercial mineral block. Three of the four groups of three steers were inoculated with combinations of four fibrolytic species isolated from kangaroo foregut contents (Table 4). The fourth group were an uninoculated control group. Samples of rumen contents were taken seven days prior and just prior to inoculation, and one, four, seven, 10, 14, 21 and 28 days after inoculation. These samples were assayed for the presence of the inoculated species using real time PCR assays.

Table 4: Experimental group and fibrolytic species inoculated

Experimental group	Inoculated strain/s
1	Control - no species inoculated
2	*Ruminococcus*-like isolate YE166
3	*Ruminococcus flavefaciens* isolate YE125 and *Butyrivibrio sp.* isolate YE161
4	*Ruminococcus sp.* isolate YE137, and isolates YE125, YE161 and YE166

3.2.2 Cannulated Steer Management

The cannulated steers (eight *Bos indicus* cross, four *Bos indicus* × *gertrudis*) were obtained on loan from University of Queensland's Mt Cotton Research Farm. The 12 animals had been grazing on pangola grass pastures for 2-3 months prior to the commencement of the trial. Initial measurement of liveweights were taken prior to departure from Mt Cotton, and liveweight ranking was used to allocate two *Bos indicus* cross and one *Bos indicus* × *gertrudis* steer to each of the four experimental groups.

Particular care was taken with handling practices and the physical layout of the pens in order to minimise the risk of spread of inoculated bacterial species between different groups of cattle. It has been demonstrated in previous work that rumen bacteria can be spread from one animal to another via airborne vectors/aerosols (FLOT.125 Final Report; R. Jones, pers. comm.; Bräu, L. *et al*, 2000). Saliva has also been reported to be an effective vector for the transmission of anaerobic rumen bacteria (Dehority and Orpin, 1988).

Steers were individually housed in 8.6 m^2 (2.3 x 3.7 m) pens in an insect-proof isolation facility. The facility was divided by a central cattle crush area into an east and west end with identical pens. The control group was housed in the east end of the facility, while three inoculated groups were housed in the west end, with an empty pen buffer or the central walkway separating the groups (Figure 1).

1	1			*Crush area*	2	2	2					
Eastern end					*Western end*							
1					3	3	3			4	4	4

Figure 1. Simplified layout of isolation facility pens, with occupied pens and crush area identified. Numbers in pens indicate experimental group, and dark grey pens were empty.

The central crush area was enclosed at either end with swinging doors, normally kept shut to minimise airflow between the two ends of the facility. Wind direction was typically from the east.

Steers were fed once daily, with the residue of the previous day's feed cleared and weighed prior to new feed being presented. The pens were cleared and steers fed in order of experimental group (group 1, followed by 2, etc), with staff changing gloves between clearing the pens and feeding the steers, and between activities with different experimental groups. The daily ration consisted of:

- Spear grass hay: an initial ration of 10 kg chaffed spear grass hay was presented to each animal, and was increased to maintain *ad libitum* intake by increasing the previous day's intake by 10% when the previous day's intake was entirely eaten.
- Cotton seed meal: 2 kg solvent extracted cotton seed meal was provided each day in a separate feed bin.
- Urea: The spear grass hay was sprinkled with concentrated liquid urea, and mixed thoroughly. The initial ration of urea was 20 g in solution, and this was increased to 40 g in solution after one week.
- Mineral block: a Rumevite Blue/White mineral stock block containing no urea (Ridley AgriProducts, Parkenham, VIC) was placed in a feed bin in each pen.

Cattle were routinely assessed during feeding and cleaning for general well-being and body condition.

Cattle were walked from their pens to the central crush area one experimental group at a time (group 1/controls first, followed by groups 2, 3 and then 4). Liveweights of the steers were recorded while they were in the crush. Each group was returned to their original pens before the next group was brought to the crush. The crush and race area were thoroughly cleaned with water between groups 2 and 3, and following group 4. The crush area was not cleaned between groups 1 and 2, and between groups 3 and 4, as there was no risk of spreading inoculated strains from group 1 to group 2, or from group 3 to group 4.

3.2.3 Inoculation with fibrolytic species.

500 mL cultures of each inoculant strain were prepared in either a rumen fluid (RF) based medium with cellobiose (YE125, YE137 and YE166) or RF based medium with birchwood xylan (YE161). Samples (1 mL) were taken from three cultures of each strain prior to inoculation. These were centrifuged, the supernatant discarded and the bacterial pellets frozen at -20ºC, for later DNA extraction and enumeration of bacterial cells by real-time PCR. Following the collection of samples of rumen contents on day 8, each animal was inoculated with a 500 mL culture of each inoculate strain according to the schedule described in table 4 using a tube inserted via the cannula into the rumen.

3.2.4 Sample collection and processing

Prior to daily feeding on days 1, 8, 9 12, 15, 18, 22, 29, and 36, samples of rumen contents were collected and strained through nylon stocking. Between 50 and 100 mL of rumen fluid was collected from each steer and placed on ice prior to further processing. Thereafter, four 1 mL aliquots of each sample were pelleted by centrifugation, the supernatant discarded and the pellet stored at -20ºC prior to genomic DNA extraction.

3.3 Identification of fibre-associated bacterial species within the kangaroo forestomach

3.3.1 Kangaroo forestomach samples

To augment the collection of samples previously used in this project, samples of forestomach contents were taken from freshly harvested Wallaroos, Red kangaroos and Eastern Grey kangaroos, from five locations throughout western and central Queensland (Table 5). From the liquid fraction of the digesta, 1 mL aliquots were taken, centrifuged, and the pelleted bacteria stored frozen at -20 ºC for subsequent DNA extraction. In addition, representative samples of entire forestomach contents, including partially digested feed material, were also stored frozen.

Table 5: Kangaroo forestomach samples collected for examination inB.NBP.0351

Location (QLD)	Red	Eastern Grey	Wallaroo	Year collected
Charleville	2	2	-	2000
Charleville	3	5	-	2002
Charleville	2	2	2	2007
Longreach	2	2	2	2007
Cloncurry	3	-	3	2007
Charters Towers	-	4	-	2007
Dingo	-	5	3	2007

Genomic DNA was extracted from stored samples following the procedure of Yu and Forster (2005). In order to remove bacteria not associated with solid material and increase the proportional representation of species associated with digesta, aliquots (0.3-0.5 g) of total forestomach contents were subjected to two cycles of vigorous agitation for 20 seconds in 20 mL 1 × Phosphate Buffered Saline (PBS), with the PBS-cell suspension decanted between cycles and replaced with fresh PBS. This washing was performed to reduce the density of bacteria not associated with plant material while not actively disrupting any biofilm on the digesta. Excess liquid was removed from the washed digesta by squeezing, and genomic DNA extracted from 0.1 g of the digesta. Extracted genomic DNA was used in subsequent real time PCR assays and for DGGE analysis.

3.3.2 Identification of dominant bacterial species

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

- The intensity of the band on the gel.
- 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 V2/V3 region was then re-amplified by PCR and a clone library of the PCR products produced in *E. coli* cells (commercial cloning kit). 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 V2/V3 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 V2/V3 PCR product migrated to the same position as the band that the stab was taken from. Five clones containing the correct insert had their V2/V3 region sequenced (Griffith University sequencing facility) and three identical sequences from different clones was deemed sufficient for assigning the sequence to a specific band (Klieve *et al*

2007). The sequence was subsequently compared with others in the GenBank database to determine species identity and phylogenetic position (Ouwerkerk and Klieve, 2001).

A second set of clone libraries was constructed from amplification of the complete 16S rRNA gene from genomic DNA, in order to allow more comprehensive sequence analysis of the bacteria represented by each clone. The V2/V3 region contains approximately 170 bases, while the complete 16S rRNA gene contains approximately 1500 bases. The complete gene, while unsuitable for DGGE analysis in its entirety, allows DGGE analysis of the V2/V3 region within the gene but also provides more data for phylogenetic analysis. The procedure followed was similar to that described above, except complete 16S rRNA genes of bacteria were amplified from extracted genomic DNA by PCR as described earlier. The amplified products were cloned, and the V2/V3 region of each clone screened by DGGE as described above.

4 Results and Discussion

4.1 Evaluation of *in vitro* persistence of fibrolytic bacteria from Kangaroos

4.1.1 Real time PCR assay development

The following primer and probe sets were designed for real time PCR (5' *Taq* nuclease) assays of each of the indicated strains.

Table 6: Primer and probe sequences identified

Target isolate	Primer/probe name	5'-3' sequence, including terminal modifications
YE125	YE125F2	TGA CCT GGG CTA CAC ACG TAC T
YE125	YE125R2	TGA GAT TCG CTC CAC ATT GC
YE125	YE125P2	**FAM-**AAT GGC AAT TAA CAA AGA G-**BHQ**
YE137	YE137F1	GAA ACG GAT GGT AAT ACC TCA TAA CA
YE137	YE137R1	GAG TCC ATC TTT GAG TGA TAA ATC TTT G
YE137	YE137P1	**FAM-**TTA GAA GGG CAT CCT TT-**BHQ**
YE161	YE161F1	GCC ACC GAT TGG CTA TGC
YE161	YE161R1	GGT AGT CCA CGC CGT AAA CG
YE161	YE161P1	**FAM-**ACC AAC AGC TAG TAT TC-**BHQ**
YE166	YE166F2	GCA ACG CGA AAA ACC TTA CC
YE166	YE166R2	GGT CCG AAG AAA AGG CTT ATC TC
YE166	YE166P2	**FAM-**ATG GAG AGG ACC GGC T-**BHQ**

Searches against the GenBank and RDP databases did not yield any complete matches to other known DNA sequences to the regions of the 16S rRNA gene targeted by the assays. The closest matches for each amplicon to sequences held in the GenBank database are presented in appendix 1.

As the 16S rRNA gene regions targeted by these assays were identical in Ruminococcus-like strains YE125 and YE137, the assay for YE125 (primers YE125F2 and YE125R2, and probe YE125P2) was used for subsequent quantitation of these two bacterial isolates.

The specificity of each real time PCR assay was evaluated against a panel of ruminal bacteria, which had been grown to maximum cell density in liquid culture, centrifuged and genomic DNA extracted. The extracted gDNA for all panel members was set to 1ng/µL (representing approx. 10^8 cell/mL). Each target strain was detected with its specific assay at the correct level and other non-specific amplification represented less than 1% of the cells present in the sample (Table 7) – for example *R. flavefaciens*-like isolate YE125 was present at levels of 5.65E+07 cells/mL in its specific real time PCR assay and at less than 1% of the cells present in the YE161 real time PCR assay (2.48E+03 cells/mL). The detectable limits were set at levels of 10^3 cells/mL.

Table 7. 5' Taq *nuclease assay specificity evaluation*

	YE125 assay	YE161 assay	YE166 assay
Bacteria	cells/mL	cells/mL	cells/mL
Bacteroides fragilis 683	0	1.17E+01	0
B. fibrisolvens AR12	0	4.51E+02	2.32E+00
B. fibrisolvens AR27	0	0	0
B. fibrisolvens AR73	0	2.00E+02	0
B. fibrisolvens ATCC 19171	0	1.51E+00	0
B. fibrisolvens YE44	0	0	0
B. fibrisolvens-like isolate YE161	0	2.97E+08	0
Butyrate producing bacterium ART55/1-like YE129	0	0	0
Clostridiidiae-like isolate YE257	0	0	1.92E+03
Clostridiidiae-like isolate YE266	0	0	1.48E+03
Clostridium butyricum YE12	1.24E+04	1.48E+04	0
Clostridium butyricum YE15	5.40E+04	7.16E+04	0
Clostridium glycolicum YE255	0	0	2.46E+04
E. coli ATCC 15766	0	0	0
E. coli YE254	0	0	0
Eubacterium ruminantium AR2	0	0	0
Eubacterium rectale YE131	0	1.09E+03	0
Fusobacterium necrophorum AR4	0	0	0
Lactobacillus sp. YE07	0	0	0
Lactobacillus sp. YE08	0	0	0
Lactobacillus sp. YE16	0	0	0
Megasphaera elsdenii YE34	0	0	0
Prevotella ruminicola ss *brevis* AR20	0	2.06E+00	0
Prevotella ruminicola AR29	0	3.75E+00	0
Prevotella sp. YE139	0	0	0
R. flavefaciens AR45	4.59E+04	0	0
R. flavefaciens-like isolate YE125	5.65E+07	2.48E+03	0
R. flavefaciens-like isolate YE137	4.70E+08	4.50E+01	0
R. flavefaciens-like isolate YE166	0	0	4.53E+08
R. productus/hansenii YE168	9.71E+03	7.53E+01	0
R. albus AR67	0	0	0
Selenomonas ruminantium AR55	0	0	0
Shigella sp. YE261	0	0	0
Streptococcus bovis AR25	0	0	0
S. bovis Sb15	0	0	0
S. bovis YE01	0	0	0
S. bovis 2B	0	0	0
S. intermedius AR36	0	0	0
Whole bovine rumen contents "66"	0	0	0

Shaded figures indicate the target species for each assay. 0 - indicates no positive signal detected.

4.1.2 *In vitro* colonisation and persistence in a rumen-based fermentation by kangaroo fibrolytic bacteria

Four duplicated fermenter-based experiments were conducted, in which the fibrolytic kangaroo foregut bacteria were inoculated into a fermenter and key digestion and fermentation parameters were evaluated. The fibrolytic bacteria were inoculated into the fermenter either singly or in combination (see table 3).

Nutritional analysis of the spear grass utilised as feed during the fermentation experiments indicated 72.3% neutral detergent fibre and 0.25 % nitrogen as a proportion of dry matter.

Effect on fibre utilisation

The effects of the addition of the fibrolytic bacteria on fibre utilisation are presented in Figures 2 and 3.

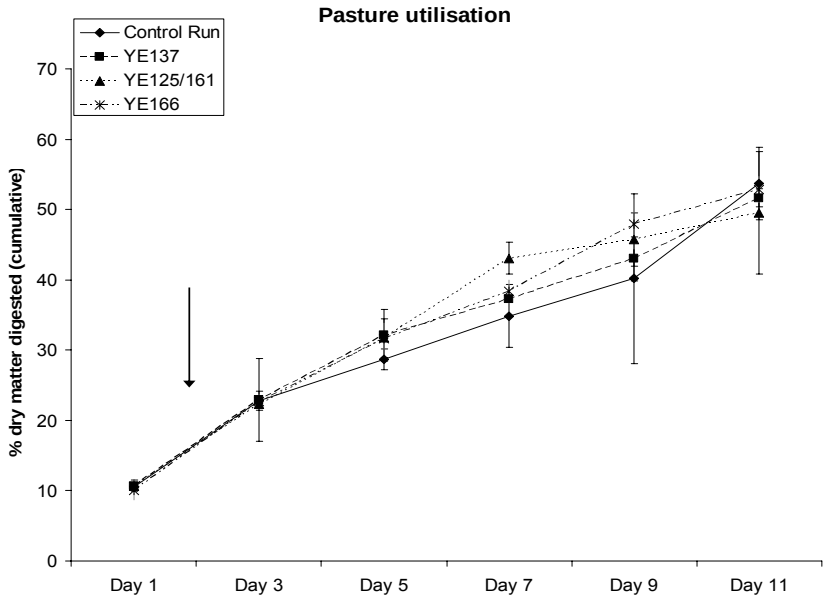


Figure 2. Percent removal of ground spear grass from nylon bags over time in the fermenter. Each data point is the average of two fermentations. Bars show standard deviation from the mean. Kangaroo fibrolytic strains were added following sample collection on day 2 (indicated by arrow).

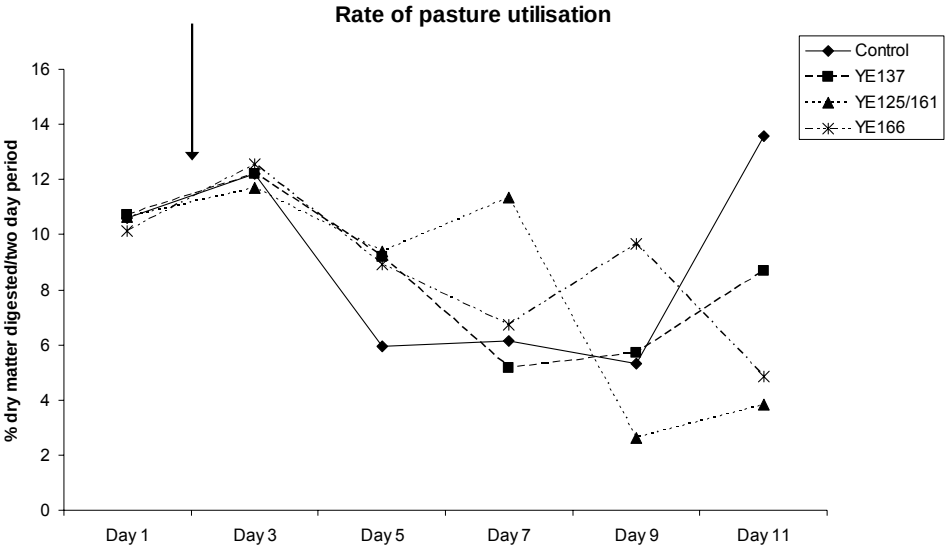


Figure 3. Percent removal of ground spear grass from nylon bags in the fermenter per sampling cycle. Each data point is the average of two fermentations. Kangaroo fibrolytic strains were added following sample collection on day 2 (indicated by arrow).

The addition of the kangaroo fibrolytic strains had a noticeable effect on the rate of dry matter disappearance within the fermenter. This effect was most evident between days 3 and 5, where rate of digestion was increased from 5.9 % loss in the control experiments to between 8.9 to 9.4 % loss in the same time period when the kangaroo isolates were present. The only fibrolytic

strain to persist in the fermenter system beyond day 6 (see below) at detectable levels was YE125 (see Figure 6.b), in which fibre digestion rates were high in the middle stages of the fermentation (days 3 to 7) before dropping dramatically following day 7. This sharp decrease in the rate of dry matter loss is likely to be a result of reaching the limit of digestibility of the substrate. All the dry matter digestion experiments reached a maximum of approximately 50% dry matter loss after 11 days, with the YE125/YE161 combination digesting 43.1% (compared to 34.9% in the controls) by day 7.

The increase in rate of dry matter digestion observed when kangaroo fibrolytic isolates were present suggests that these marsupial bacteria may play a more significant role in a continuous fermentation system such as the rumen, where feed material is constantly removed and replaced with fresh material at a rate of approximately 2% per hour on low quality forage (Bowen, 2003). An increased rate of digestion would allow a faster rate of throughput (i.e. increased intake), and therefore more of the cellulosic material in the feed to be converted to microbial protein and VFA's in the rumen, improving feed utilisation efficiency. However, between fermentation variability in digestibility was high and differences relatively small.

VFA production

The daily production of total VFA, acetate, propionate and butyrate are presented in Figure 4.

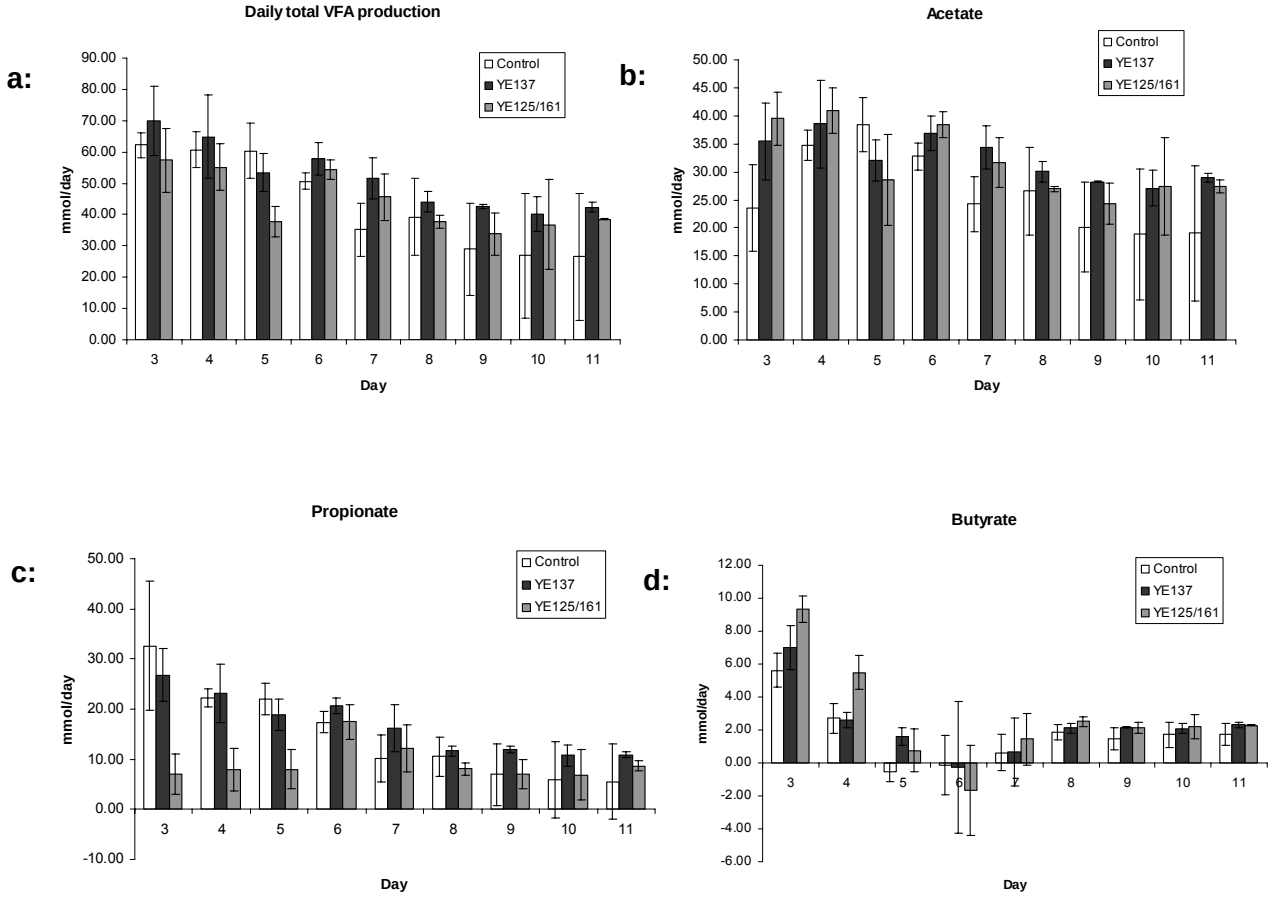
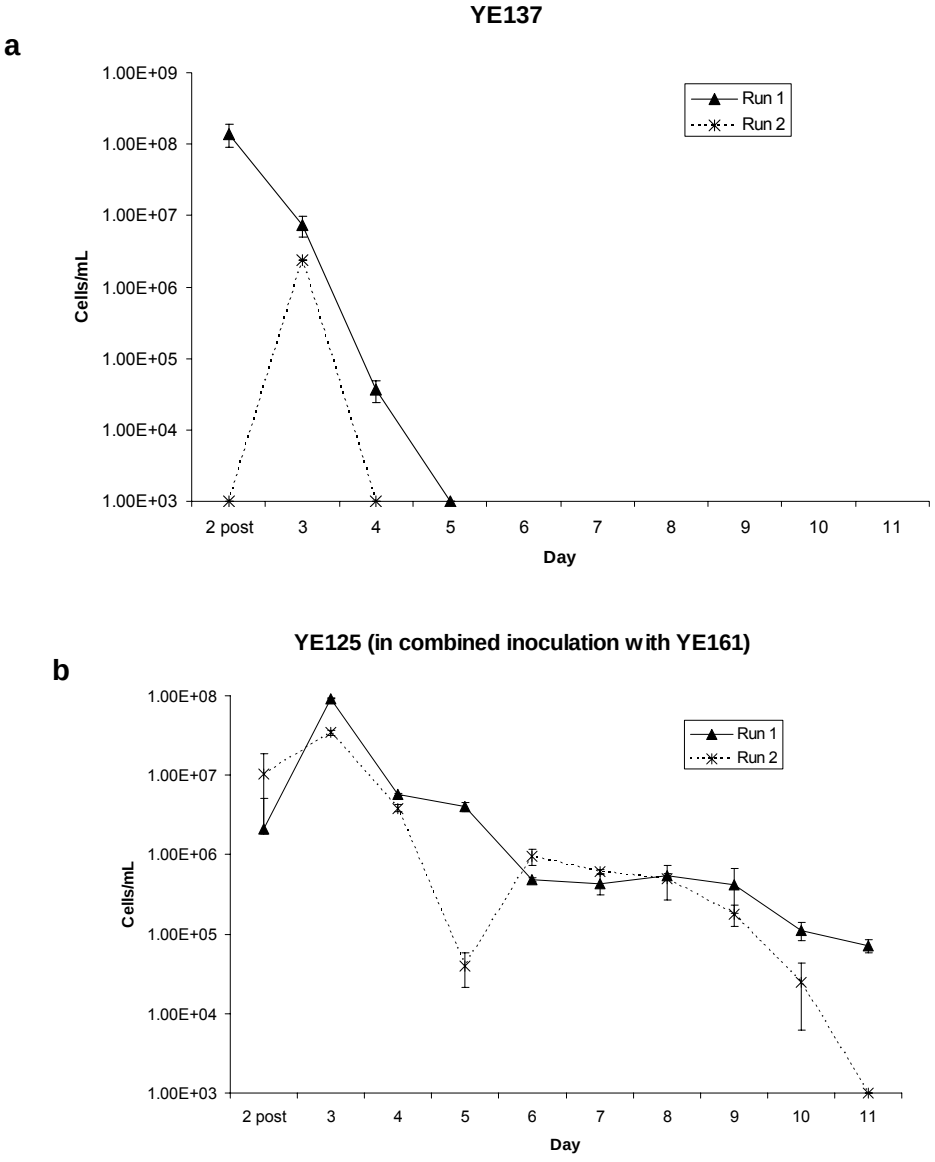


Figure 4. Daily average total VFA (a), acetate (b), propionate (c) and butyrate (d) production. Results of two fermentations for each bacterial isolate were averaged. Bars represent one standard deviation. Bacterial isolates were inoculated on day 2.

VFA production was variable between experiments, but there was a noticeable trend towards increased acetate and decreased propionate production, particularly early in the fermentations when kangaroo isolates were added. This would correlate with the increased rate of fibre digestion during this period as *Ruminococci* typically produce acetate from cellulose digestion (Mackie *et al*, 2002). Again, between fermentor run variability was high.

Bacterial population density

The total numbers of the kangaroo fibrolytic isolates persisting in fermentations, as determined by real-time PCR, are presented in Figure 5. The control experiments were assayed for the four kangaroo isolates, and none were detected at any stage of the control fermentations.



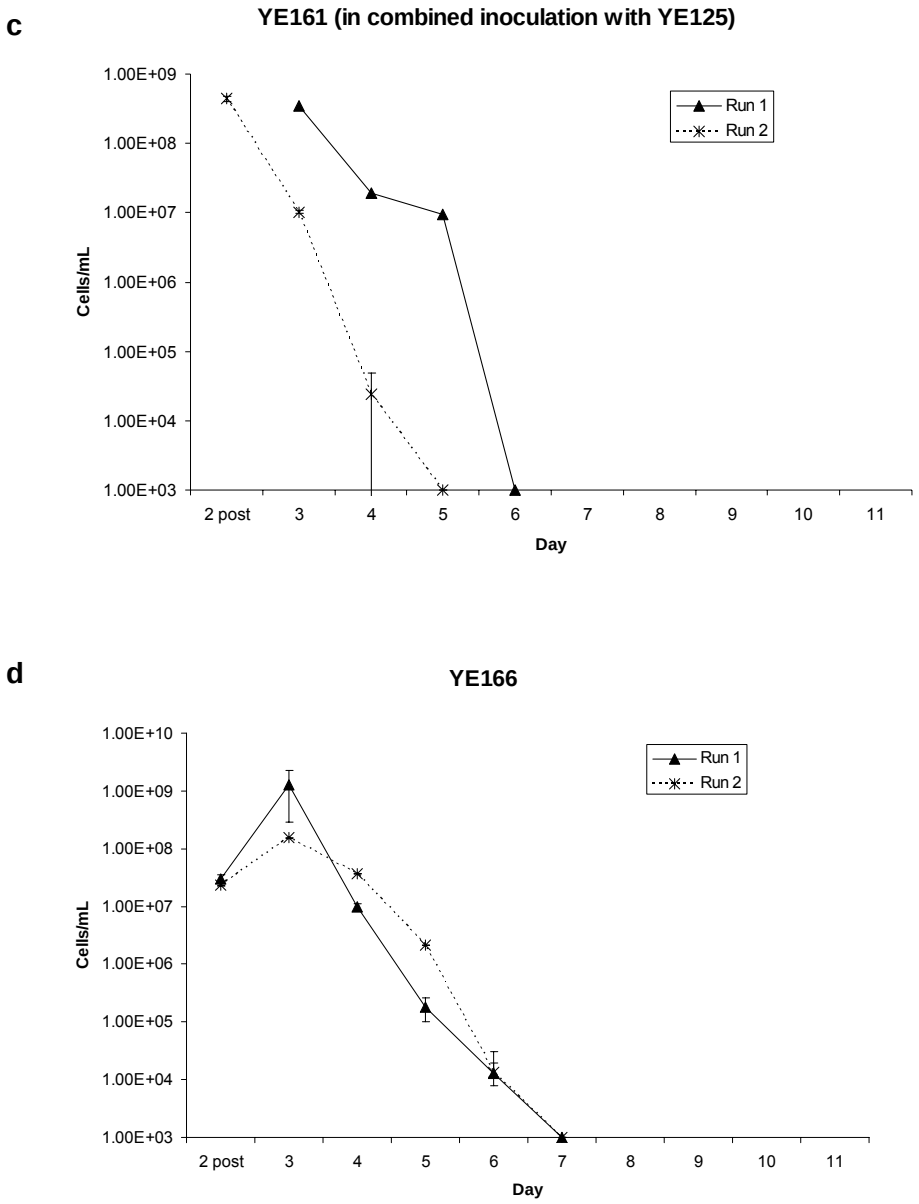


Figure 5. Bacterial cell equivalents in fermentation liquor. Cultures of the fibrolytic isolates were added to the initial population on day 2, or day 3 for YE125/YE161 run 1. a: enumeration of Ruminococcus sp. *YE137; b: enumeration of* R. flavefaciens *YE125; c: enumeration of* Butyrivibrio sp. *YE161; d: enumeration of* Ruminococcus*-like YE166.*

Most isolates did not establish well in the fermenter and followed a consistent pattern of reduction to numbers below detectable levels over the course of 3 to 5 days following inoculation. The exception to this trend was *R. flavefaciens* isolate YE125, which stabilised at between 10^5 to 10^6 cells/mL following an initial increase in cell density. The isolates that apparently did not establish in the fermenter (YE137, YE161 and YE166) may have been maintained at a low population density ($<10^3$ cells/mL).

4.2 Pen trial – inoculation of cannulated steers with kangaroo-derived highly fibrolytic bacteria

Although most of the isolates did not establish populations in a fermentor with rumen-based microbial populations, it was decided to determine whether the isolates would establish in the rumen of cannulated steers, where they would receive a better mix of nutrients than was available in the fermentations that were undertaken.

4.2.1 Enumeration of fibrolytic species by real time PCR

Enumeration by real time PCR of the 1 mL aliquots taken from the inoculant cultures, immediately prior to inoculation, gave the following estimates of target species concentration: YE125: 5.64×10^7 cells/mL; YE137: 1.69×10^8 cells/mL; YE161: 2.64×10^8 cells/mL; YE166: 6.67×10^7 cells/mL;

The density of cells in the inoculate cultures suggest that the initial inoculation would have been well within detectable limits by real time PCR (i.e. in excess of 10^3 to 10^4 cells/mL) once it had been dispersed throughout the rumen. This process will have diluted the initial inoculant density by a factor of approximately 100 (dependent upon the volume of the individual animal's rumen), giving an initial density in the range of 10^5 to 10^6 cells/mL for the strains inoculated into the steers. Previous work has shown that real time PCR can detect and enumerate inoculant bacterial species shortly after inoculation (Klieve *et al* 2003; FLOT.125 Final Report).

However, real time PCR of rumen fluid samples collected throughout the trial, both pre- and post-inoculation, did not detect YE125, YE137 or YE161 in any sample. Results from the YE166 real time PCR assay showed consistent estimates of 10^4 to 10^5 cells/mL for all animals, including the control group, both pre- and post-inoculation. Investigations retesting log dilutions of a number of samples in the YE166 real time PCR assay gave increased C_T values of between 3 and 3.5 for each log dilution, results indicative of template-dependent amplification. These results suggest that the estimates obtained through YE166 assay are not due to non-specific degradation of the probe, but are rather due to detection of a target sequence. Increasing the annealing/extension temperature from 66 ºC to 68 ºC increased the observed C_T values by approximately five cycles and this increase is consistent with an inefficient amplification of target template/s that are similar but not identical in sequence to the target YE166. These results suggest the presence of a *Ruminococcus*-like species, related to YE166, in the steers as part of their established bacterial community. The presence of related strains had not been detected in the specificity evaluations (which included rumen fluid from a steer) of the real time PCR assay for YE166 (Table 7). The estimates of 10^4 cells/mL obtained for this YE166-like species are going to be significantly lower than the actual concentration of this species, due to the aforementioned inefficient detection.

The inability to detect the isolates in samples taken on the day following inoculation (inoculation on day 8, sample collected 24 hours later on day 9) was surprising, given that a turnover of approximately 2 rumen volumes of the liquid phase of the rumen contents could be expected over the course of 24 hours (Beveridge and Lang, 1981), and turnover of the solid phase (and attached bacteria) is at a lower rate. The rapid decrease from the initial cell density to below detectable limits within 24 hours suggests that washout and cell death far exceeded any replication of the inoculant bacteria that may have taken place.

Previously, it was shown that YE136, YE161 and YE166 were not able to colonise and establish significant populations in a rumen-like fermentation, although YE125 did establish a sustainable population in similar experiments. This inability to establish a viable population was, at that time, attributed to the harshness of the feed material fed to the fermentor and the lack of other nutrients that could be expected to be found in the rumen. This was not the case with inoculation of the steers. In addition, unlike the aforementioned strains, YE125 did establish in the fermentor but fared no better than the other strains when inoculated into the rumen proper. During the course of these fermentations, the populations of the former three isolates declined to below detectable limits over the course of 3 days (YE137 and YE161) or 5 days (YE166).

The high levels of fibrolytic activity displayed by the selected kangaroo isolates indicate a role in the digestion of insoluble feed particles, which requires attachment to the feed particles. At this stage, no data is available to indicate why the kangaroo isolates did not establish, however, subsequent investigations into possible reasons for the complete lack of any establishment of the strains in the rumen environment found that a range of mechanisms are used to facilitate bacterial attachment to feed, including non-specific mechanisms and specific adhesins and receptors that are subject to regulation (Miron *et al*, 2001). It has also been shown that growth of cellulolytic rumen bacteria in media containing cellobiose rather than plant cell walls reduces the

ability of the strain to attach to, and degrade plant cell walls (Kim *et al*, 1999; Miron and Ben-Ghedalia, 1993). Unfortunately, all fibrolytic strains used in the fermenter studies and inoculated into the rumen were grown in cellobiose. This may have resulted in the down-regulation of adhesins in the inoculated strains, potentially contributing to the inability of the kangaroo bacteria to attach to substrate, which could explain their total lack of survival. Adherant bacteria typically form digestive consortia as part of a biofilm providing a micro-environment for the consortia. Growth of the fibrolytic strains on media containing the native grass of interest could increase their ability to establish stable, surface-associated populations.

There are several other possible reasons for the lack of colonisation and survival of the fibrolytic strains, these include predation by protozoa, or susceptibility to bacteriocins secreted by the ruminal microflora. The fact that the introduced strains would have had to compete with and displace existing established fibrolytic populations is a big hurdle to overcome. Prior to weaning, calves have a rumen microbial population that is distinct from mature cattle and which undergoes a transition to the mature population when the calf starts to feed on plant material (Dehority and Orpin, 1988). Therefore, populations of plant fibre degrading bacteria do not become fully developed until shortly after weaning. Inoculation of calves at weaning with kangaroo fibrolytic bacteria may aid in the introduction and establishment of these bacteria in the rumen ecosystem.

4.3 Variation to project objectives

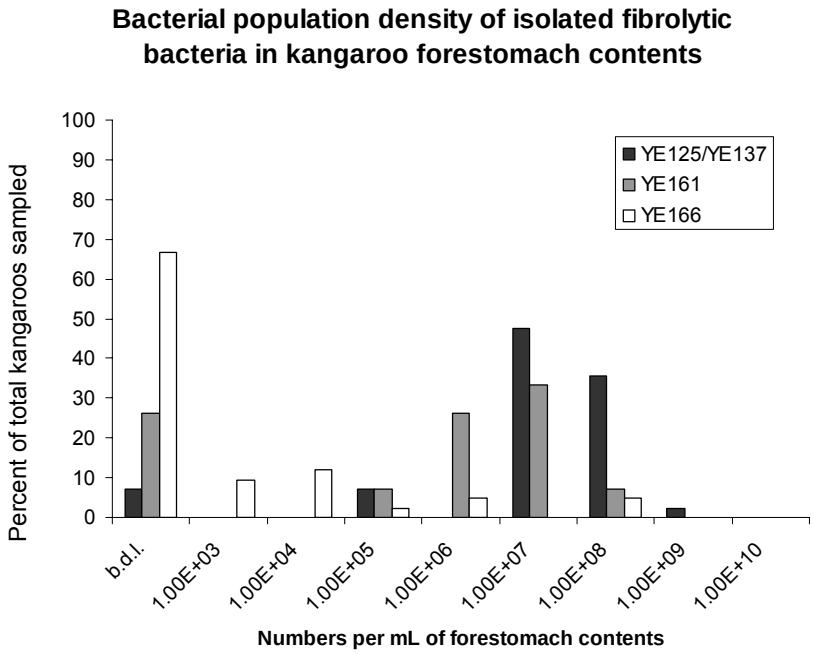
Although one species of culturable fibrolytic bacteria from the kangaroo forestomach did show some persistence *in vitro,* none colonised the rumen of cannulated cattle in the feeding experiment. Therefore, it was agreed that the remainder of the project would be restructured with the primary aim of determining the most important plant fibre degrading species in the kangaroo foregut and which species interact with each other.

4.4 Importance of culturable fibrolytic kangaroo bacteria and identification of yet-to-be cultured fibre-associated species within the kangaroo forestomach

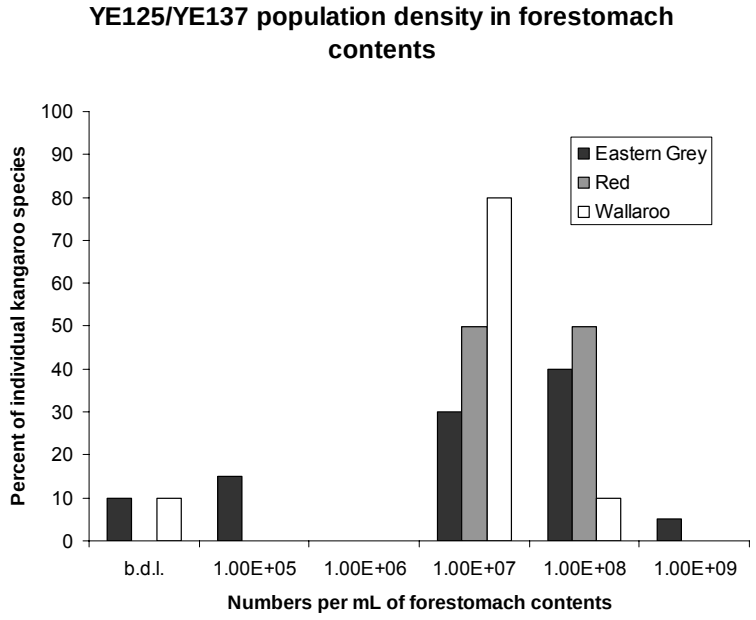
4.4.1 Enumeration of previously isolated kangaroo fibrolytic bacteria

The population densities of the four fibrolytic bacteria isolated previously in this project were determined, by real time PCR, in forestomach content samples collected from 42 kangaroos (Table 5). Results are presented in Figure 6. Data are presented as frequency graphs with the percentage of sampled kangaroos that had specific bacterial populations within each log value range (e.g. numbers of bacteria between 10^5 and 10^6 per mL) being plotted.

a


Bacterial population density of isolated fibrolytic bacteria in kangaroo forestomach contents
Percent of total kangaroos sampled
100
90
80
70
60
50
40
30
20
10
0
YE125/YE137
YE161
YE166
b.d.l.
1.00E+03
1.00E+04
1.00E+05
1.00E+06
1.00E+07
1.00E+08
1.00E+09
1.00E+10
Numbers per mL of forestomach contents

b


YE125/YE137 population density in forestomach contents
Percent of individual kangaroo species
100
90
80
70
60
50
40
30
20
10
0
Eastern Grey
Red
Wallaroo
b.d.l.
1.00E+05
1.00E+06
1.00E+07
1.00E+08
1.00E+09
Numbers per mL of forestomach contents

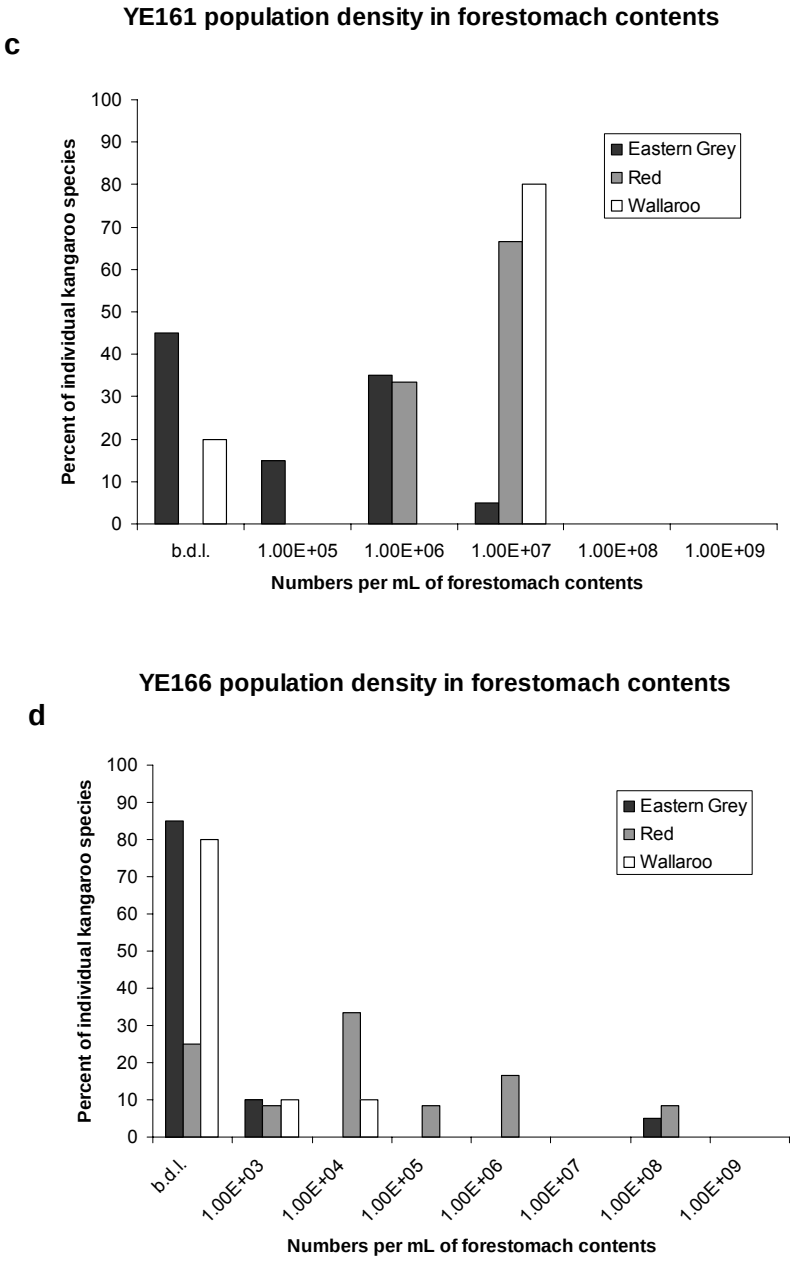


Figure 6. Frequency of occurrence of fibrolytic isolates YE125/YE137, YE161 and YE166 at given population densities in sampled wallaroos and kangaroos. b.d.l – below detectable limits. Scale is based on grouping on a logarithmic population density basis, for example, 1.00E+05 means population density was between 10^5 and 10^6 target cell equivalents per mL of rumen contents.

The population densities of the bacteria in the kangaroo forestomach samples suggest that these fibrolytic species are probably not the dominant fibrolytic species within the kangaroo forestomach. *Ruminococcus flavefaciens* YE125 and YE137, two isolates with virtually identical 16S rRNA genes (and inseparable by real-time PCR) but different cellulolytic activities, are probably the most useful and were present in the majority of samples at reasonable densities (10^7-10^9 cells/mL), and present in most individuals from all three species of kangaroo. *Butyrivibrio* sp YE161 was detected at lower densitiy (10^6 to 10^8 cells per mL), and not at all in 11 animals, 9 of which were Eastern Greys. This species appears to be of greater value to Wallaroos and Red kangaroos than in Eastern grey kangaroos. The overwhelming majority of samples (>80% of Wallaroos and Red kangaroos) did not have *Ruminococcus*-like isolate YE166 at detectable densities.

4.4.2 Identification of dominant fibre associated bacterial species

Overall bacterial cell densities for Eastern Grey kangaroo forestomach samples have been reported as high as 76×10^{10} g^{-1} (Dellow *et al*, 1988). The populations of the isolated fibrolytic bacteria (*Ruminococcus flavefaciens* YE125 and YE137, *Butyrivibrio* sp YE161, and *Ruminococcus*-like isolate YE166) would form less than 0.1% of the total bacterial population at best (data presented in 4.4.1). The small proportion that these isolates comprise of the total bacterial population (0.1% to below detectable limits) suggests that the four fibrolytic isolates currently in culture are unlikely to be a part of the fibrolytic "functional core" of bacteria in kangaroos, as they are not present in sufficient densities or frequencies to be key members of a conserved fibrolytic consortium. Other, yet-to-be identified or cultured bacteria probably play a more significant role in fibre degradation in these animals.

The increasing gulf between the dominant fibrolytic bacteria identified by isolation and through non-culture dependent methods suggests a bias during isolation for particular taxa, including *Ruminococcus*, the genus to which YE125, YE137 and YE166 belong. It has been suggested that a significant proportion of rumen cellulolytic activity may be due to novel, currently uncultured cellulolytic bacterial species (Stevenson and Weimer, 2007), as intensively studied and culturable ruminal bacterial species are a small proportion of the total bacterial populations in the rumen. This also appears to be the case with the kangaroo bacteria. The identification of the dominant bacterial species associated with plant material in kangaroos should allow the elucidation of a "functional core" for fibre digestion in kangaroos. These bacteria have not previously been identified and the culture-independent DNA profiling methodology of DGGE was used for this purpose.

Plant material from 30 forestomach samples collected in 2007 was washed with PBS, reducing the density of bacteria not associated with plant material while not actively disrupting any biofilm on the digesta. The hypervariable V2/V3 region of the 16S rRNA gene was amplified from genomic DNA extracted from this washed solid material, and analysed using DGGE, with dominant (intense) bands that were present in a large proportion of samples being selected for further investigation. DGGE profiles from 15 of these samples are presented in Figure 7.

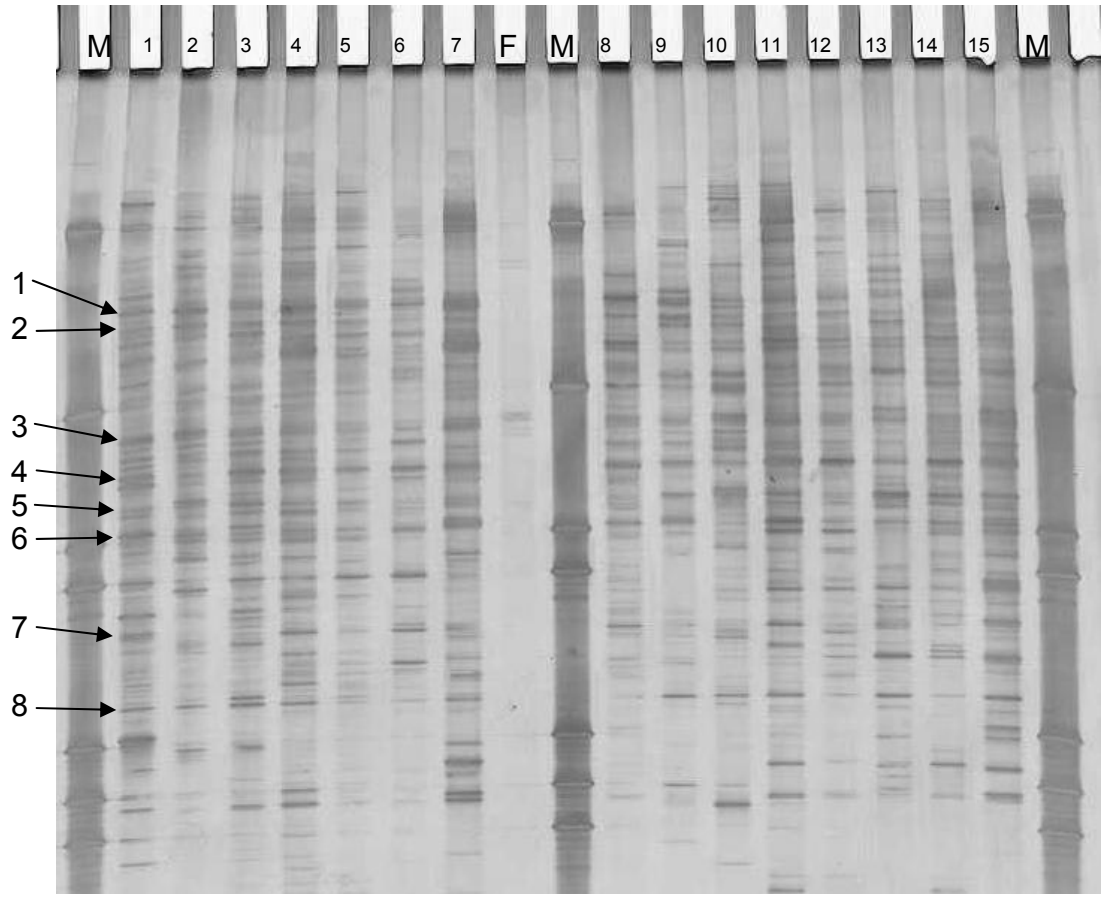


Figure 7. DGGE of 16S rRNA gene V2/V3 region of plant-associated bacterial gDNA extracted from kangaroo and wallaroo forestomach contents. M: DGGE markers; F: Fibrolytic isolates YE125, YE166 and YE161; 1-15: Kangaroo forestomach samples. Samples 1, 6, 7, 8, 13 and 15 are from Wallaroos, samples 2, 3, 9 and 10 are from Eastern Grey kangaroos, and samples 4, 5, 11, 12, and 14 are from Red kangaroos.

The absence of dominant bands corresponding to the fibrolytic isolates YE125, YE137, YE161 and YE166 further supports the real time PCR enumeration data that these bacteria are unlikely to play a significant role in plant fibre degradation. A total of eight dominant bands, corresponding to eight bacterial species, were identified as being present in all samples. These bands were selected for species identification by DNA sequencing and analysis.

Clone libraries of 16S rRNA genes were constructed from a representative animal of each species (a Grey Buck, a Red Buck and a Wallaroo Doe). The V2/V3 region was amplified from 226 16S rRNA gene clones and analysed by DGGE. Clones which displayed the same motilities as the dominant bands observed in the DGGE of the original forestomach genomic DNA were sequenced. Sequence data of the complete 16S rRNA gene from each selected clone was analysed for homology to other clones with similar motility, and to identify the closest phylogenetic relatives in the GenBank and Ribosomal Database Project databases.

A second set of clone libraries, derived from amplification of V2/V3 bands (bands 1 to 6) excised from gels and re-amplified, were constructed. Clones were screened by DGGE for the same motility as their source band, and selected clones sequenced. Sequence data from these libraries was compared to that from the complete 16S rRNA gene clone libraries (Table 8).

Table 8: Number of clones obtained that correspond to each identified dominant DGGE band, and nearest relative as indicated by DNA sequence analysis

Band	Number of 16S clones	Number of V2/V3 clones (from band excision)	Nearest relative
1	6	0	Bacteroides capillosus and Lachnospiraceae bacterium 19gly4 (94% over 172 bases)
2	3	2	*Pseudobutyrivibrio ruminis* CF1b (99% over 170 bases)
3	8	1	*Dorea formicigenerans* (99% over 170 bases)
4	6	5	*Clostridium polysaccharolyticum/C. populeti* (100% over 169 bases)

Band 1, was represented by 6 similar clones from both a red buck and a wallaroo doe, which shared 100% identity across the V2/V3 region. Across this region, the 6 clones each aligned equally with *Bacteroides capillosus* and Lachnospiraceae bacterium 19gly4 on both GenBank and RDP II databases. This is not a strong phylogenetic relationship with these species, however, its closest relatives are involved in fibre digestion (pectinolytic) and therefore it could be a member of the fibrolytic core.

The three clones of the 16S gene and two clones of the V2/V3 region which correspond to band 2 were identified as *Pseudobutyrivibrio ruminis. P. ruminis* is a butyrate producing rumen bacterium that is phenotypically similar to *Butyrivibrio fibrisolvens*, and is associated with hemicellulose degradation.

The eight 16S clones and one V2/V3 clone from both a grey buck and a wallaroo doe that represent band 3 were identified as being strains of the obligate anaerobe *Dorea formicigenerans*, originally isolated from human faeces. *D. formicigenerans* is a non-cellulosic bacterium, utilising a range of carbohydrates (fructose, glucose, lactose, maltose and galactose), and producing formic, acetic and lactic acids, and ethanol (Taras *et al*, 2002).

Band 4 was represented by six 16S clones and five V2/V3 clones from a Grey buck and a red buck, and share 100% identity across the V2/V3 region with *Clostridium polysaccharolyticum* and *C. populeti*. *C. polysaccharolyticum* is a member of the phylogenetic Clostridium cluster XIVa, and like most of the clostridiae in this cluster, is cellulolytic (Warnick *et al*, 2002). *C. polysaccharolyticum* is also xylanolytic, and produces formate and butyrate.

None of the identified dominant bands were related to *Ruminococci*, unlike three of the highly fibrolytic isolates used throughout this project (YE125, YE137 and YE166). This data is consistent with the observed bias in culture-based isolation towards certain taxa in ruminants, and is strong evidence that the cellulolytic members of a functional core of fibre utilisers in kangaroos are likely to belong to different taxa.

The identity of remaining dominant bands is still to be confirmed and additional clones and further sequencing will be required before the identity of the eight main bands is defined. A number of 16S rRNA gene and V2/V3 hypervariable region sequences have been determined for the remaining four dominant bands, although there is insufficient replication to rigorously describe the principal species contributing to the remaining dominant bands.

5 Success in Achieving Objectives

The initial objectives of the project were achieved, the ability of the isolated fibrolytic bacteria from kangaroos to colonise and persist was established both *in vitro* (within a fermentation vessel) and in the rumen of cannulated cattle. Unfortunately, these bacterial isolates failed to

establish in the rumen. The major objective following revision of the project was to define a "functional core" of fibre digesting microbes in the foregut of kangaroos using non-culture dependant (DNA based) methods. These objectives have been partially met in that the importance of the culturable isolates *in vivo* has been determined and some members of a probable "functional core" of microbes for fibre digestion have been identified. To complete the latter objective will require further detailed study. This objective was ambitious and challenging and the amount of work required was underestimated.

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

There will be no immediate impact from this work on the Meat and Livestock Industry. Further research would be required before any tangible benefits would flow to the industry. However, if the functional core of microbes that digest native grasses in the kangaroo forestomach can be elucidated and mechanisms allowing them to establish in the rumen developed, benefits to the industry could begin to flow within five years.

7 Conclusions and Recommendations

7.1 Conclusions

1. Current fibrolytic bacterial isolates from the kangaroo foregut are not competitive in the rumen ecosystem.
    - Four fibrolytic bacterial isolates (three cellulolytic Ruminococci, and one xylanolytic *Butyrivibrio*) from kangaroo forestomach contents were evaluated for their ability to colonise a fermentation system based on bovine rumen contents. Three isolates did not persist for more than 5 days although one did persist and establish a stable population.
    - None of the bacterial isolates established populations in 12 cannulated steers (adult animals).
2. Conventional bacterial isolation appears to have been biased towards bacterial groups (especially Ruminococci), which are not representative of dominant *in vivo* fibrolytic species.
    - Analysis of kangaroo forestomach contents from 42 kangaroos by quantitative real time PCR assay and Denaturing Gradient Gel Electrophoresis indicated that the three cellulolytic *Ruminococci* and one xylanolytic *Butyrivibrio* bacteria isolated in the preliminary stage of the project were not dominant or conserved in all species or across the geographical range.
3. Using non-culture dependant molecular techniques (DGGE, clone libraries and DNA sequence analysis) an initial appraisal of the core of fibrolytic bacteria in kangaroos has identified bacteria related to cellulolytic clostridia, a hemicellulolytic rumen bacterium and carbohydrate utilising bacteria. The identification of these dominant, fibre-associated bacteria will guide isolation to allow evaluation of their ability to colonise and improve native pasture digestion in ruminants.

7.2 Recommendation

It is recommended that further research is undertaken to understand the microbial ecology of fibre degradation in the forestomach of kangaroos to identify communities that may have value in increasing the rate and extent of utilisation of native pastures by ruminants. Improving the rate of degradation of plant fibre in native pasture would result in increased feed intake and liveweight

gain. We estimate that an increase in gain by 300 g/d is not unreasonable. The flow on effects of this would be ability to meet market specifications earlier with younger animals, improved reproductive performance and more efficient utilisation of feed resources. Fibrolytic inoculants may be particularly valuable during dry seasons when pasture quality is low and the proportion of fibre in native grasses (and introduced C_4 grasses) is high. Improved economic returns would be from a non-chemical, non-antibiotic and non-GMO mechanism.

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9 Appendices

9.1 Appendix 1

Appendix 1: Results of BLASTn searches for the target amplicon for 5' *Taq* nuclease assays:

Target strain	Similar sequences	Non-matched assay primer/probes
YE125	Uncultured bacterium clone CATTLE_26 16S gene GenBank accession AY854288.1, 95% over 73 bases	YE125R2
	Uncultured bacterium clone Ad-C2-75 16S gene GenBank accession AY816654.1, 95% over 73 bases	YE125R2, YE125P2
	Uncultured bacterium clone Ad-C2-49 16S gene GenBank accession AY816653.1, 95% over 73 bases	YE125R2, YE125P2
YE129	Uncultured equine intestinal eubacterium 16S gene, clone ML29 GenBank accession UEQ408175, 95% over 69 bases	YE129F1, YE129P1
	Lachnospira pectinoschiza isolate M83 16S gene GenBank accession AY699279.1, 100% over 53 bases	YE129F1
	Lachnospira pectinoschiza isolate M60 16S gene GenBank accession AY699278.1, 100% over 53 bases	YE129F1
YE137	Ruminococcus flavefaciens strain R13e2 16S gene partial sequence GenBank accession AF104847, 92% over 75 bases	YE137F1, YE137P1
	Uncultured bacterium clone p4f04ct-1 16S gene partial sequence GenBank accession AY578655.1, 90% over 66 bases	YE137P1, YE137R1
	Uncultured rumen bacterium clone for 16S rRNA, partial clone:U29-D09 GenBank accession AB185779.1, 89% over 67 bases	YE137P1, YE137R1
YE161	Uncultured bacterium clone cvf63067 16S gene GenBank accession AY100587.1, 96% over 56 bases	YE161F1, YE161P1, YE161R1
	Uncultured bacterium partial 16S rRNA gene GenBank accession UBA400250, 94% over 57 bases	YE161F1, YE161P1
	Uncultured bacterium clone BREC-40 16S gene GenBank accession AY338344.1, 92% over 57 bases	YE161F1, YE161P1
YE166	Uncultured bacterium clone Ad-H2-92 16S gene GenBank accession AY816506.1, 91% over 69 bases	YE166P2, YE166R2
	Uncultured bacterium clone YRCE20 16S gene GenBank accession AY311728.1, 89% over 69 bases	YE166P2, YE166R2
	Uncultured bacterium clone BB93 16S gene GenBank accession AY985169.1, 89% over 69 bases	YE166F2, YE166P2, YE166R2