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Using kangaroo bacteria to reduce emissions of methane and increase productivity

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Abstract

This project investigated the roles of reductive acetogenesis and methanogenesis in the foregut of kangaroos to determine whether, in the future, reductive acetogenesis could be used to reduce methane emissions from sheep and cattle and increase productivity. It was reaffirmed that most kangaroos don't generate methane and shown that when methanogenesis is inhibited reductive acetogens can survive in a rumen-like environment. The reductive acetogens in culture represent a fraction of the diversity present and there are unusual archaea, whose role is still to be defined, associated with kangaroos. Further research is recommended, if the pivotal mechanisms and the microbes involved in reductive acetogenesis in kangaroos could be determined and understood, it is possible that methane emissions from cattle and sheep could be markedly reduced and productivity and profitability increased.

Executive summary

Methane is a by-product in the digestion of plant material by all cattle and sheep. Effectively it is wasted feed material and energy that could otherwise be available for animal production. Methanogenesis is a microbiological mechanism that removes hydrogen (produced during the fermentation of feed) from the rumen. It has been estimated that, if three quarters of the methane generated could be channelled into animal product, instead of lost to the atmosphere, 10% of Australia's greenhouse gas emissions could be permanently eliminated. At the same time $150 million worth of production annually (calculated as beef cattle equivalents) could be generated by Queensland's primary producers alone.

Methane is also a major greenhouse gas contributing heavily to global warming with a warming potential 21 times greater than carbon dioxide. Cattle and sheep contribute 53% of Australia's total methane emissions (3 million tonnes annually or the equivalent of 63 million tonnes of carbon dioxide) (NGGIC, 2007) and 14% of the nation's total greenhouse gas emissions. Methane has been specifically identified as a greenhouse gas for which policies and strategies are required to be developed to reduce emissions

Methane is generated in the rumen by methanogenic archaea that utilise hydrogen to reduce CO_2, and is a significant electron sink in the rumen ecosystem. However, reductive acetogenesis has been suggested as an alternative. Not only does this reduce or nullify methane emissions, it can also supply a considerable proportion of the energy needs of the animals. If methane was wholly replaced by acetate in cattle and sheep this would represent an energetic gain of 4 - 15% to the animal.

In Australia, marsupials had evolved to fill the niche occupied predominantly by sheep and cattle elsewhere, and like the ruminants, kangaroos developed an enlarged complex forestomach for fermentation of plant material. However, unlike sheep and cattle, kangaroos emit very little methane and appear to possess an alternative mechanism to methanogenesis although their digestive process is analogous to sheep and cattle.

In earlier work investigating the bacterial species present in kangaroos we were able to demonstrate, with a limited number of Red and Eastern Grey kangaroos, that this mechanism was likely to be reductive acetogenesis and a variety of reductive acetogens were isolated that may be of value in reducing methane emissions from sheep and cattle.

Initially, the purpose of the current project was to evaluate and compare the ability of reductive acetogens from the kangaroo foregut to colonise rumen-like environments, out-compete methanogens and reduce methane generation both *in vitro* (fermentation apparatus) and *in vivo*. However, while some *in vitro* results were encouraging, it became apparent that the lack of basic knowledge of the microbiology of the kangaroo forestomach and the processes that enabled reductive acetogenesis to outcompete methanogenesis in this environment would limit progress. The project was then refocussed to build our knowledge of hydrogen utilisation in kangaroos and determine the factors that enable reductive acetogenesis to outcompete methanogenesis and how we can get reductive acetogens to persist in a rumen–like environment.

Through a range of fermentor based experiments the ability of the kangaroo reductive acetogens to survive in a rumen-like environment was determined, with and without the inhibition of methanogenesis. Fermentors were also used to investigate various aspects of fermentations that were started with kangaroo forestomach contents and their ability to support reductive acetogenesis. Parameters that were measured included methane production. A wide variety of DNA based

molecular techniques were also developed and used to examine populations of archaea (which include methanogens) and the cultivable and yet-to-be-cultured species of reductive acetogens, both in the fermentor and in foregut contents, from 42 Red and Eastern Grey kangaroos and wallaroos.

The main findings and conclusions from the work were:

- When methanogenesis was inhibited some kangaroo reductive acetogens can maintain populations in a rumen-like environment. Of three species of reductive acetogens from the kangaroo foregut, YE257 was able to maintain its' population density when methanogenesis was inhibited. Without inhibition of methanogenesis the three species were not able to maintain populations.
- Fermentations initiated with kangaroo foregut contents resulted in very different outcomes that were not predictable. Two fermentations were run, one remained acetogenic but also contained dense populations of apparently non-methanogenic archaea while in the other, reductive acetogens rapidly disappeared and sometime after they had become undetectable the fermentation turned methanogenic. Why these fermentations were so different was not apparent but could be important as some of the keys to maintaining an acetogenic versus methanogenic ecosystem could lie in the differences that occurred.
- A study of culturable reductive acetogens, archaea and methanogens in 42 kangaroos and wallaroos from throughout Queensland was undertaken. In many kangaroos and particularly Red kangaroos, archaea (including all methanogens) are not present. When present they were at lower densities than in cattle and sheep and mostly did not possess methane production genes. They are also, to a large extent, genetically quite distinct from known methanogens. Archaeal communities appear to be very diverse and novel. Culturable reductive acetogens, except *Cl. glycolicum,* were present at reasonable densities in all kangaroos.
- Further evidence that these populations produce little if any methane (except possibly some Wallaroos) has been generated by observing the comparative emissions when gut contents were incubated *in vitro*. Cattle rumen contents generated considerable methane whereas virtually none was generated by the kangaroos and only a small amount from the Wallaroos.
- DNA sequencing has shown that the reductive acetogens that are in culture represent only a very small proportion of the diversity of reductive acetogens in kangaroos and the most important species, or consortia of species, may be yet to be cultured. DNA sequence analysis of archaea show that those related to methanogens had more similarities to *Methanosphaera stadmanii* (of human gut origin) than to the *Methanobrevibacter sp.* commonly found in cattle. Other sequences were related to sequences from Tammar wallabies and to non-methanogenic archaea (*Thermoplasma sp.*). Some sequences were so unusual as to defy alignment with any previously described sequences.
- A number of kangaroos had McrA sequences that were closely related to a group of methane oxidising archaea (anaerobic methanotrophs). Their role in the ecosystem remains to be determined but it is possible that they also contribute to lowering methane emissions.

There will be no immediate impact from this work on the Meat and Livestock Industry. This project has reaffirmed that most kangaroos are unlikely to generate much methane. The microbiology within the foregut of kangaroos is novel and to fully appreciate the hydrogen economy in these animals and how reductive acetogenesis, and other hydrogen utilising pathways, dominate over methanogenesis will take considerably more strategic research. We recommend that this be done as this is a microbial ecosystem that functions in a similar fashion to the rumen but produces very little methane. If the pivotal mechanisms and the microbes involved could be determined and understood, it is

possible that these principals and microbes, when applied to the rumen of cattle and sheep could markedly reduce methane emissions and increase productivity and profitability.

Contents

1 Background

Methane is a by-product in the digestion of plant material by all cattle and sheep. Effectively it is wasted feed material and energy that could otherwise be available for animal production. Methanogenesis is a microbiological mechanism that removes hydrogen (produced by the fermentation of feed) from the rumen. It has been estimated that, if three quarters of the methane generated could be channelled into animal product, instead of lost to the atmosphere, 10% of Australia's greenhouse gas emissions could be permanently eliminated. At the same time $150 million worth of production annually (calculated as beef cattle equivalents) could be generated by Queensland's primary producers alone. This would represent a major boost to rural economies and communities.

Methane is also a major greenhouse gas contributing heavily to global warming with a warming potential 21 times greater than carbon dioxide. Cattle and sheep contribute 53% of Australia's total methane emissions (3 million tonnes annually or the equivalent of 63 million tonnes of carbon dioxide) (NGGIC, 2007) and 14% of the nation's total greenhouse gas emissions. Beef cattle alone contribute 50% of these emissions.

Globally, livestock are responsible for the generation of 90 million tonnes of methane (equivalent to 1.9 billion tonnes of carbon dioxide) annually.

Australia, as a signatory to the Kyoto protocol, is required to limit total greenhouse emissions and methane has been specifically identified as a greenhouse gas for which policies and strategies are required to be developed to reduce emissions (Howard, 1997). In addition, from a global warming perspective, reducing methane emissions is seen as highly advantageous in delivering near-term climate benefits as methane has a high radiative forcing potential (21 times that of CO_2) but a relatively short atmospheric life, approx. 10 years. Therefore, reductions in methane emissions could be expected to result in measurable reductions in the concentration of atmospheric methane and slow the rate of climate change within decades (Weitang, 2003).

Methane is generated in the rumen by methanogenic archaea that utilise hydrogen to reduce CO_2, and is a significant electron sink in the rumen ecosystem. However, reductive acetogenesis has been suggested as an alternative electron sink (Mackie and Bryant 1994; Joblin 1999; Nollett *et al.* 1997). Despite methanogenesis predominating in the rumen, acetogenesis predominates in other anaerobic gut ecosystems (Breznak and Switzer 1986). Not only does this reduce or nullify methane emissions, it can also supply a considerable proportion of the energy needs of the animals. In the termite, reductive acetogenesis has been calculated to produce enough acetate to account for 33% of the animals total energy requirement (Breznak and Switzer 1986). If methane was wholly replaced by acetate in ruminants this would represent an energetic gain of 4 - 15% to the animal (Joblin 1999; Nollet *et al.* 1997).

The geographic isolation of Australia has meant that large domestic herbivores were not present on the continent until the arrival of Europeans 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. However, unlike sheep and cattle, kangaroos emit very little methane (Kempton *et al.,* 1976) and appear to possess an alternative mechanism to methanogenesis although their digestive process is analogous to sheep and cattle. An investigation of the bacterial species present in kangaroos (see below) demonstrated

that this mechanism was likely to be reductive acetogenesis and a variety of reductive acetogens were isolated that may be of value in reducing methane emissions from sheep and cattle.

1.1 Experimental outcomes supporting B.NBP.0354

Department of Primary Industries and Fisheries, Queensland undertook a study to form a collection of culturable bacteria from kangaroo forestomach contents for future use in reducing methane emissions from cattle and sheep. This project (completed in April 2004) provided the foundations for the current project undertaken by Queensland Primary Industries and Fisheries (QPIF), formally known as Department of Primary Industries and Fisheries (DPI&F), as part of the Department of Employment, Economic Development and Innovation (DEEDI).

The presence of methanogens and reductive acetogens in the forestomach of kangaroos (and sheep) was examined using the molecular biology based technique of the Polymerase Chain Reaction (PCR). A sheep that had previously been shown by Real-Time PCR to contain methanogens was used as a positive control. Samples of total DNA from foregut contents were obtained from eight kangaroos. All were negative for the presence of methanogens but positive for bacteria in general. DNA from sheep rumen contents was positive for both. A literature search unveiled the presence of primers that would enable a PCR assay for the functional genes that would identify the presence of reductive acetogens. This assay was developed and used to show the presence of reductive acetogens in the eight kangaroos that lack methanogens.

From samples of kangaroo forestomach contents a variety of reductive acetogens were isolated and made available for B.NBP.0354. Three distinct species had a very high affinity for hydrogen, with two appearing to be obligate acetogens only producing acetate from fermentation while the other produced copious acetate plus branched chain fatty acids, presumably through the fermentation of amino acids.

1.2 Project revision

The original objectives for project B.NBP.0354 were to evaluate and compare the ability of reductive acetogens from the kangaroo foregut to colonise rumen-like environments, out-compete methanogens and reduce methane generation both *in vitro* (fermentation apparatus) and *in vivo*.

While some *in vitro* results were encouraging, they were not as conclusive as hoped. The pure culture reductive acetogens and the *Desulfovibrio* mix appeared to reduce methane emissions and methanogen numbers while at the same time increasing total VFA production through increased acetate and propionate production. However, the kangaroo acetogens were not able to maintain population density in the fermentor.

In consultation with MLA, it was agreed to restructure B.NBP.0354 to increase our knowledge of hydrogen utilisation in macropod marsupials and in particular a knowledge of how and why reductive acetogenesis out competes methanogenesis in these animals and how we can get reductive acetogens to persist in a rumen–like environment.

2 Project objectives

2.1 Original objectives

The initial purpose of the project was to determine whether reductive acetogens isolated from the foregut of Red and Grey Kangaroos were capable of competing with ruminal methanogens to reduce methane emissions and increase productivity per unit feed intake, by:

1) Evaluating and comparing the ability of reductive acetogens from the kangaroo foregut to colonise and out-compete methanogens in an *in vitro* fermentation apparatus and determine the extent of reduction in methane generation, and
2) Evaluating and comparing the ability of selected reductive acetogens to colonise the rumen of cattle, reduce methane generation and improve growth rate.

2.2 Revised objectives

Following revision of the project it was deemed unlikely, given our lack of knowledge of the hydrogen economy in kangaroo foregut contents that Objective 2 above could be met and objectives were modified to increase our knowledge of the processes and microbes involved in hydrogen utilisation in kangaroos. New objectives replacing Objective 2 were:

1) To evaluate the ability of reductive acetogens to out-compete methanogens in the kangaroo foregut through direct measurement on culled wild kangaroos and *in vitro* fermentations, and
2) To investigate factors required to enable kangaroo reductive acetogens to persist in a rumen-like environment.

3 Methodology

3.1 Molecular biology based methodologies for identifying, enumerating and monitoring archaeal and reductive acetogen populations

3.1.1 DNA extraction from fermentor and foregut samples and Denaturing Gradient Gel Electrophoresis (DGGE) of archaea

Total genomic DNA was extracted from thawed samples of fermentor liquor or kangaroo foregut contents, using a bead-beating protocol (initially as reported by Whitford *et al.*(1998), then as modified by Yu and Forster (2005)). Partial 16S rRNA genes of archaea were amplified from extracted genomic DNA by PCR. A nested PCR procedure was used with primers Arch46F (Ovreas *et. al*. 1997) and Arch1017R (Barns et. al., 1994) in the first round of amplification. The resulting PCR product was used as template in the second PCR which amplified across the variable regions 2 and 3 (V2V3) of the 16S rRNA gene using the primers Arch344F-GC (Raskin *et. al*., 1994) and Univ522R (Amann *et. al*. 1995). Denaturing Gradient Gel Electrophoresis (DGGE) through a 30 – 60% gradient was used to separate the resulting PCR products to show which DNA bands (corresponding to methanogen species) were present in the fermentor. This methodology has been described previously (Ouwerkerk *et al.,* 2008).

3.1.2 Real-time PCR assay to enumerate total archaea

Total methanogen numbers were estimated using a real-time PCR assay (Takai and Horikoshi, 2000) directed towards the domain archaea. The primers used were Arch349F (5' – GYGCASCAGKCGMGAAW - 3') and Arch806R (5' - GGACTACVSGGGTATCTAAT - 3'). The probe used was 6-FAM labelled Arch516F (5' – GYCAGCCGCCGCGGTAAHACCVGC - 3'). Standards were made by direct counting cells of *Methanobrevibacter ruminantium* ATCC35063 or *Mbb. smithii* DSM 861 grown in broth culture. The cells were counted using a Petroff-Hauser Bacteria Counter. The real-time PCR assay was run on a Corbett RotorGene 3000 or 6000 under the following conditions - 1 cycle at 94^{o}C for 1 min; then 45 cycles at 94^{o}C for 10 sec, 64^{o}C for 30 sec and 30^{o}C for 1 min. The specificity of the archaeal real-time PCR was checked against the bacterial panel (Table 1) as described by Ouwerkerk *et al.* (2002).

3.1.3 Real-time PCR assay for enumeration of culturable reductive acetogens

Primers and probes for specific real-time PCR assays were developed to target unique regions of the FTHFS or 16S rRNA genes. Primer and fluorescent 3' DNA probe design was conducted using Primer Express ver. 2 (Applied Biosystems, Inc.). The assays were conducted in 25 µl volumes, using RealMasterMix probe mix (Eppendorf) or RealMasterMix Probe Mix (5Prime) 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 thermal profile was 94ºC for 1 minute, followed by 40 cycles of denaturation at 94ºC for 10 s and annealing/extension at 60ºC for 30 s.

Specificity of the primers and probes were evaluated *in silico* using BLASTn and Ribosomal Database Project (RDP) searches against GenBank and the RDP database. Assay specificity for all 3 assays was evaluated against the rumen bacterial isolates presented in Table 1, as well as the reductive acetogen isolates used in this study.

Table 1: 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
Clostridium butyricum	YE12
Clostridium butyricum	YE15
Clostridium glycolicum	YE255
Eschericia coli	ATCC 15766[1]
Eschericia coli	YE254
Eubacterium ruminantium	AR2
Eubacterium cellulosolvens	YE257
Eubacterium rectale	YE131
Fusobacterium necrophorum	AR4
Lactobacillus sp.	YE07
Lactobacillus sp	YE08
Lactobacillus sp	YE16
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
Megasphaera elsdenii	YE34
Sheep rumen gDNA	681
Bovine rumen gDNA	M95

[1] strain obtained from the American Type Culture Collection

Genomic DNA (10 ng) from each strain or isolate was assayed using generic reaction conditions, and assays which displayed non-specific detection were modified by increasing the annealing/extension temperature to 64ºC in order to eliminate non-specific detection.

Three series of quantitative standards were prepared for each strain using pure cultures enumerated by direct count in a Petroff-Hauser chamber. A log dilution series was prepared in both TE and rumen fluid in TE from 10^{10} to 10^{2} cells/ml. Genomic DNA was prepared from 1 ml of each dilution, and resuspended in 500 µl sterile water. A log dilution series was prepared from genomic DNA from 10^{10} cells in TE down to the equivalent of 10^{2} cells. All three series of standards were assayed, and log concentration vs C_T was plotted.

Due to non-specific background detected in the initial fermentor control runs and a lack of ability to differentiate between isolates, these real-time PCR assays to enumerate the kangaroo reductive acetogens were modified. The complete, revised, sets of primers and probes used in subsequent work are presented in Table 2.

Table 2: Primers and probes used in real-time PCR assays to detect reductive acetogens and methanogens

Target isolate	Primer/probe name	5'-3' sequence, including terminal modifications
YE255	YE255AF1	GGA GTT GAA CTT GCA GAG GAA GTA T
YE255	YE255AR1	TCG TAG CAG AAT TGG AAG TCA TTT
YE255	YE255AP1	**FAM-**AAG ACT TTG CGA ACA AG-**BHQ**
YE257	YE257F1	CCA CGC CGT AAA CGA TGA A
YE257	YE257R1	GCG GCA CCG ACC ATC TT
YE257	YE257P1	**FAM-**ACT AGG TGT TGG CCA TC-**BHQ**
YE266/YE273	YE266F1	GCA GAA ATC GCA TTA GTA GAG AAG AA
YE266/YE273	YE266R1	CGC CTT TTG CCC ATA CTT CA

YE266/YE273	YE266P1	**FAM-**CTT GGC GTA AAT GTT AAA CT-**BHQ**
Methanogens	Arch349F	GYGCASCAGKCGMGAAW
Methanogens	Arch806R	GGACTACVSGGGTATCTAAT
Methanogens	Arch516	**6FAM**-TGYCAGCCGCCGCGGTAAHACCVGC-**tamra**

3.1.4 Molecular techniques and assays used to investigate microbial populations in kangaroo forestomach contents.

Total genomic DNA was extracted from stored samples following the procedure of Yu and Forster (2005). Extracted genomic DNA was used in subsequent real-time PCR assays (for specific reductive acetogens, total archaea and the functional gene in methanogenesis – McrA), conventional PCR assays (ARCH 1 and mcrA3) and clone libraries, to better define the core of hydrogen utilisation in kangaroos. Only those assays not previously described are detailed in this section.

The presence of *McrA* genes was detected by PCR using the primer set MLf (5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3') and MLr (5'-TTCATTGCRTAGTTWGGRTAGTT-3') of Luton *et. al*. (2002).

To determine numbers of *McrA* genes present a SYBR Green quantitative real-time PCR assay (Denman *et al.* 2007) was used. The primers used were qmcra-F (5' – TTCGGTGGATCDCARAGRGC – 3') and qmcra-R (5' - GBARGTCGWAWCCGTAGAATCC – 3'). Standards were made by direct counting cells of *Methanobrevibacter smithii* DSM 861 grown in broth culture. The cells were counted using a Petroff-Hauser Bacteria Counter. The real time PCR assay was run on a Corbett RotorGene 6000 under the following conditions - 1 cycle at 50^{o}C for 2 mins; 95^{o}C for 10 mins then 40 cycles at 95^{o}C for 15 secs, 60^{o}C for 60 secs. High resolution melt curve analysis, with a temperature increase of 1^{o}C every 30 secs over a range from 60^{o}C to 95^{o}C, was applied to completed reactions to determine amplicon specificity.

The real time PCR assays were run on a Corbett RotorGene 6000 under the following conditions – 1 cycle at 94^{o}C for 1 min; then 40 cycles at 94^{o}C for 10 sec, 64^{o}C (YE255), 66^{o}C (YE257, YE266/YE273) for 30 sec. The specificity of the real-time PCR was checked against the bacterial panel as described by Ouwerkerk *et al.* (2002).

In addition to the qualitative and quantitative PCR assays, clone libraries of kangaroo forestomach FTHFS genes were constructed in order to define the true diversity of reductive acetogens in kangaroos.

A 1000 bp fragment of the FTHFS gene was amplified by PCR from seven kangaroo forestomach samples using the primers used by Leaphart and Lovell (2001): FTHFSf: 5' TTY ACW GGH GAY TTC CAT GC and FTHFSr: 5' GTA TTG DGT YTT RGC CAT ACA.. PCR products were purified using QIAgen PCR spin columns (Qiagen Pty Ltd, Australia) and cloned using the TOPO-TA cloning kit (Invitrogen Corporation, USA). Plasmids containing the cloned DNA were purified using the QIAprep spin miniprep kit (Qiagen Pty Ltd, Australia). Clones were then sent for DNA sequencing. DNA sequencing was performed using the ABI PRISM BigDye 3.1 cycle sequencing kit (Applied Biosystems, USA). Cloned inserts were sequenced using the T7 & T3 primers supplied with the TOPO-TA cloning kit. Sequence fragments were assembled using Sequencher Ver 4.8 (Gene

Codes Corporation, Ann Arbor, MI, USA). Assembled 16S rRNA gene sequences were compared with GenBank, EMBL, and DBJJ non-redundant nucleotide databases using the Gapped BLAST database search program (Altschul *et al.* 1990) at the National Centre for Biotechnology Information (NCBI). Similar 16S rRNA gene sequences were included in the phylogenetic analysis and the sequences aligned using ClustalW (Ludwig *et al.* 2004). The alignments were optimised manually and phylogenetic analyses carried out using PAUP*, Ver 4.0b8 (Swofford, 2002). Trees were generated using maximum parsimony (P), maximum likelihood and distance matrix (D) analyses. Before constructing the trees a series of likelihood (L) ratio tests were performed using Modeltest ver 3.7 (Posada and Crandall, 1998) to determine the most suitable nucleotide substitution model to use for the L and D analyses. The model selected was a general time reversible model (GTR) with estimates of invariant sites (I) and among site heterogeneity (G) (summarised as GTR +I+G) for the 1290 bases of 16S rRNA gene sequence used to produce the trees. Bootstrap resampling (P, 1000 replicates; D, 1000 replicates; L, 100 replicates) was used to assess the branch support of all inferred trees.

To clarify the types of archaea in kangaroos, several clone libraries were constructed in order to define these archaea. Qualitative and quantitative PCR products from assays described above were used in the clone libraries. The cloning and DNA sequencing procedures were the same as those described for FTHFS gene clone libraries above.

3.2 Fermentor based studies - General

The methodology associated with using the fermentor 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 native pasture (predominately spear grass) at Brian Pastures Research Station. Fermentor liquor (50 mL aliquots) was combined with an equal volume of rumen fluid/glycerol medium and stored frozen at -20^{o}C (or -80^{o}C). Each fermentor 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 3 L of a rumen-fluid-based (RF) medium. At commencement of the fermentation 45 g of finely milled spear grass (or Mitchell grass) was added as substrate for the fermentor. On the second day of fermentation the amount of milled hay added daily was reduced to 15 g. Each day, half of the fermentor liquid was removed and replaced with anaerobic culture medium that had been modified by the removal of most nutrients to leave a balanced salts solution. The fermentor 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.

Each day, at the time of replacement of fermentor liquor with fresh salts solution, samples of fermentor liquor were collected for determination of volatile fatty acid (VFA) production (as per Ouwerkerk and Klieve, 2001), to enumerate inoculant bacteria, culturable kangaroo acetogens and total methanogens (by real-time PCR) and to profile methanogen populations (DGGE). To determine effects on plant fibre digestion in the ruminal environment, 12 nylon bags on a metal support were inserted into the fermentor. Each bag contained a pre-weighed amount of milled native (spear or Mitchell grass) pasture hay (approx 1 g). At zero time and at two day intervals, two bags were removed to determine dry matter disappearance. Methane concentrations in the outflow gas from the fermentor were measured continuously throughout the experiments using an Environmax NDIR Methane analyser (Liston Scientific). Total fermentation time per experiment was 11 days.

3.2.1 Addition of kangaroo reductive acetogens without inhibiting methanogenesis

In total, four fermentor runs were performed without any additions to act as controls and establish base-line comparative data.

In non-control fermentations, on the second day of each experimental run either a pure culture of one of the reductive acetogens or a mixed culture (Table 3) was inoculated into the fermentor following replacement of fermentor liquor with balanced salts solution. The mixed culture removed hydrogen as well as the reductive acetogens but a pure culture could not be isolated. Upon further investigation it was discovered that the primary member of the mix that was likely to remove hydrogen was not a reductive acetogen but *Desulfovibrio desulfuricans,* which removes hydrogen by reducing sulphur compounds. As this mechanism was distinctly different from that used by the reductive acetogens it was decided to trial this mixture in the fermentor as well to determine whether they could out compete methanogens and reduce methane.

Table 3: Schedule of kangaroo cultures inoculated into the fermentor

Experiment ID	Inoculated strain/s
Control	No isolate inoculated
YE255	*Clostridium glycolicum* YE255
YE266	Kangaroo reductive acetogen YE266 (not closely related to any previously cultured bacterium).
YE257	Kangaroo reductive acetogen YE257 (not closely related to any previously cultured bacterium)
Reducing bacterial mix	Mixture of mainly three bacteria, *Desulfovibrio desulfuricans*, *Escherichia coli,* and *Enterococcus sp.* that utilise hydrogen

3.2.2 Addition of kangaroo reductive acetogens following inhibition of methanogenesis

These fermentor experiments were similar to those reported in 3.2.1. Bromoethanesulphonic acid (BES) was used to inhibit methanogenesis. Once methane production from the fermentor was well established, day 5 for BES run 2 and day 4 for all other runs, BES was added with the fresh culture medium. 10 mL of 2M BES was added to all runs except BES run 2 where 5 mL was added, to initially suppress methane production and then each day 5 mL of 2M BES was added to maintain suppression. Two initial fermentations where only BES was added to the fermentations were performed and used as inhibition positive controls. For the acetogen runs, the day after BES was added 100 mL of each of isolates YE255, 257 and 266 were added into the fermentor.

3.2.3 Fermentations with whole kangaroo gut contents only

Two different fermentors were used in this work, to expedite progress in the work. This was not intended to be for comparative purposes but to determine whether a fermentation with whole kangaroo gut contents as the starter culture would maintain reductive acetogenic conditions or not.

3.2.3.1 Operation of fermentor 1 and sampling

The fermentor run was initiated with forestomach contents (approx. 100 mL) from a Red kangaroo (designated Red doe 4).

At commencement of the run, the kangaroo forestomach contents were added to 2 L of a rumen-fluid-based (RF) medium and 30 g of finely ground Mitchell grass (*Astrebla lappacea*) was added as substrate for the fermentor. 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 fermentor liquid was removed and replaced with an anaerobic balanced salts solution. The fermentor 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.

To determine the types of bacteria that colonised plant material and the rates and extent of colonisation, in the kangaroo forestomach, nylon bags on a metal support were inserted into the fermentor. Each bag contained a pre-weighed amount of ground Mitchell grass (approx 1 g). At zero time and at daily intervals, one bag was removed and the contents washed with a phosphate buffer, aliquots were removed for DNA extraction. Daily maintenance of the fermentor and sampling were the same as for other fermentations. Total fermentation time was 10 days.

On days 9 and 10, 5 mL of fermentation liquor was removed, placed into a Hungate tube, the head space replaced with CO_2/H_2, the tube sealed and incubated at 39ºC for 2 hours. The headspace gas was analysed for the presence of methane by gas chromatography.

3.2.3.2 Operation of fermentor 2 and sampling

This was essentially the same as for fermentor one except the amount of feed and salts supplied daily was scaled up for a 3 litre fermentation (as per previously reported fermentations) instead of 2 litres. In addition, methane concentrations in the outflow gas from the fermentor were measured continuously throughout the experiment using an Environmax NDIR Methane analyser (Liston Scientific). The fermentation was initiated with forestomach contents from an Eastern Grey kangaroo (designated Grey doe 7). Extra nylon bags were included to determine dry matter digestibility in the fermentation. Total fermentation time was 11 days.

3.2.4 Addition of sample of whole kangaroo gut contents to a rumen based fermentation following inhibition of methanogenesis

These fermentor experiments were similar to those reported in 3.2.2. The day after BES was added to suppress methane production, 100 mL of forestomach contents from Grey Buck 27 was added.

3.3 Studies on archaeal and reductive acetogen populations in wild kangaroos

3.3.1 Kangaroo forestomach samples

Originally, 12 samples of kangaroo gut contents were available and these had been collected and stored prior to the current project. To widen the selection of samples, particularly from kangaroos from other geographical areas within Queensland, this collection was augmented with samples of forestomach contents from freshly harvested wallaroos, Red kangaroos and Eastern Grey kangaroos, from five locations throughout western and central Queensland (all available samples are documented in Table 4). 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.

Table 4: Kangaroo forestomach samples collected for examination in B.NBP.0354

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

To determine the importance of archaea, microbes with methane generating genes and the culturable reductive acetogens in the hydrogen economy of kangaroo foregut contents, a range of PCR-based assays were used with all 42 kangaroo foregut samples. These assays are detailed in section 3.1.4.

3.3.2 *In vitro* methane production analysis

Samples of bovine rumen contents and kangaroo forestomach contents from 6 cattle and 6 kangaroos (2 red, 2 grey and 2 wallaroos), stored at -20ºC in an equal volume of 1:1 v/v RF+/glycerol, were defrosted on ice. The samples from cattle were obtained via stomach tube and thereafter treated and stored in exactly the same manner as had been done with the kangaroo samples. An aliquot (3-5 mL) of each sample was filtered through a nylon stocking into a sterile container under anaerobic conditions. Four replicates of 0.5 mL of the filtrate were inoculated in 5 mL RF+, and replicates incubated for either 0 or 7 hours at 39ºC prior to chilling on ice for 20 minutes. Chilled samples were stored at 4ºC.

The headspace (10.7 mL) of each tube was analysed by GC for methane, and data correlated to standards with a known methane concentration.

Genomic DNA was extracted from 1 mL aliquots of each tube following the procedure of Yu and Forster (2005), and used in real-time PCR assays for total archaeal and mcrA gene numbers, as described previously.

4 Results and discussion

4.1 Fermentor based experiments

4.1.1 Addition of kangaroo reductive acetogens without inhibiting methanogenesis

4.1.1.1 Methane production

Daily methane produced by the fermentations is presented in Figure 1.

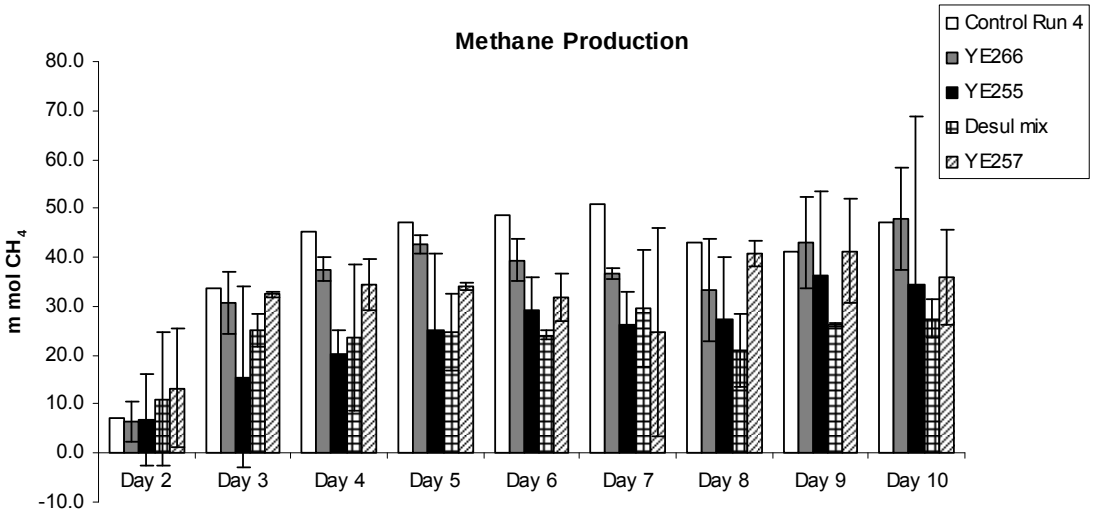

Figure 1. Daily methane production from fermentations with kangaroo bacteria added. The bacteria were added on day two and data is from duplicate fermentations, except for the control where reliable data was available from one fermentation only. Bars show standard deviation from the mean.

Some trends did appear in the data, i.e., YE266 and YE257 reduced methane emission marginally to day seven or eight but then there was no difference to the controls, YE255 reduced emissions throughout but more so up to day 7 (up to 50% reduction), the *Desulfovibrio* mix reduced emissions throughout by at least 50%. However, there were very large variations in emissions from duplicate fermentations.

4.1.1.2 Persistence of reductive acetogens in fermentations

The persistence of kangaroo reductive acetogens YE266, YE257 and *Clostridium glycolicum* YE255 are presented in Figure 2. The control fermentations were assayed for YE266, YE255 and YE257 and they were not detected at any stage.

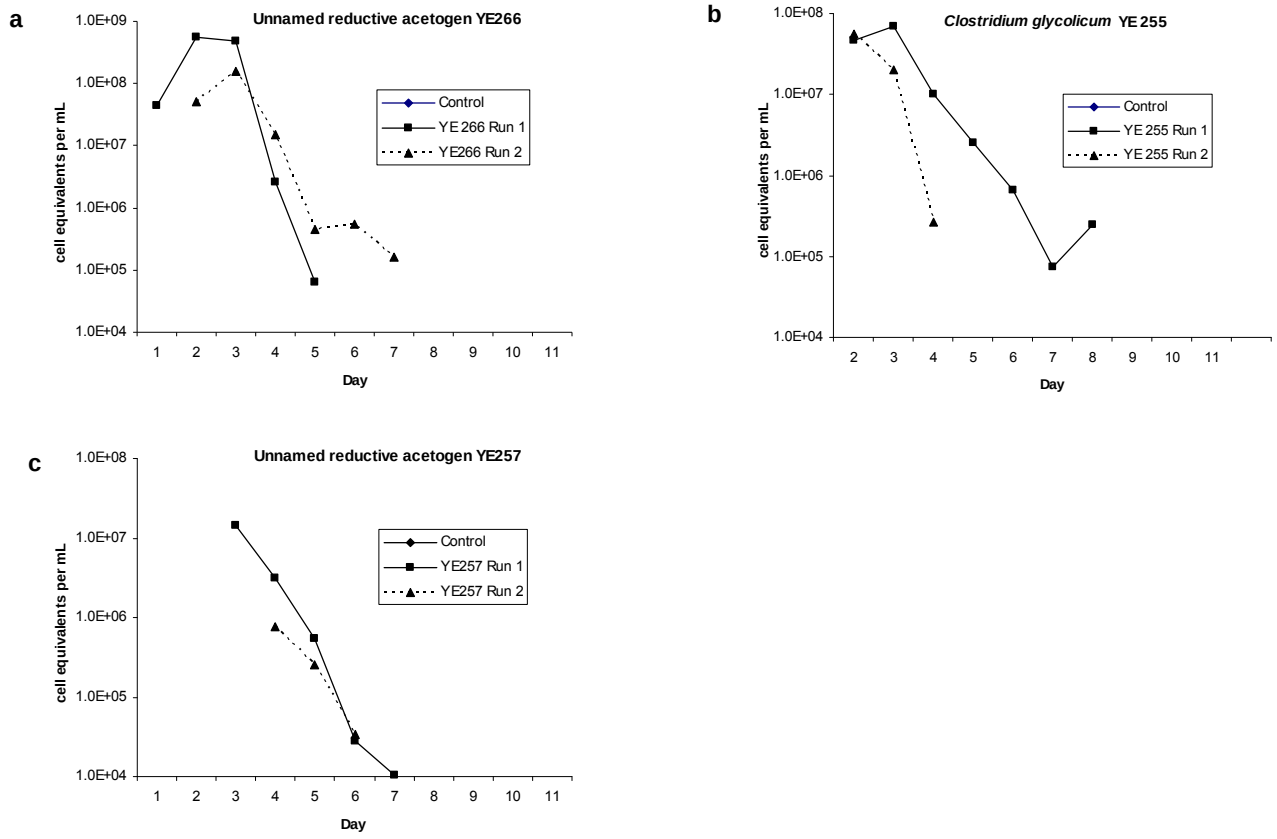

Figure 2. Population density of the unnamed kangaroo reductive acetogen YE266 (a), Clostridium glycolicum *YE255 (b) and unnamed kangaroo reductive acetogen YE257(c) in fermentations with ruminal methanogens.*

None of the kangaroo reductive acetogens persisted at high densities for the entire period of fermentation (11 days).

4.1.1.3 Methanogen population density and diversity

The total numbers of methanogens, as determined by real-time PCR, present in the fermentations are presented in Figures 3 and 4.

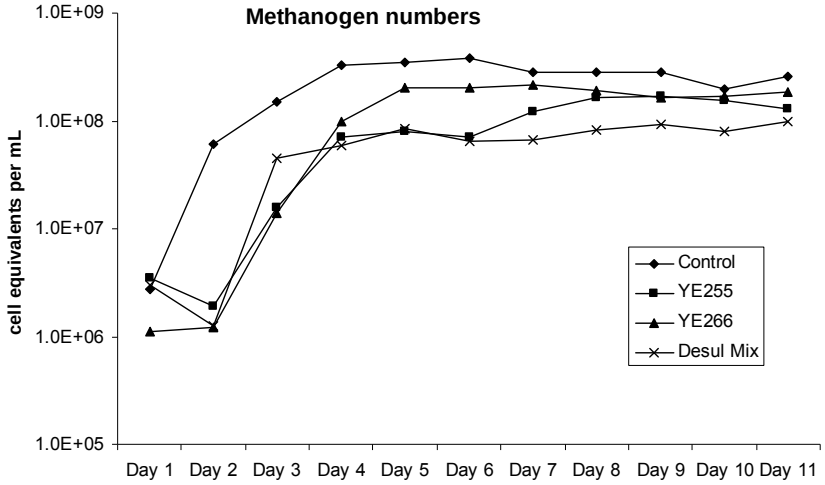

Figure 3. Cell equivalents of total archaeal numbers in fermentation liquor. Curves are averages of three (control) and two fermentations.

The impact of kangaroo reductive acetogens and the bacterial mix on total methanogen numbers remains equivocal. Although the numbers of methanogens were reduced in these fermentations and quite markedly with the *Desulfovibrio sp.* containing mix, these fermentations appear to have differed to the controls in that there was a 24 hour lag before populations of methanogens began to establish. This did not occur in the first two control fermentations but did in the third. When the fermentations with kangaroo bacteria are compared with the third control fermentation only, a different picture arose (Figure 4).

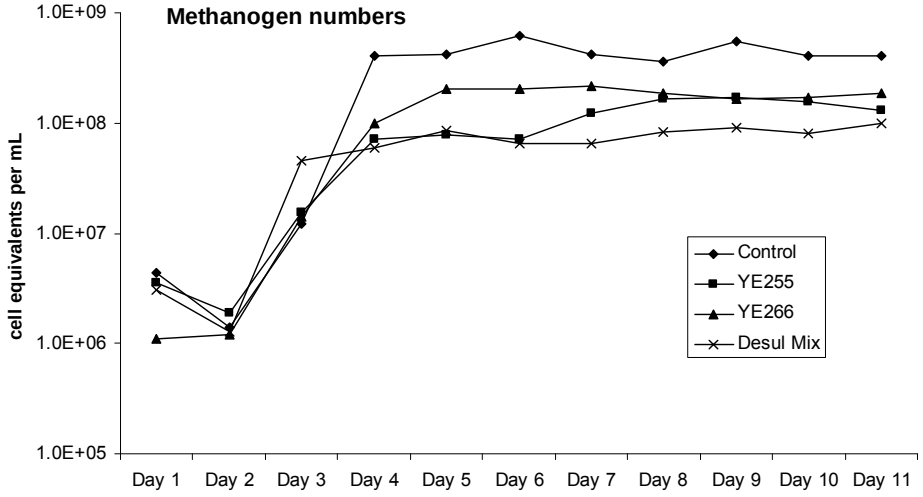

Figure 4. Cell equivalents of total archaeal numbers in fermentation liquor. Curves are averages of two fermentations except for the control which is only for control run 4.

From Figure 4 it appears that the numbers of methanogens in fermentations with kangaroo bacteria were fewer, particularly with the *Desulfovibrio sp.* containing mix, than in this control fermentation. However, as the other two control fermentations did not have a lag in establishment of the methanogen community it is difficult to directly compare these fermentations with others. In addition, there was considerable variability between duplicate fermentations. Fermenter runs for YE257 cannot be directly related to previous runs due to different extraction methods for samples and

different standards being available. The YE257 runs showed that methanogen numbers once established stayed stable at approximately 10^8 cells per mL.

A comparison between Figures 1 and 4 show a reasonably good general agreement between fermentations with the highest methanogen numbers and the highest methane emissions.

DGGE gels typical of the archaeal community within the fermentor during a control fermentation and fermentations in which YE266 or YE257 were added are presented in Figure 5.

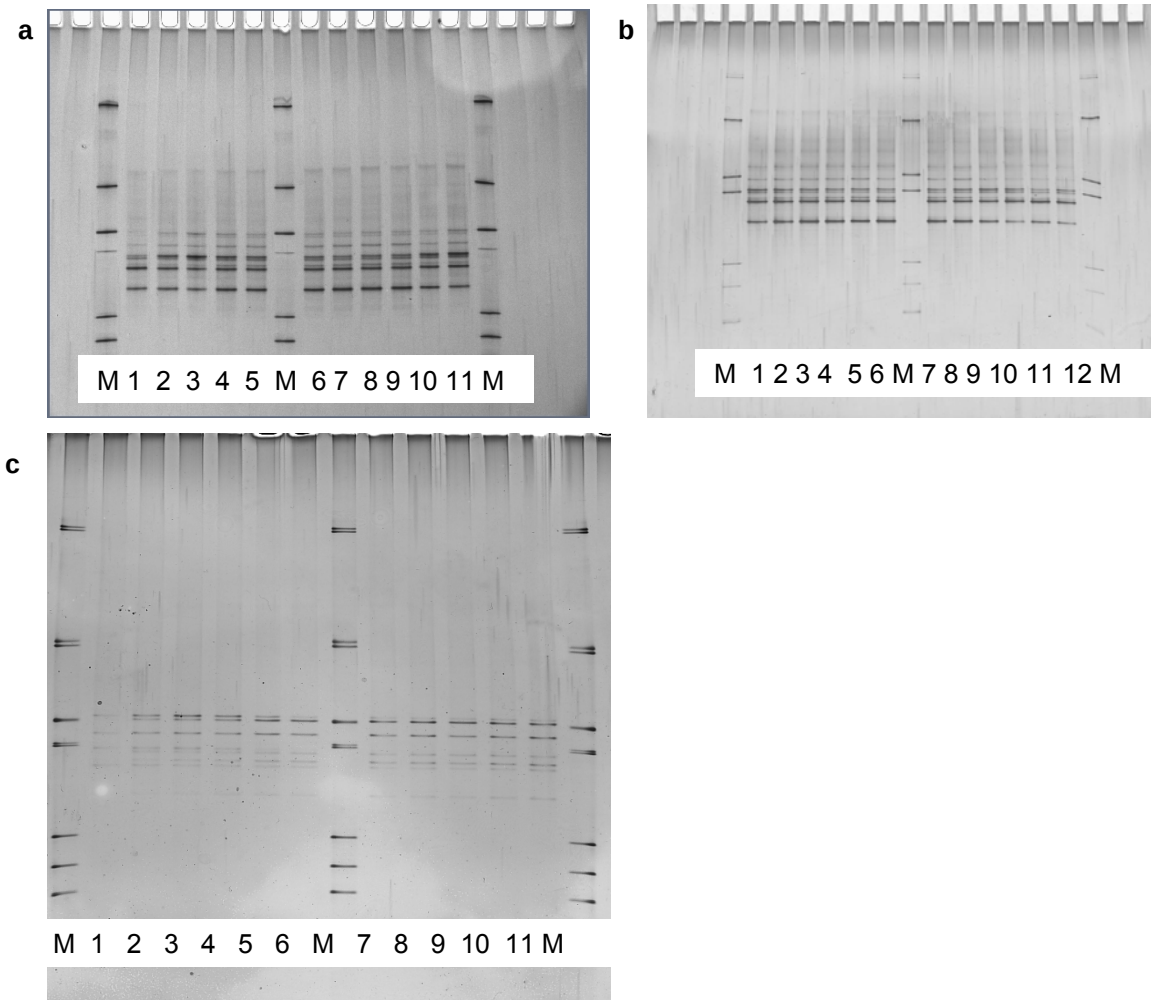

Figure 5. DGGE profiles of the archaeal community in fermentors without addition of kangaroo bacteria (a) with addition of YE266 (b)and with addition of YE257 (c). M – Markers. Numbers correspond to day of fermentation.

The diversity amongst the methanogen community changed very little throughout each fermentor run with and without the addition of kangaroo bacteria. Although not presented, all of the other fermentor runs gave similar results to the gels in Figure 5. Previously (Project B.NBP.0352), we determined that the three most dominant bands represented 96% of the community and were represented by three species of methanogens that were closely related to two different *Methanobrevibacter sp.* (currently unnamed), one isolated in Western Australia and the other in New Zealand. The closest related named species was *Mbb. thauri*.

4.1.1.4 VFA production

The daily production of total VFA, acetate, propionate and butyrate are presented in Figure 6.

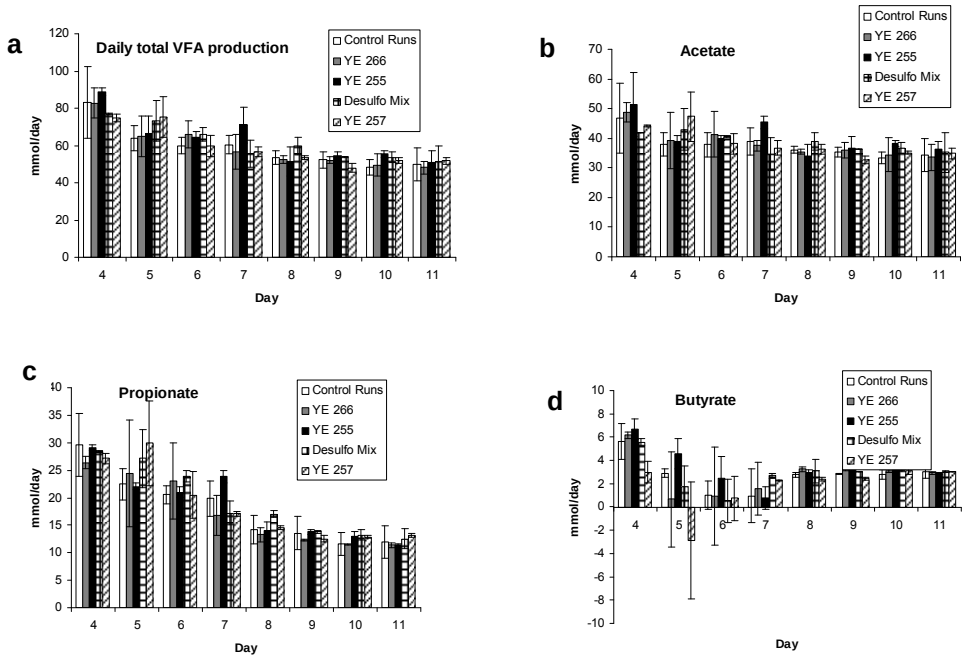

Figure 6. Daily average total VFA (a) acetate (b) propionate (c) and butyrate (d). Results of two fermentations (three for control). Bars represent one standard deviation.

There was a high degree of variability between fermentations in total VFA production and in the proportion of acetate, propionate and butyrate. Overall, the addition of the kangaroo bacteria appeared to increase total VFA production slightly and this appeared to be mainly from increased acetate (as expected) and propionate. The impact was more pronounced early to mid (up to day 8) fermentation when, in most cases, reductive acetogens were above detectable limits.

4.1.1.5 Effect on fibre utilisation

Summary results are presented in Figure 7.

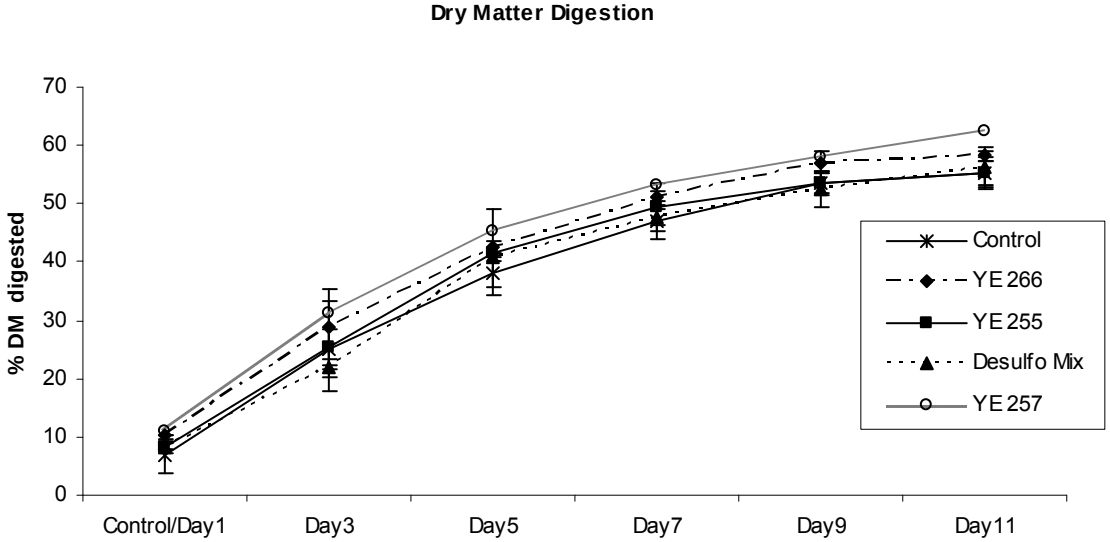

Figure 7. Percent removal of milled spear grass from nylon bags in the fermentor per sampling cycle. Each data point is the average of two fermentations. Bars show standard deviation from the mean. Strains of alternative hydrogen utilising bacteria from the kangaroo foregut were added following sample collection on day 2.

The inclusion of reductive acetogens and a mixture of bacteria including *Desulfovibrio sp.* appears to have had neither a positive or negative impact on the ability of fibrolytic bacteria to digest fibrous plant material. Although fermentations with YE257 and YE266 appear to have digested marginally more material than the control fermentations, the percentage of dry matter digested was more from the outset and the pattern was parallel to the controls throughout.

These results were not unexpected. As long as hydrogen is being removed from the fermentation fibre digestion shouldn't be inhibited and reductive acetogens or methanogens *per se* play no other role in fibre digestion than removing hydrogen.

4.1.2 Addition of kangaroo reductive acetogens following inhibition of methanogenesis

4.1.2.1 Methane suppression

The use of BES to suppress methane was virtually immediate and complete (Figure 8). In all experiments, methane was reduced to undetectable levels (on a 0 – 50 ppm scale) within half an hour of addition and remained at a very low level for the remainder of the experiment. In experiment two, BES was added a day later than in the other experiments as it took an extra day for the methane concentration to rise in this experiment. The addition of reductive acetogens had no noticeable effect on methane concentrations.

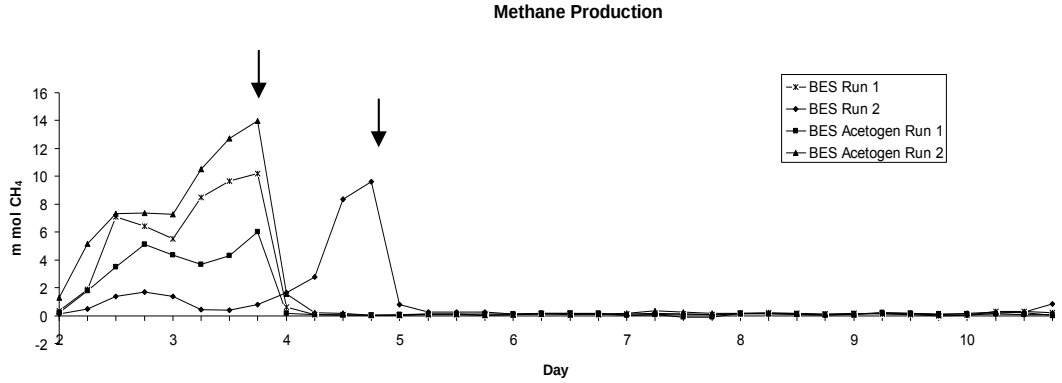

Figure 8. Six hourly methane production from fermentations with BES added, with and without kangaroo reductive acetogens added. Arrows indicate when BES was added, kangaroo reductive acetogens were added the day after BES was added.

4.1.2.2 Methanogen population density and diversity

Total methanogen numbers were markedly reduced by the addition of BES (Figure 9). Methanogen populations peaked at around 5 x 10^8 cells per mL immediately prior to BES addition (Day 4). Following BES addition there was a steady decrease in numbers down to approximately 1 x 10^7 cells per mL, or a 90 – 95% reduction from peak numbers. The addition of reductive acetogens did not appear to influence this pattern and BES appeared to be the major factor influencing methanogen numbers.

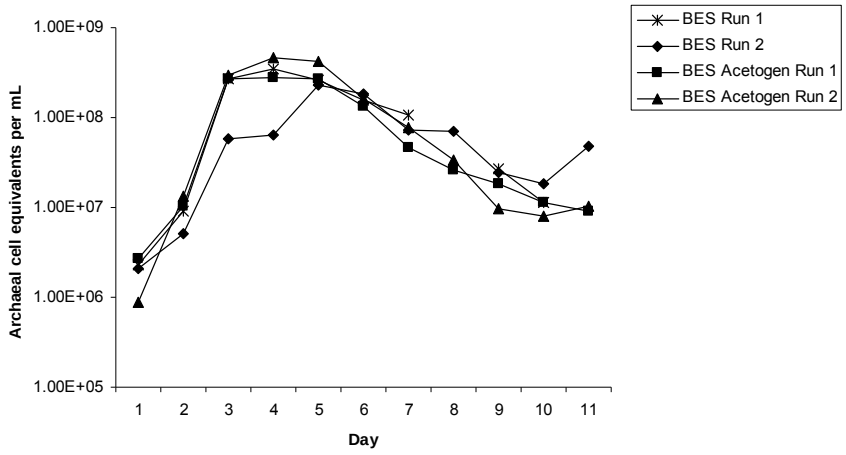

Figure 9. Cell equivalents of total archaeal numbers in fermentation liquor. Data for days 8 and 11 BES Run 1 were omitted for technical reasons.

The population structure of the methanogen community and how this changed with BES addition and with the addition of the reductive acetogens was investigated by DGGE and results are presented in Figure 10. Figure 10a is from the earlier work reported above without the addition of BES and has been included for comparative purposes. Without BES addition (Figure 10a), the population structure was very stable throughout the fermentation.

When BES was added the stability of the system changed markedly, the major change being the disappearance of band 3b and increases in numbers and density of bands toward the top of the profile which represent species with a higher ratio of AT to GC in their DNA. The *Methanobrevibacter sp.* represented by bands 1b and 2b don't appear to be as heavily inhibited as 3b but they may be more heavily affected than appears on first inspection and the apparent increase in dominance of the AT rich species may be an artefact relating to these species being less affected by BES. Given that the bands on a DGGE gel represent relative abundance and not actual numbers, then a decrease in the dominant species will allow other species that are not affected to appear to be gaining in dominance. As the total methanogen numbers have declined by 90 – 95%, then the latter argument is likely and populations of the three normally dominant *Methanobrevibacter sp.* may have been decimated with species 3b being the most affected. Whether the AT rich species would increase in numbers and return methane production to measurable quantities remains to be determined. The resistance of the AT rich species to BES and lack of methane production may suggest that some of these methanogen species have means of survival other than through the production of methane.

The methanogen population changes were very similar with and without the addition of reductive acetogens, again suggesting that the overwhelming factor involved in producing these changes was the addition of BES.

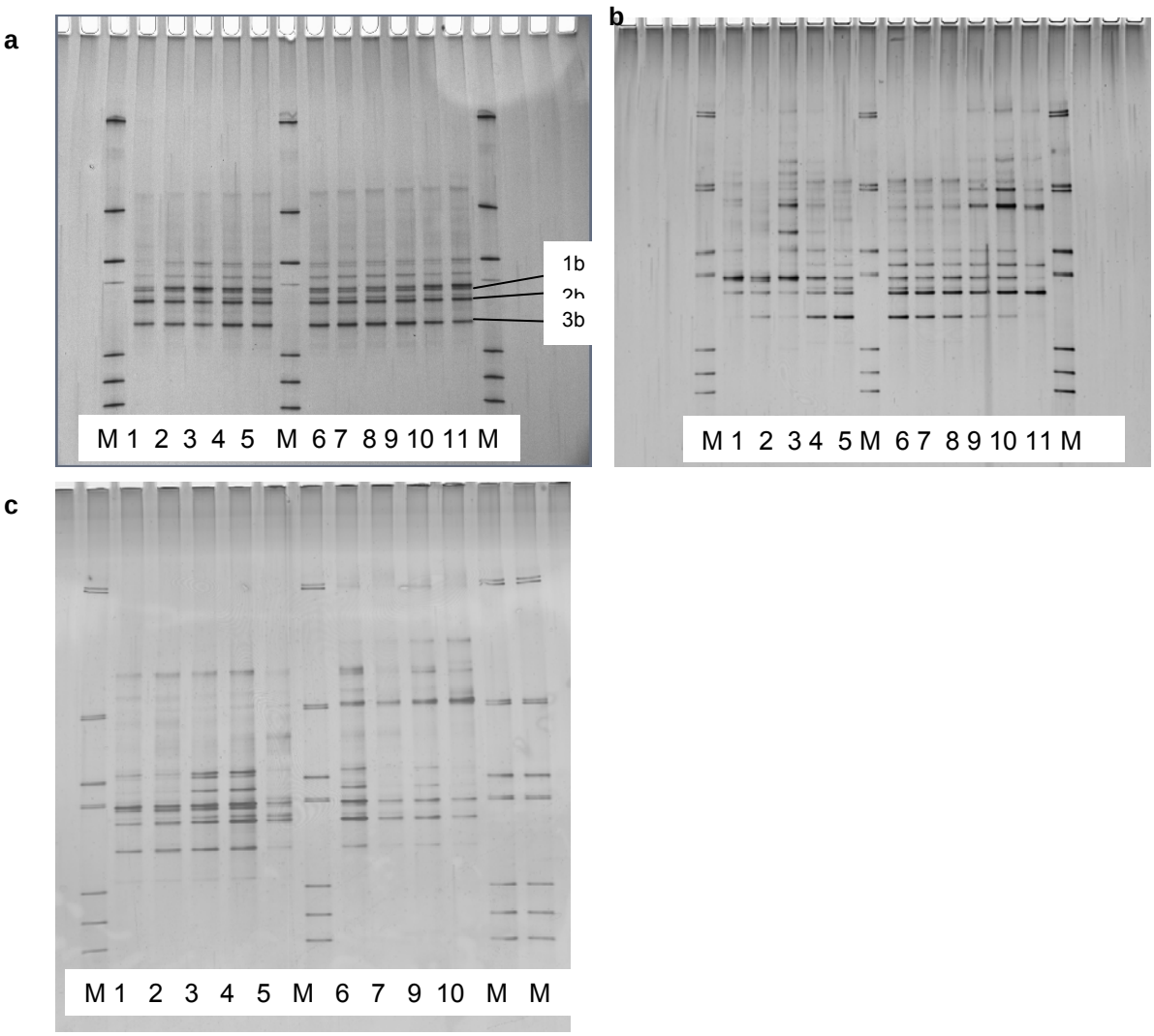

Figure 10. DGGE profiles of the archaeal community in fermentors without addition of BES (a) with the addition of BES (b) and with the addition of BES and reductive acetogens YE255, 257 and 266 (c).

4.1.2.3 Persistence of reductive acetogens in fermentations

The persistence of kangaroo reductive acetogens *C. glycolicum* YE255, YE257 and YE266/YE273 when methanogenesis was inhibited are presented in Figure 11. The BES only control fermentations were assayed for *C. glycolicum* YE255, YE257 and YE266/YE273 and these species were not detected at any stage.

Without inhibition of methanogenesis by BES (Section 4.1.1.2), the reductive acetogens did not persist in the fermentations and declined to undetectable levels. In the current work, *C. glycolicum* YE255 declined at a similar rate as it had previously without inhibition of methanogenesis. YE266/YE273 also declined but the rate of decline was considerably slower than when methanogenesis wasn't inhibited and this species was still detectable by the end of the experiments. Reductive acetogen YE257, on the other hand, shows considerable promise for future use as an alternative hydrogen utiliser, as long as methanogenesis can be inhibited. With BES addition YE257 was able to maintain its' population density throughout the fermentation. This suggests that YE257 is more robust than the other reductive acetogens, as had been hypothesized with the observation that

YE257 was present in both Red and Eastern Grey kangaroos whereas YE266/YE273 was only present in Eastern Grey kangaroos (Ouwerkerk *et al.,* 2007).

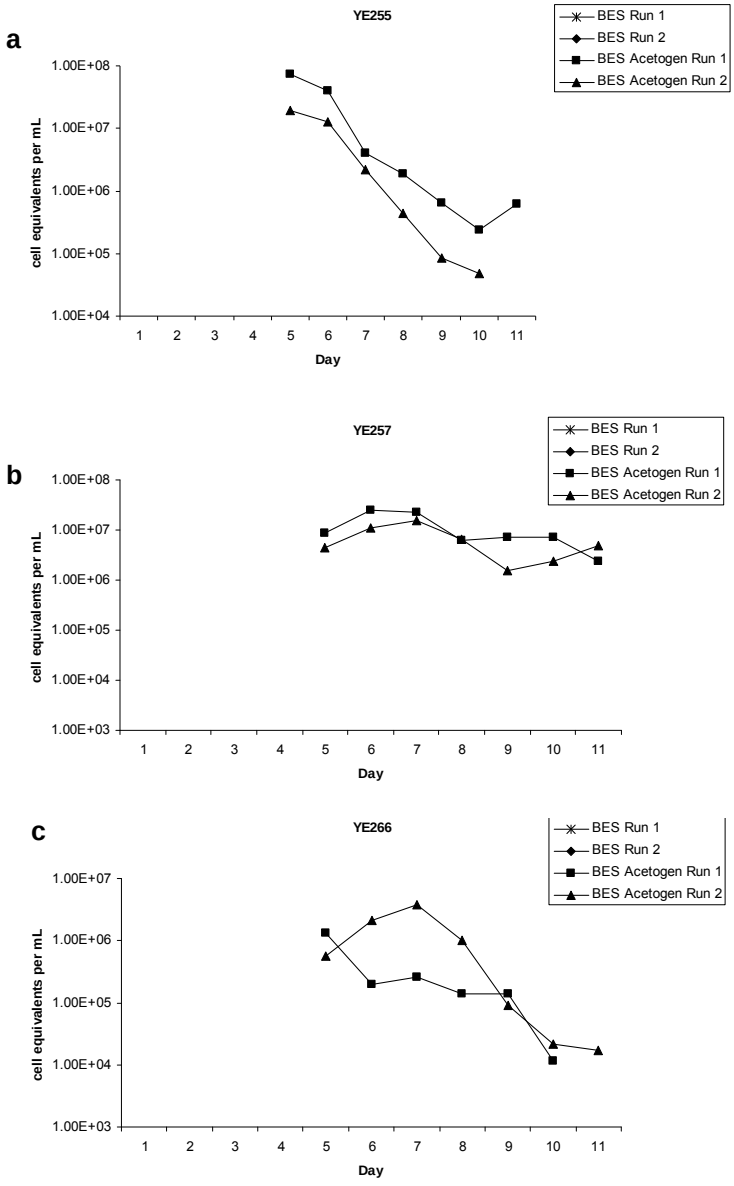

Figure 11. Population density of the kangaroo reductive acetogen Clostridium glycolicum *YE255 (a) YE257 (b) and YE266/YE273 (c) in fermentations with BES inhibition of methanogenesis.*

4.1.2.4 VFA production

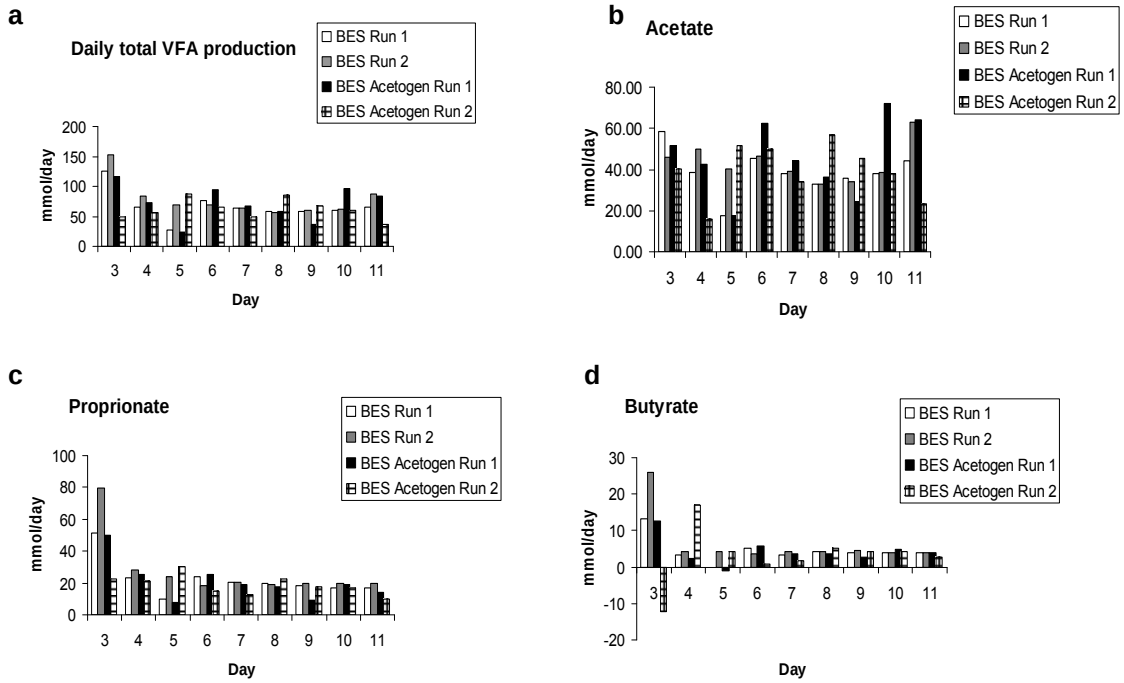

Figure 12. Daily average production of total VFA (a), acetate (b), propionate (c) and butyrate (d).

Between experiment variability in VFA production (Figure 12) was too great to be able to make any real conclusions from the data to date. Interestingly the acetate data is the most variable and on individual days, following the inoculation with reductive acetogens, acetate was considerably higher with reductive acetogens added than without, particularly in the first run. It is tempting to hypothesize that these spikes in acetate represent an additional contribution from the presence of reductive acetogens but more data would be required to support this assertion.

In conclusion, it appears that the inhibition of methanogenesis using BES markedly decreases methanogen numbers and appears to be more inhibitory to the normally dominant *Methanobrevibacter sp.* than methanogens with a higher ratio of AT to GC in their genome. The kangaroo reductive acetogens appear to survive better when methanogenesis is inhibited and isolate YE257 was able to maintain its population density under those conditions.

4.1.3 Fermentations with whole kangaroo gut contents only

4.1.3.1 Methane production

Daily methane produced in fermentation 2 as compared with the average control bovine fermentation (Section 4.1.1.1) is presented in Figure 13.

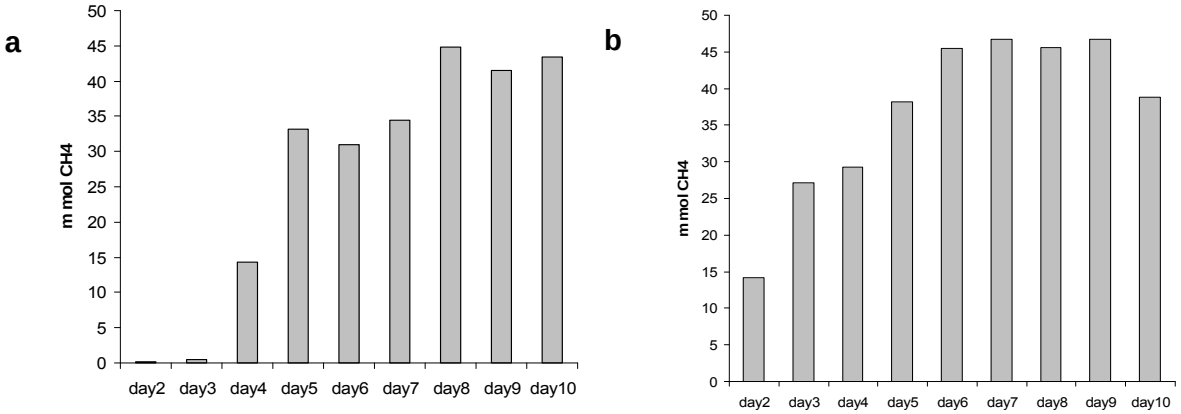

Figure 13. Daily methane production from a fermentation initiated with kangaroo foregut content (a) and the average of three control bovine fermentations (b).

In addition, in fermentation 1, the production of methane following incubation of 5 mL of fermentor liquor at 39ºC for 2 hours on day 10 (final day of fermentation) was measured and compared with two other fermentors being run concurrently, one producing leucaena inoculum and the other investigating the impact of 5% (on an oil content basis) cottonseed on methane reduction. The results are presented in Figure 14.

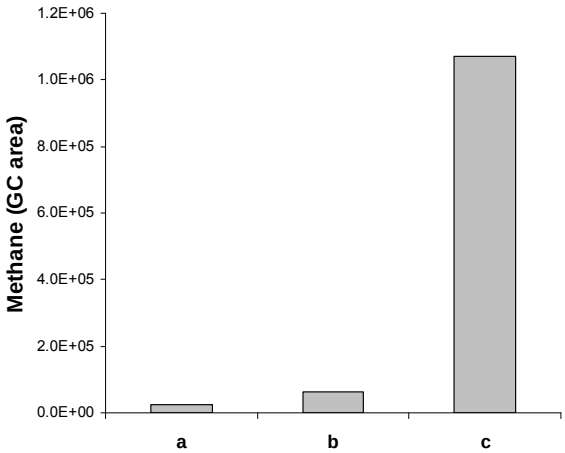

Figure 14. GC analysis of methane in headspace gas of incubated fermentor liquor from a kangaroo fermentation (a), leucaena (b), and a fermentation derived from cattle rumen contents with 5% cottonseed included in the diet (c) .

From Figure 13 the production of methane from the fermentation initiated with forestomach contents from the Grey kangaroo (fermentation 2) did not produce any methane until day 4 of the fermentation but then produced methane in a similar pattern and amount as in control fermentations initiated with cattle rumen contents. The lag in methane production was very interesting and suggested that something may have been inhibiting the methanogens in the early stages of the fermentation and/or that the original density of methanogens was very low. Fermentation 1 appears

not to have become methanogenic at all, although as methane was not measured throughout the fermentation but only from an aliquot of liquor taken and incubated at the end of the fermentation, this will require further investigation. The leucaena fermentor also did not appear to produce a large amount of methane but it was later found that this fermentation had a very distinct pattern of diurnal variation in emission, with a major increase in emissions following feeding (the incubated samples were taken prior to feeding) that was probably linked to the highly digestible nature of the leucaena leaf that it was being fed. This issue would not affect either of the other two fermentors and these should be more directly comparable suggesting that kangaroo fermentation 1 produced little if any methane.

4.1.3.2 Persistence of reductive acetogens and archaea in fermentations

The persistence of reductive acetogens and archaea in the two kangaroo fermentations was also very different to each other.

In fermentor 1 (Figure 15), the numbers of reductive acetogens was initially high and remained so throughout the fermentation. Interestingly, even higher numbers were found associated with the pasture hay (from day 2 onwards) than free in the liquor. Archaea were also present in this fermentation and while they decreased in population density towards the end of the fermentation in the liquor they increased in density on the pasture hay from the nylon bags. There are some other very unusual features about these archaeal populations, as well as the association with the plant fibre, particularly at a time when most of the digestible material had been removed and populations of other fibre associated bacteria were in decline. There are some anomalies in the data that need to be addressed, on day 2 no archaea could be detected and none could be detected in the liquor on day 10. Fresh duplicate samples were used to repeat the analyses and the results were the same. More interesting were the apparent lack of methane production and the co-existence of a large population of reductive acetogens and archaea. In addition, it is possible that these archaea were not methanogens (see DGGE; Section 4.1.3.3) and this will require further study as it would be both very novel and could be a factor involved in preventing methanogenesis in the kangaroo forestomach.

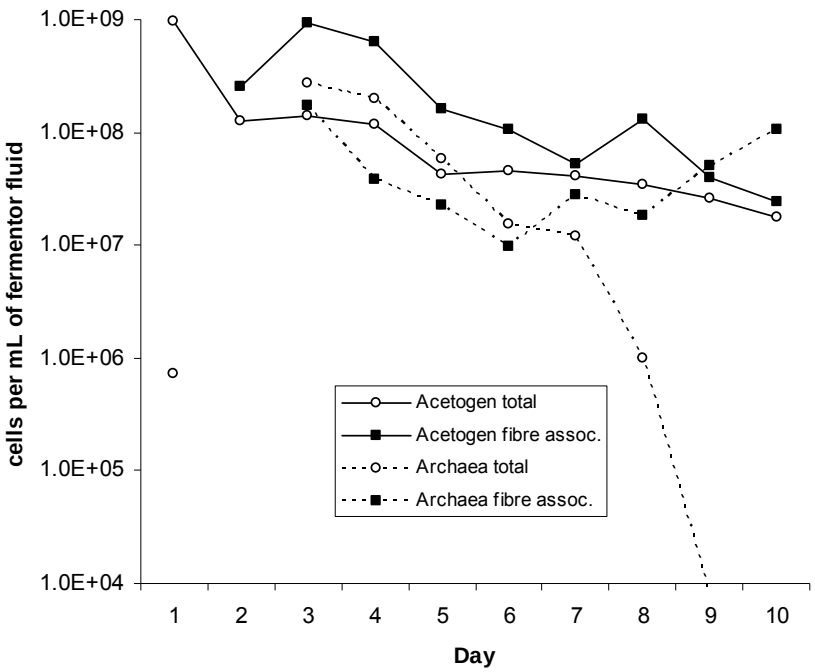

Figure 15. Population densities of reductive acetogens and archaea in kangaroo fermentation 1, initiated with contents from a Red kangaroo.

Fermentor 2 (Figure 16) was entirely different. The acetogens, initially present in the foregut material, were below detection limits by the second day of the fermentation and never recovered. Archaea on the other hand were not detected before day 4, then rapidly increased in numbers and reached a plateau density of around 10^8 per mL. The density of archaeans and the production of methane were fairly well parallel and the anomalies in Fermentation 1 were not apparent in this fermentation suggesting that the archaea present were methanogens and only methanogens. It is interesting to note that the methanogens did not become dominant until after the demise of the acetogens, indicating that in kangaroos the acetogens are dominant and prevent methanogenic populations developing. How this is achieved and why the acetogens died out so rapidly in fermentation 2 but not in fermentation 1 is a key question.

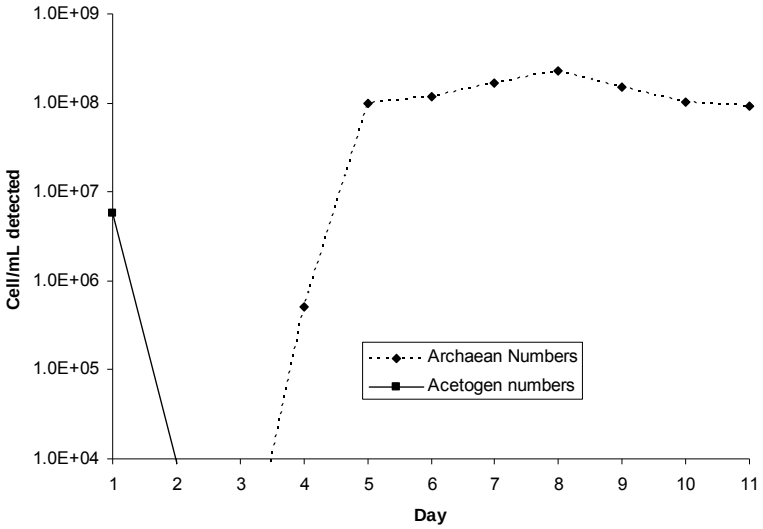

Figure 16. Population densities of reductive acetogens and archaea in kangaroo fermentation 2, initiated with contents from an Eastern Grey kangaroo.

4.1.3.3 DGGE

The first PCR step in the DGGE process is specific for archaea and if a product cannot be visualised then the populations are deemed to be below detection limits. Therefore, archaea were below detection limits in the original foregut content samples from both kangaroos, from all the samples from Fermentor 1, and from the first three days of Fermentor 2. The latter is in close agreement with real time PCR data for archaea and methane emission data for fermentation 2. The DGGE gel (Figure 17) of the archaeal community within the fermentor from days 4 to 11, is noticeably simpler than previous cattle rumen derived fermentations (Section 4.1.1.3) and it is likely that a single species, or at least one main species is present.

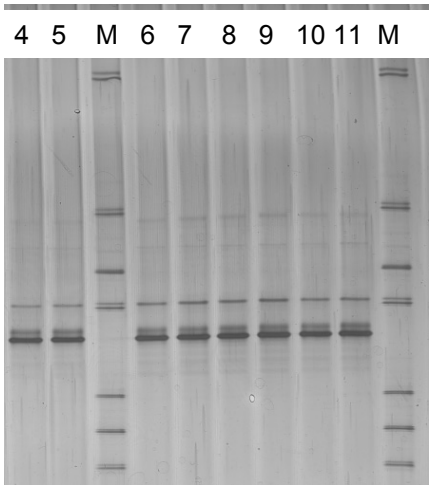

Figure 17. DGGE profile of the archaeal community in fermentor 2 from day 4 to 11. M – Markers. Numbers correspond to day of fermentation.

There appears to be a major anomaly with regard to archaeal populations in fermentation 1. Normal PCR suggests that there were no archaea present in the fermentation (which, if they were methanogens, would agree with the limited methane emission data), whereas, real time PCR

suggests that there was a large population of archaea present and that they tended to preferentially associate with the fibrous fraction of the diet. This same assay was used with fermentation 2 and all previous methane based fermentations and has shown a good correlation between the presence and absence of archaea and the presence and absence of methane. So there is little reason to suspect that there is a technical issue with the assay. If the assay was detecting something other than true archaea this should have been obvious previously. However, the two assays (conventional and real time PCR) do use different primers and it is possible that the primers used to amplify methanogens for DGGE are more specific than those in the real time assay, suggesting the intriguing possibility that there may be non-methanogenic archaea in the foregut of at least some kangaroos. Based on these results a more detailed study of the archaea in kangaroos was instigated (Section 4.2).

4.1.3.4 VFA production

The production of total VFA, acetate, propionate and butyrate are presented in Figure 18.

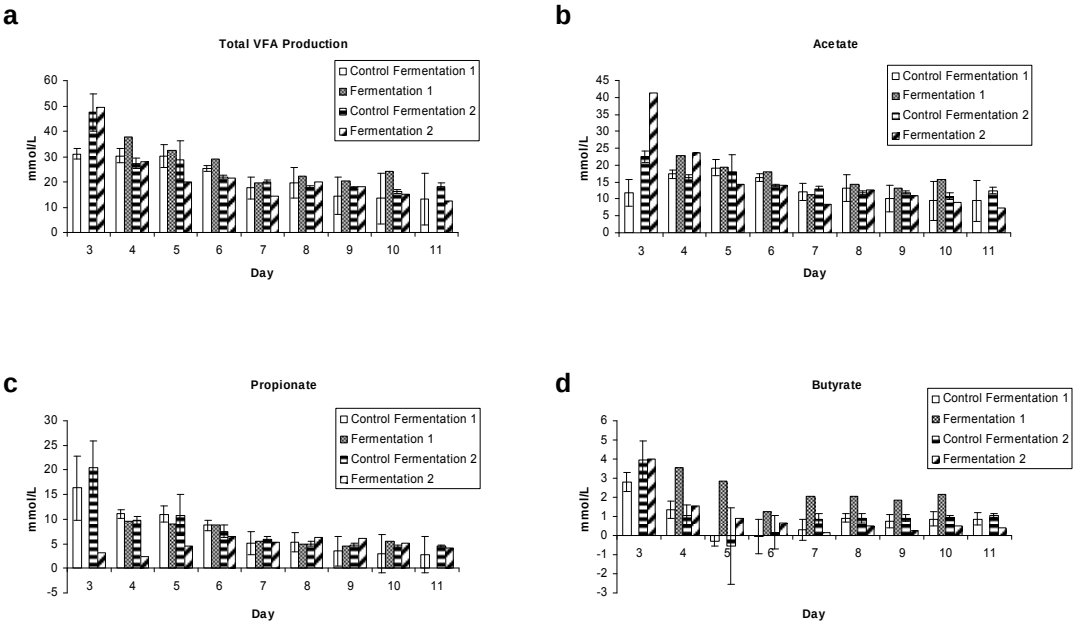

Figure 18. Total VFA (a) acetate (b) propionate (c) and butyrate (d). Control fermentation one (average of two fermentations), control fermentation two (average of three fermentations). Fermentation one and two, one fermentation each. Bars represent one standard deviation. In fermentation one, samples were not taken for day 3 and fermentation was complete by day 10.

There was a degree of variability between fermentations in total VFA production and in the proportion of acetate, propionate and butyrate. Fermentation one produced the highest levels of VFA's out of all fermentations, with higher levels of acetate and markedly higher levels of butyrate. Fermentation two had very low levels of propionate for the first five days and high acetate production, corresponding to the period when archaea were below detectable limits.

4.1.3.5 Effect on fibre utilisation

Results for fermentor 2 are presented in Figure 19.

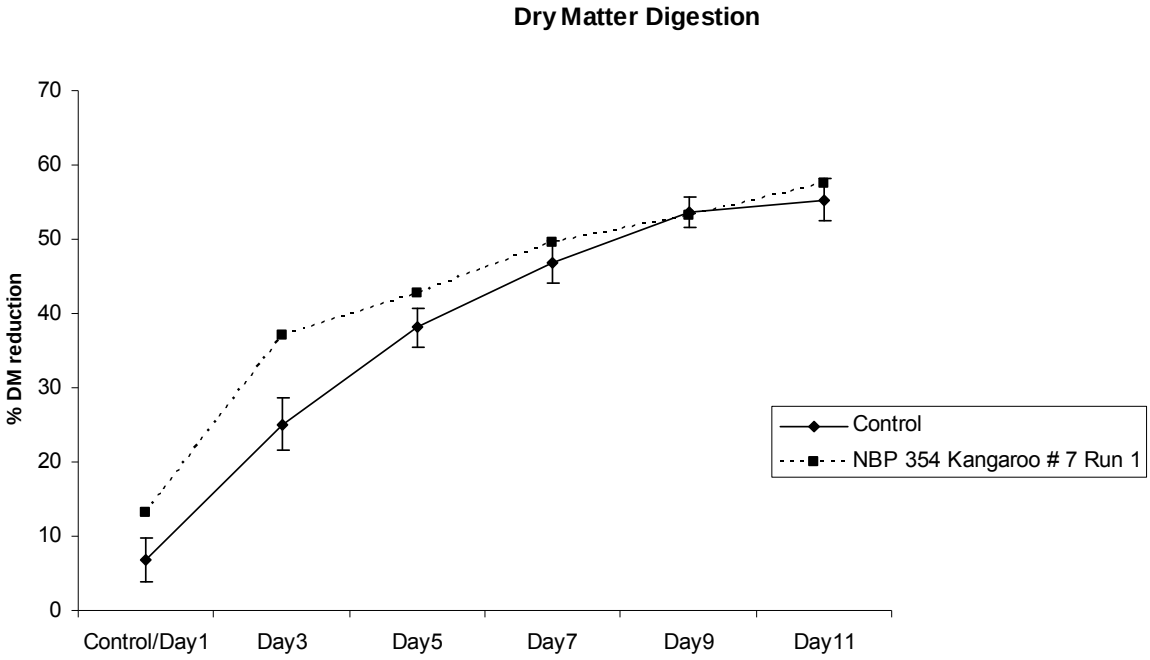

Figure 19. Percent removal of milled native pasture grass from nylon bags in the fermentor per sampling cycle. Bars show standard deviation from the mean for three control bovine-rumen-fluid based fermentations.

The kangaroo foregut content initiated fermentation appeared to digest native pasture grass faster than the cattle rumen derived fermentations. Both types of fermentations had similar end points with just above 50% of the dry matter being removed.

4.1.4 Addition of sample of whole kangaroo gut contents to a rumen based fermentation following inhibition of methanogenesis

4.1.4.1 Methane suppression

The use of BES to suppress methane as outlined in section 4.1.2.1 was again implemented here with similar outcomes.

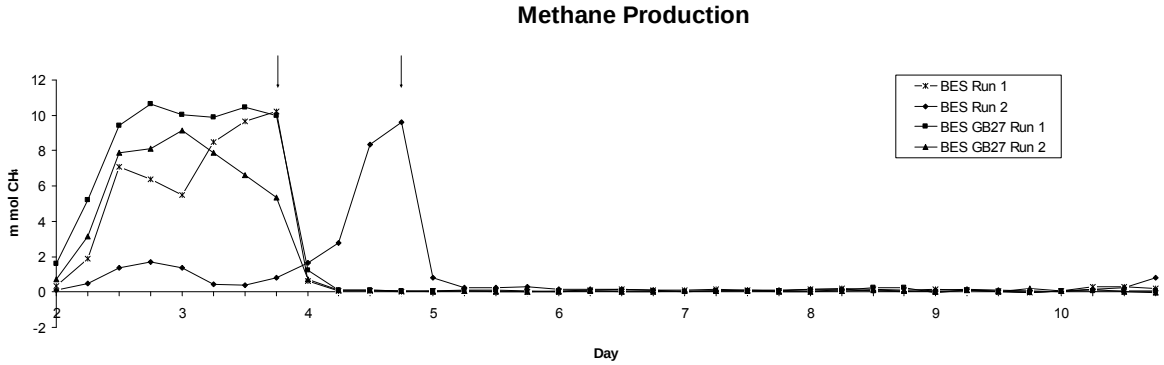

Figure 20. Six hourly methane production from fermentations with BES added, with and without kangaroo forestomach contents added. Arrows indicate when BES was added, kangaroo forestomach contents were added the day after BES was added.

4.1.4.2 Methanogen population density and diversity

Total methanogen numbers were markedly reduced by the addition of BES (Figure 21). Methanogen populations peaked at around 10^8 cells per mL immediately prior to BES addition (Day 4), following addition there was a steady decrease in numbers down to approximately 10^7 cells per mL, or a 90% reduction in numbers. The addition of whole kangaroo forestomach contents did not appear to influence this pattern and BES appears to be the major factor influencing methanogen numbers.

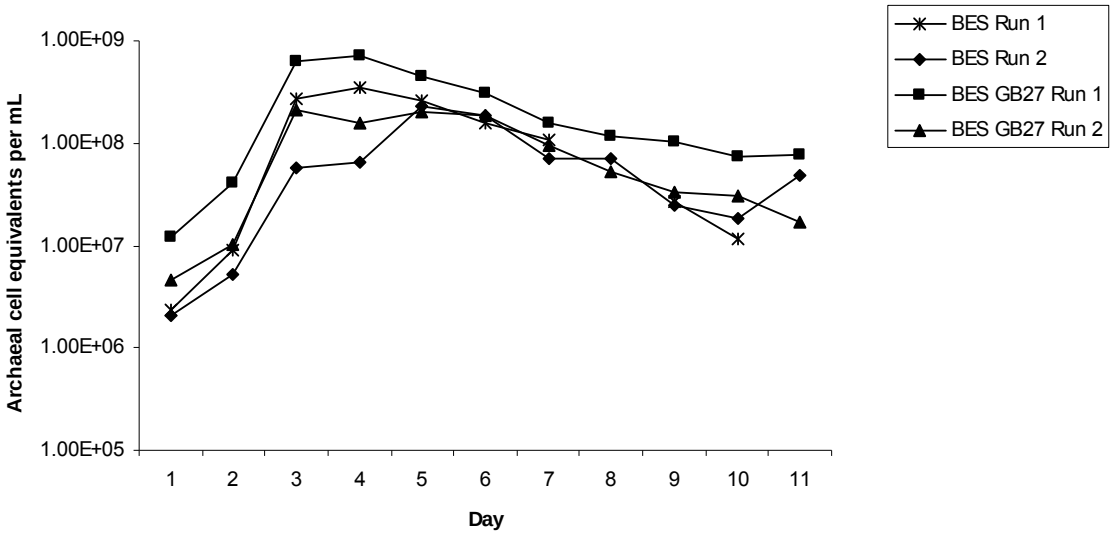

Figure 21. Cell equivalents of total archaeal numbers in fermentation liquor. Data for days 8 and 11 BES Run 1 were omitted for technical reasons.

As previously noted the addition of BES had a marked impact on the structure of the methanogen populations in the fermentor as identified from changing DGGE profiles (Figure 22). Also in keeping with the earlier work when cultures of the culturable acetogens were added, the addition of kangaroo forestomach contents appears to have had little impact on these changes and the major factor involved appears to be the addition of BES.

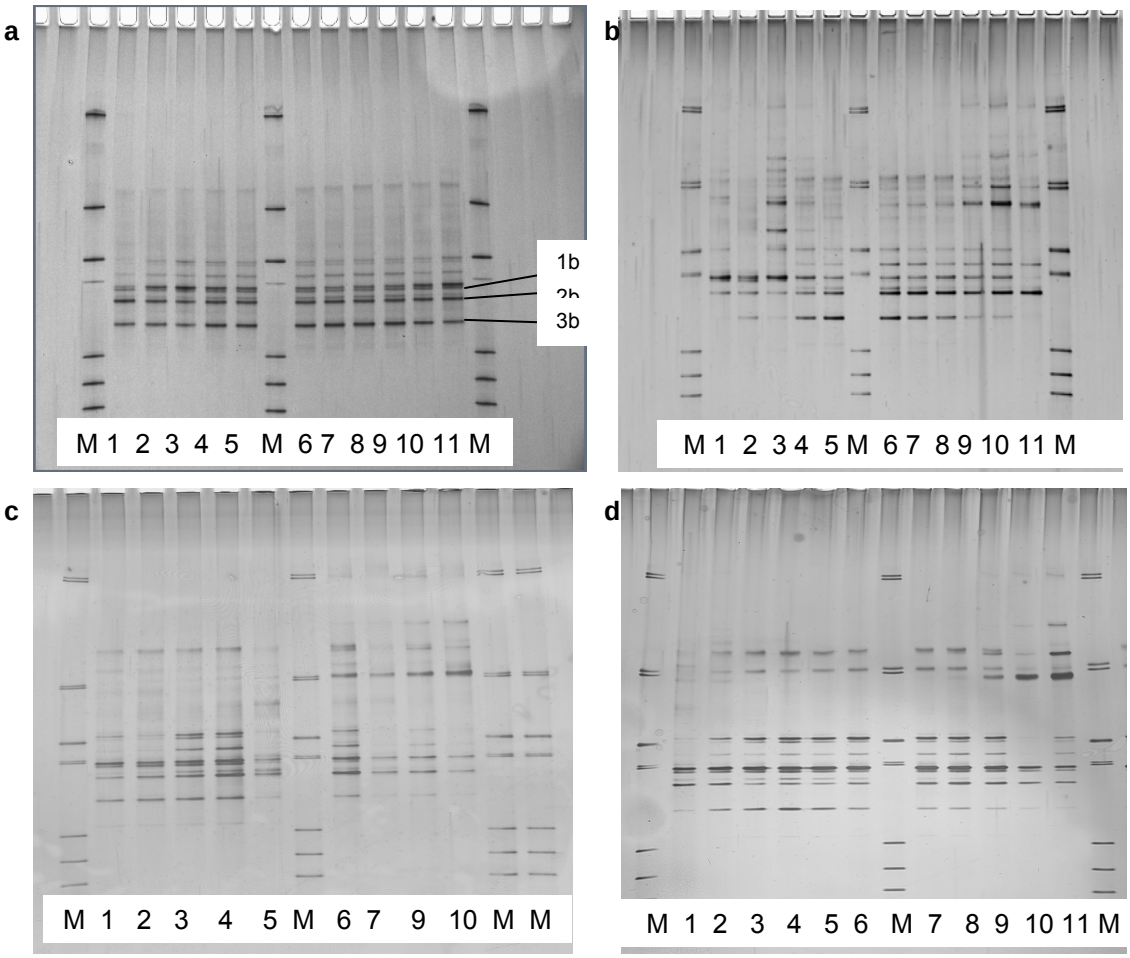

Figure 22. DGGE profiles of the archaeal community in fermentors without addition of BES (a) with the addition of BES (b), with the addition of BES and reductive acetogens YE255, 257 and 266 (c) and with the addition of BES and Grey Buck 27 (d).

4.1.4.3 VFA production

The addition of kangaroo foregut contents appears to have had a marked impact on VFA profiles in the fermentor (Figure 23). From 48 to 72 hours after addition total VFA increased 2 to 3 fold which was probably carried over from the inoculum but interestingly this wasn't additional acetate as may have been expected but a major increase in propionate and butyrate (up to 10 fold for propionate and above this for butyrate). Acetate concentrations were not consistently elevated by the addition of forestomach contents. This is a perplexing result that suggests that bacteria that produce propionate and butyrate as fermentation end products in the kangaroo forestomach could be more significant than in cattle and as propionate production consumes hydrogen these bacteria may play a role in the hydrogen economy in kangaroos and aid the reduction in methane emissions.

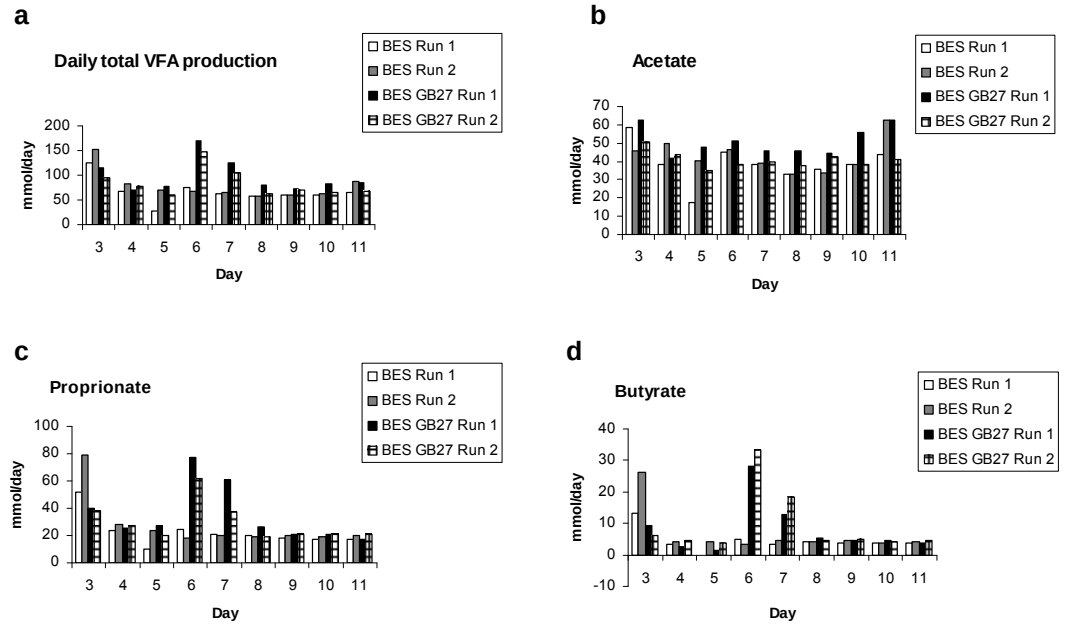

Figure 23. Daily average production of total VFA (a), acetate (b), propionate (c) and butyrate (d).

4.2 Defining the microbial core of hydrogen utilisation in kangaroos

4.2.1 Hydrogen utilising microbes in wild kangaroos

4.2.1.1 Presumptive archaeal populations

Results of assays to define archaeal populations are summarised in Table 5.

Table 5: Results of qualitative and quantitative archael PCR assays

Animal ID	ARCH 1 PCR	Band pattern for ARCH 1*	ARCH 516 Real time PCR	McrA PCR	McrA Real time PCR
Grey buck 1 (2000)	++	Both	-	+++	3.27E+05
Grey buck 2 (2000)	+++	1	-	+++	1.29E+08
Red doe 3 (2000)	+++	Extra	3.03E+07	+++	-
Red doe 4 (2000)	+	Extra	-	+++	-
Grey doe 1 (2002)	+/-	2	-	+/-	-
Red doe 2 (2002)	+	Both	3.11E+07	+++	-
Grey doe 3 (2002)	+/-	Both	7.02E+06	+	2.05E+05
Red buck 4 (2002)	+	Both	2.50E+06	+++	-
Red buck 5 (2002)	+/-	Both	-	-	-
Grey buck 6 (2002)	+/-	Both	-	+/-	1.53E+05
Grey buck 7 (2002)	+/-	Both	-	+/-	1.00E+06
Grey doe 8 (2002)	+/-	Both	9.33E+05	++	1.44E+06
Wallaroo buck 9 (2007)	+++	Extra	-	-	-
Grey doe 10 (2007)	+/-	Both	-	++	-
Grey buck 11 (2007)	+	2	-	-	1.48E+05
Red doe 12 (2007)	+/-	Both	-	+/-	-
Red buck 13 (2007)	+/-	Both	-	++	-
Wallaroo doe 14 (2007)	+++	Both	-	+++	-
Wallaroo doe 15 (2007)	+	Both	3.42E+06	+++	-

Animal ID	ARCH 1 PCR	Band pattern for ARCH 1*	ARCH 516 Real time PCR	McrA PCR	McrA Real time PCR
Wallaroo buck 16 (2007)	++	Both	-	+++	-
Grey doe 17 (2007)	+++	Both	4.57E+05	+++	-
Grey buck 18 (2007)	+	Both	3.01E+06	+++	1.05E+06
Red doe 19 (2007)	+	Extra	-	+	-
Red buck 20 (2007)	+	Extra	-	+	-
Wallaroo buck 21 (2007)	+	Both	-	+/-	-
Red buck 22 (2007)	+/-	Both	-	-	-
Wallaroo doe 23 (2007)	+	Both	-	-	-
Red buck 24 (2007)	+/-	Extra	-	-	-
Red doe 25 (2007)	+/-	Extra	-	-	-
Wallaroo buck 26 (2007)	+	Both	6.52E+05	++	-
Grey buck 27 (2007)	+/-	Both	-	+/-	1.62E+05
Grey buck 28 (2007)	+/-	Both	-	+/-	-
Grey doe 29 (2007)	+/-	Both	-	+/-	7.05E+05
Grey doe 30 (2007)	+/-	1	-	+++	-
Grey buck 31 (2007)	+/-	Both	-	+	-
Grey doe 32 (2007)	+/-	Both	-	+/-	-
Grey doe 33 (2007)	+/-	Both	2.04E+06	+	1.34E+07
Grey buck 34 (2007)	+/-	Both	7.49E+06	++	4.44E+07
Grey buck 35 (2007)	+/-	Both	-	+	-
Wallaroo doe 36 (2007)	++	Extra	1.37E+08	+++	2.44E+09
Wallaroo doe 37 (2007)	++	Extra	8.80E+07	+++	2.96E+10
Wallaroo buck 38 (2007)	++	Extra	5.80E+07	++	2.82E+7
Sheep 686	+++	1	9.67E+09	+++	1.73E+10
Sheep 791	+++	1	1.86E+10	+++	3.02E+10
Sheep 680	+++	1	4.81E+10	+++	4.30E+10
Steer 892	++	1	1.83E+08	+++	1.96E+08
Steer 574	++	1	1.41E+08	+++	2.35E+08
Steer 871	++	1	2.11E+08	+++	1.75E+08

Qualitative PCR results; -= below detectable limits; +/- indicates marginal positive; + indicates weak positive; ++ indicates postive; +++ indicates strong postive.
See Figure 24. Banding patterns. Band **1 indicates a band of a size expected for the archaeal (methanogen) 16S rRNA gene; band **2** indicates the presence of another common band that differs significantly in size to Band 1 (see Figure 24); **Both** indicates that Band 1 and 2 were both present in the sample; **Extra** indicates that additional bands of differing size were also present.*

Four distinct assays were used to try to better define archaeal populations in kangaroos (with three sheep and three steers included for comparison). The ARCH 1 PCR was qualitative and demonstrated the presence of archaea. The ARCH 516 real-time assay enumerated all archaea and has a broader spectrum than the ARCH 1 PCR. The McrA PCR detects a key gene in the methanogenesis pathway and the McrA real-time PCR assay enumerates the number of cells that possess this gene. Different primer sets were used in these assays, although this shouldn't have been an issue if the archaea present were related to methanogens with known mcrA genes.

However, the results were both extremely interesting and perplexing. All the rumen samples were exactly as expected, all were strongly positive for archaea, produced a PCR product (16S rRNA gene) of expected size (Figure 24), had very high numbers of archaea and very high numbers of cells with McrA genes, indicating that the archaea were most likely methanogens (as would be expected). Results from the kangaroos were much more diverse and contradictory. Most of the kangaroos were positive for the archaeal 16S rRNA gene but many only marginally positive to weakly positive (indicating very low numbers). However, only two kangaroos had PCR product of

only the expected size (as shown in the sheep and cattle, Figure 24) and almost all had a second band of larger than expected size with many having even more additional bands. Many of the animals that had a marginal or weak positive result in the ARCH 1 PCR were also below detectable limits with the ARCH real-time PCR, but there were a lot of anomalies (e.g. Grey buck two, strong positive but below detectable limits by real time PCR, Grey buck 35, marginal but by real time PCR above 10^5 cells per mL). Fourteen kangaroos (including 50% of the Wallaroos) did have detectable numbers of archaea, however, at the most these populations were at densities between 10 and 1000 fold fewer than sheep and cattle. An exception was Wallaroo doe 36 which had population densities similar to ruminants. The additional PCR products also tend to suggest that there are novel archaea present in kangaroos that are not present in ruminants.

The McrA assays (Table 5) complicate the picture further and with more anomalies. While many kangaroos that didn't possess archaea also were not positive for the McrA gene nor had detectable numbers of cells with this gene, just as many without archaea were either positive for the gene (Figure 25), many strongly so, or by real time PCR had appreciable numbers with McrA genes. There was also a lot of anomaly between the two McrA gene PCR assays. No such anomalies were seen in the sheep and cattle. As these assays prime to different positions along the gene it is possible that the anomalies represent the presence of genes that are similar in places to known McrA genes but are quite different elsewhere. These genes may not even be associated with methanogenesis. As many kangaroos were positive for McrA "related" genes but lacked archaea this would appear to be the case. The fact that many kangaroos had archaea that lacked McrA genes also indicates that in kangaroos the presence of archaea cannot be equated to the presence of methanogens or methanogenesis. Of further interest is the melting point (temperature denaturation of the PCR amplicon in real-time PCR assays) of the McrA amplicon from kangaroo forestomach contents and of McrA genes from pure cultures of known methanogens (Figure 26). The amplicons from the kangaroos positive for the presence of McrA genes had similar melt temperatures but these were different from those of known culturable methanogens. This indicates that the amplicons differed in DNA sequence.

While methanogenesis appears uncommon or absent from many kangaroos, it cannot be entirely dismissed. Of particular interest were three Wallaroos (36, 37, 38), all from the Dingo area of Queensland, that had consistent profiles similar to ruminants. These animals were positive to strongly positive in qualitative assays and had high numbers of both archaea and cells with McrA genes. The size of the 16S rRNA gene amplicons in these animals was more complex than with ruminants and they had much higher reductive acetogen populations (Table 6). Whether these animals were methanogenic would require further study.

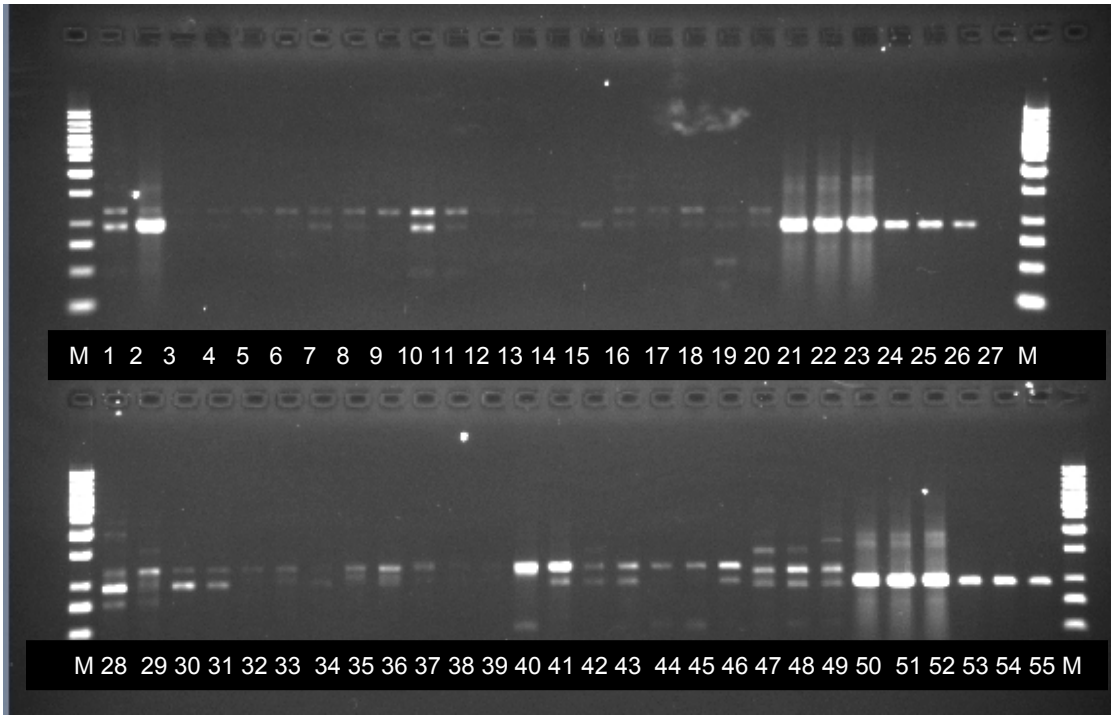

Figure 24. ARCH 1 PCR products from gut contents from Grey kangaroos (lanes 1-20), Sheep (lanes 21-23), Steers (lanes 24-26), Red kangaroos (lanes 28-39), Wallaroos (lanes 40-49), Sheep (lanes 50-52) and Steers (lanes 53-55). Negative control - lane 27. M = DNA size Marker

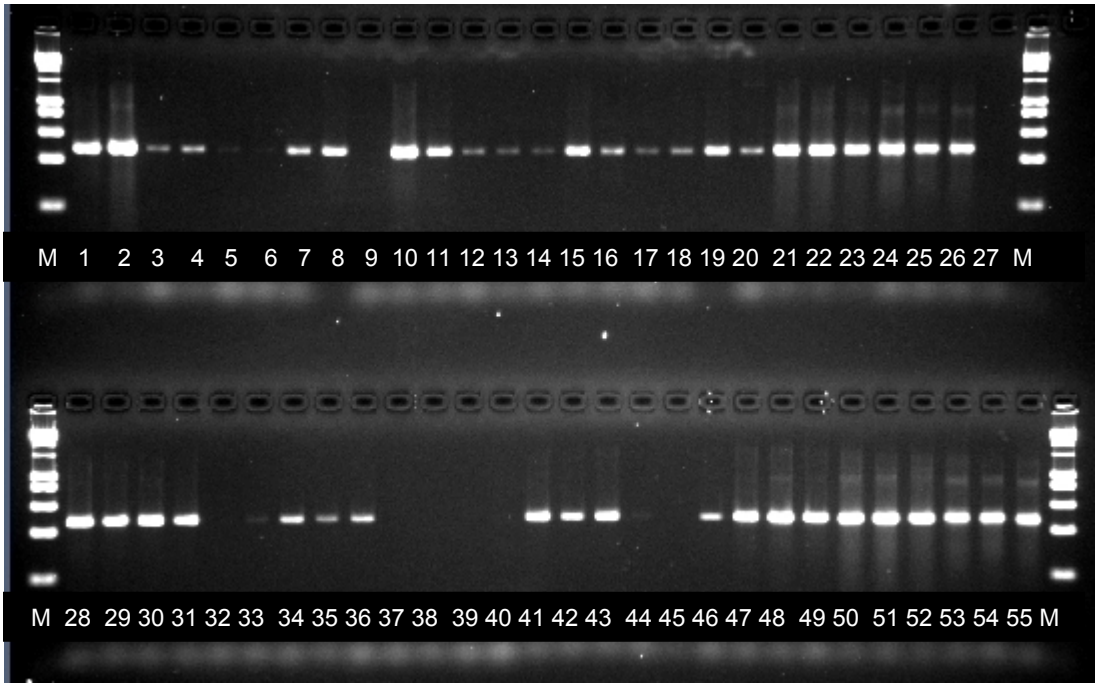

Figure 25. McrA PCR products from gut contents from Grey kangaroos (lanes 1-20), Sheep (lanes 21-23), Steers (lanes 24-26), Red kangaroos (lanes 28-39), Wallaroos (lanes 40-49), Sheep (lanes 50-52) and Steers (lanes 53-55). Negative control - lane 27. M = DNA size Marker.

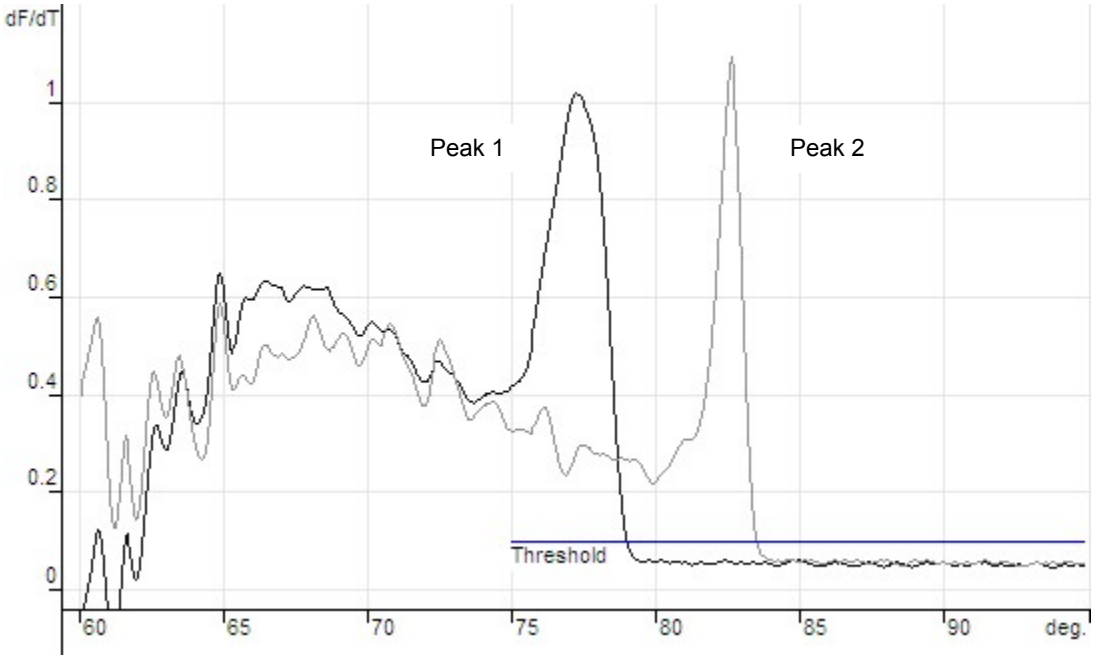

Figure 26. Melt peaks of McrA genes from Methanobrevibacter smithii (*Peak 1) and a kangaroo (Peak 2).*

4.2.1.2 Reductive acetogen populations

Results of assays to enumerate reductive acetogens isolated from kangaroo foregut contents in the foregut of all 42 kangaroos and three cattle and three sheep are presented in Table 6.

Table 6. Real-time PCR enumeration of Clostridium glycolicum YE255 and reductive acetogens YE257 and YE266/YE273

Animal ID	YE255	YE257	YE266/YE273
Grey buck 1 (2000)	-	9.57E+07	1.41E+08
Grey buck 2 (2000)	-	1.14E+07	8.83E+04
Red doe 3 (2000)	-	1.28E+07	2.78E+05
Red doe 4 (2000)	9.01E+04	1.36E+08	8.17E+06
Grey doe 1 (2002)	-	1.76E+06	1.18E+06
Red doe 2 (2002)	-	5.82E+05	3.74E+04
Grey doe 3 (2002)	-	5.22E+05	7.24E+04
Red buck 4 (2002)	-	4.32E+06	1.78E+05
Red buck 5 (2002)	-	1.34E+07	7.26E+04
Grey buck 6 (2002)	-	1.07E+06	1.72E+06
Grey buck 7 (2002)	-	1.64E+06	7.29E+05
Grey doe 8 (2002)	-	3.79E+05	3.00E+05
Wallaroo buck 9 (2007)	-	6.67E+06	8.81E+05
Grey doe 10 (2007)	-	8.56E+06	3.65E+06
Grey buck 11 (2007)	-	1.66E+07	2.05E+06
Red doe 12 (2007)	-	4.36E+06	2.55E+06
Red buck 13 (2007)	-	3.92E+06	2.29E+05
Wallaroo doe 14 (2007)	-	1.45E+05	6.70E+05
Wallaroo doe 15 (2007)	-	1.69E+05	1.01E+05
Wallaroo buck 16 (2007)	-	1.09E+06	3.46E+05
Grey doe 17 (2007)	-	1.15E+06	8.38E+05

Animal ID	YE255	YE257	YE266/YE273
Grey buck 18 (2007)	-	8.74E+05	2.89E+06
Red doe 19 (2007)	-	2.02E+06	5.96E+05
Red buck 20 (2007)	-	1.96E+06	2.08E+05
Wallaroo buck 21 (2007)	-	4.22E+06	3.66E+05
Red buck 22 (2007)	-	1.85E+07	1.08E+06
Wallaroo doe 23 (2007)	-	1.51E+06	7.98E+06
Red buck 24 (2007)	-	1.84E+06	2.62E+05
Red doe 25 (2007)	-	2.80E+06	2.33E+05
Wallaroo buck 26 (2007)	-	1.36E+06	5.38E+04
Grey buck 27 (2007)	-	3.61E+06	1.41E+06
Grey buck 28 (2007)	-	4.30E+06	4.03E+06
Grey doe 29 (2007)	-	1.05E+06	1.49E+06
Grey doe 30 (2007)	-	9.13E+05	3.17E+06
Grey buck 31 (2007)	-	1.53E+07	9.17E+06
Grey doe 32 (2007)	-	5.98E+05	1.21E+06
Grey doe 33 (2007)	-	9.28E+06	3.69E+06
Grey buck 34 (2007)	-	3.68E+06	1.27E+06
Grey buck 35 (2007)	-	3.81E+07	2.04E+07
Wallaroo doe 36 (2007)	-	9.23E+05	1.04E+07
Wallaroo doe 37 (2007)	-	1.35E+08	1.97E+06
Wallaroo buck 38 (2007)	-	1.98E+08	3.75E+06
Sheep 686	-	3.30E+04	2.43E+04
Sheep 791	-	4.43E+04	7.77E+03
Sheep 680	-	3.28E+05	3.97E+03
Steer 892	-	-	-
Steer 574	-	1.46E+04	-
Steer 871	-	-	-

-- = Below detectable limits

All except one kangaroo were negative for YE255. As previously observed, this species appears to be a minor species *in vivo* but is rapidly selected for in *in vitro* culture. All kangaroos were positive for YE257, YE266/273 and mostly in relatively high numbers. In comparison, in the ruminants only one steer was positive for YE257, in low numbers, all other steers were below detectable limits for both assays. Sheep had very low numbers of these (or related) species. It appears that these species (not YE255) of reductive acetogens are common to all kangaroos and usually at relatively high numbers. In comparison with the archaeal/methanogen data, reductive acetogenesis appears to play a greater role than methanogenesis. Interestingly, the Wallaroos (36, 37, 38) that had high density populations of archaea and cells with McrA "related" genes also supported high density populations of reductive acetogens, particularly YE257.

4.2.1.3 FTHFS clone library results

Despite the density of these acetogens, we know that the reductive acetogens that we have isolated to date represent only a small proportion of the diversity of reductive acetogens present in the forestomach of kangaroos. To better establish the breadth of this diversity and to identify groups and species of reductive acetogens that are important to try to isolate for future work we constructed clone libraries of PCR amplified FTHFS genes from kangaroo foregut contents, sequenced the DNA and compared with sequences from known reductive acetogens. Clone libraries of partial FTHFS genes from 15 kangaroos, three from Grey does, three from Grey bucks, two from Red does, three from Red bucks, three from Wallaroo does and one from a Wallaroo buck have been established and sequence data obtained from 161 clones. Approximately 1000 bp of DNA sequence was

obtained from each clone which translates into approximately 330 amino acid residues. The phylogenetic tree of partial FTHFS sequences (Figure 27) shows that no clones matched 100% to FTHFS DNA sequences of acetogens from other environments. Clones from Grey Doe 8 and Grey Buck 7 matched to kangaroo acetogen isolates YE273 and YE266 and a clone form Red buck 5 matched to acetogen isolate YE257. No clones were obtained that matched to isolate YE255 (*Clostridium glycolicum*). A large number of clone sequences (50 clones from 13 kangaroo samples), formed a large clade that did not match any known sequences. To reduce the overall size of the tree this clade was compressed to one representative clone marked with an underline on the phylogenetic tree.

Figure 27. Phylogenetic relationships between FTHFS genes cloned from the kangaroo foregut and from cultured reductive acetogens (bolded).

This study has confirmed that the extent of uncultured diversity of reductive acetogens in the kangaroo forestomach is very large and highlighted a clade comprising many species for which we currently have no culturable representatives.

4.2.1.4 Clone libraries constructed with archaeal PCR amplicons

To further add to the information on the archea present in kangaroos, several clone libraries have been constructed in order to define the archea being detected. Qualitative and quantitative PCR products (Table 5 and Figures 24 and 25) were used in the clone libraries.

Figure 28 shows the phylogenetic analysis of 903 bp of archaeal 16S rRNA gene sequence with clones from two kangaroos, grey doe 17 (5 clones) and red doe 2 (4 clones) forming a well supported clade with *Methanosphaera stadtmanae*, a methanogen isolated from human intestine (Miller and Wolin, 1985) that can only generate methane by reduction of methanol with H_2. Sequencing of *Methanosphaera stadtmanae's* genome has revealed that it lacks many of the protein-encoding sequences that are required to reduce CO_2 to methane, resulting in it having one of the most restricted energy metabolisms of all methanogens (Fricke *et al.* 2006). Another two clones from wallaroo buck 36 form a well supported clade with *Thermoplasma acidophilum*, a thermoacidophilic archaea originally isolated from a coal waste pile (Darland *et al*. 1970). *Thermoplasma acidophilum* is able to gain energy anaerobically through sulphur respiration and has adapted to scavenging nutrients from the decomposition of other micro-organisms in its environment (Ruepp *et al*. 2000).

A larger piece of DNA produced in the Arch PCR using gDNA extracted from forestomach contents from kangaroos was cloned for a number of kangaroos including wallaroo buck 9 (four clones); grey doe 17 (one clone) and red doe 4 (two clones). Approximately 1000 bases of DNA sequence obtained from these clones showed over 95% similarity over the full length between the clones from different kangaroos. However, when the DNA sequences were submitted via the BLAST program at NCBI for comparison to sequences in the GenBank database they did not show significant similarity to any sequences in the database.

Figure 29 shows the phylogeny of partial amino acid sequence translated from the functional gene methyl coenzyme M reductase A (McrA) PCR product. Approximately 151 amino acids were aligned and subjected to parsimony analysis with 100 bootstraps. Clones obtained from cattle, sheep and one kangaroo (grey buck 2) were placed within the genus Methanobrevibacter, a group of methanogens found to be dominant in the cattle rumen.

A second group of clones from kangaroos and cattle formed a well supported clade with a group of clones representing anaerobic methane oxidizers (AMNE) from the sulphate-methane interface in the Gulf of Mexico (Lloyd *et al.* 2006). These archaea are regarded as anaerobic methanotrophs and catabolise methane back into carbon dioxide and hydrogen, the opposite reaction to their methanogenic relatives. McrA gene sequences have been identified in enrichments of methane oxidizers from two subgroups (AMNE1 and AMNE2) (Hallam *et al.* 2003, 2004) and McrA is considered a useful and specific marker gene for detecting and identifying anaerobic methane oxidizers, as well as methanogens in the environment (Hallam *et al.* 2004, 2004, Lloyd *et al. 2006*). Several clones obtained from a Tammar Wallaby by Evans *et al.* (2009) also cluster within this clade.

A third cluster of McrA clones from kangaroos and some sheep form a strongly supported clade with *Methanosphaera stadtmanae* and clones from the tammar wallaby study. The clade included clones from grey doe 17 and red doe 2 reflecting the 16S rRNA data also obtained for these animals.

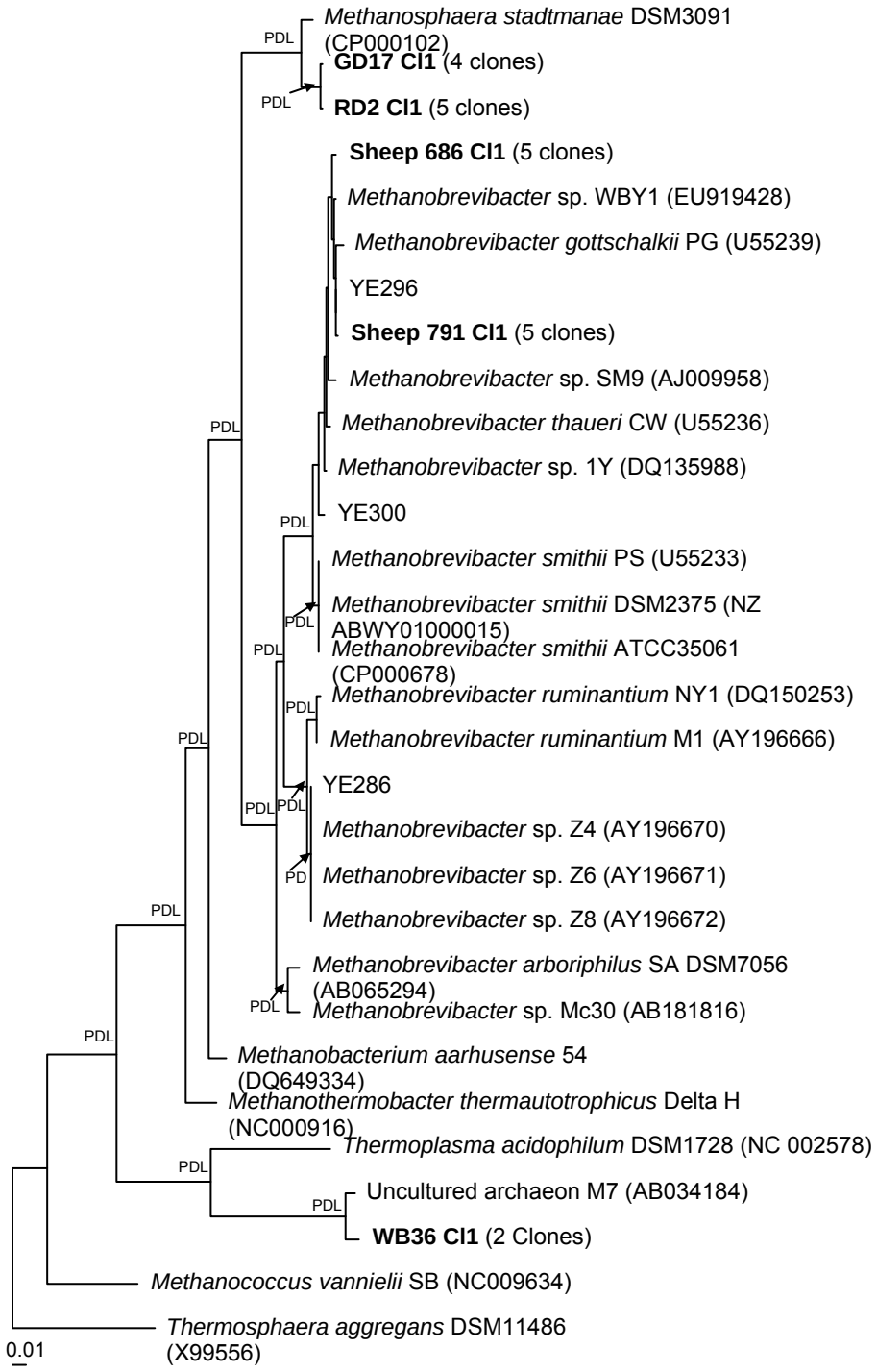

Figure 28: A parsimony (P) tree constructed from 903 bases of 16S rRNA gene sequence indicating the positions of the clones to related species and other known methanogenic archaea. Bootstrap values for parsimony (P, 1000 replicates), distance (D, 1000 replicates) and likelihood (L, 100 replicates) analyses are indicated with a corresponding letter if they exceeded 80% support. *Thermosphaera aggregans* was used as the out-group. The bar represents a sequence divergence of 1%.

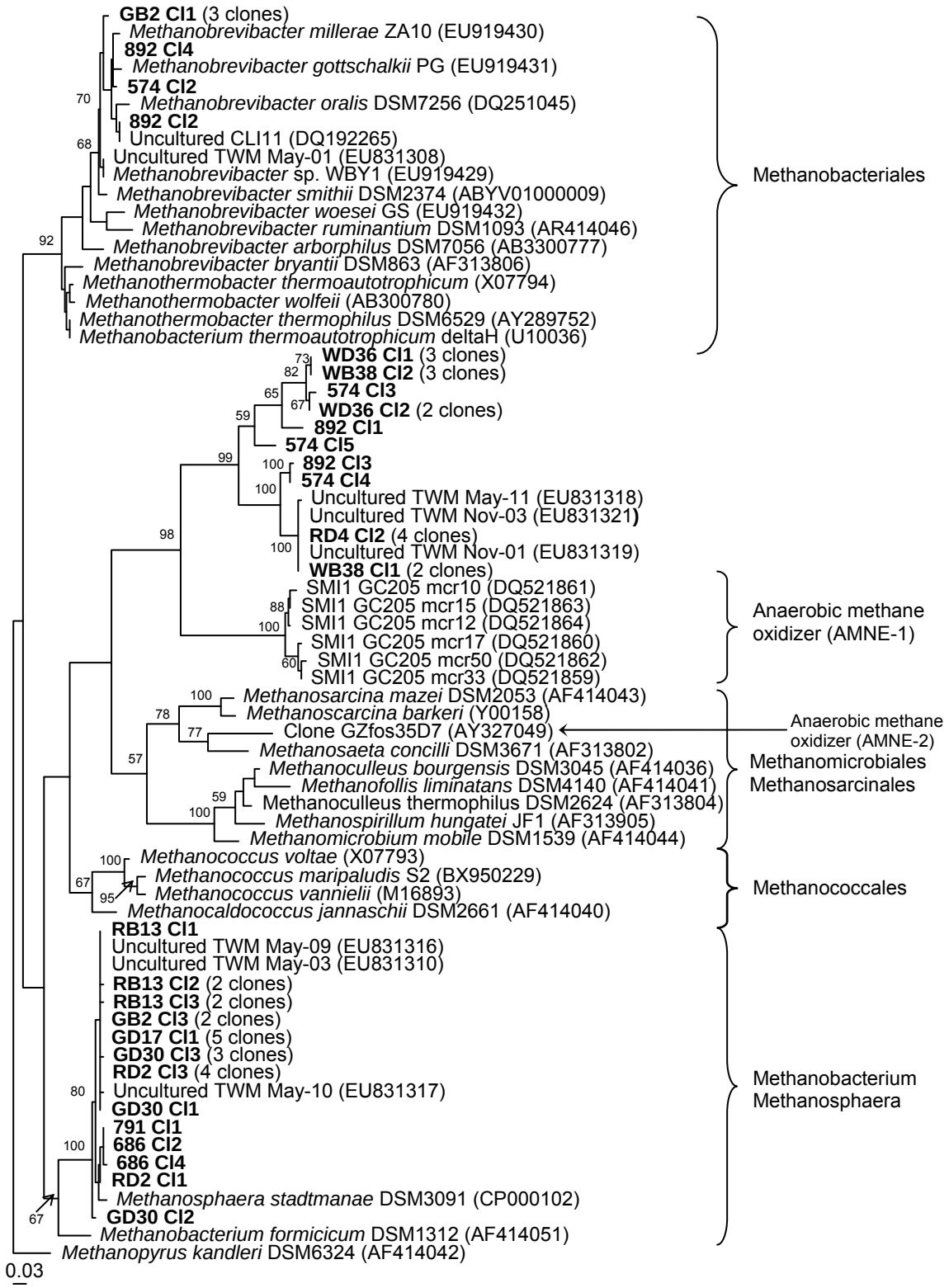

Figure 29: A parsimony (P) tree constructed from 154 amino acids translated from the methyl-coenzyme M reductase alpha subunit (mcrA) gene sequence indicating the positions of the clones to related species and other known methanogenic archaea. Bootstrap values for parsimony (P, 100 replicates), are indicated with a corresponding letter if

they exceeded 50% support. *Methanopyrus kandleri* was used as the out-group. The bar represents a sequence divergence of 3%.

4.2.1.5 In vitro *methane production analysis*

A comparison of methane generation of kangaroo foregut contents and rumen contents when incubated in fresh medium for seven hours was undertaken with samples from six cattle and six kangaroos. Significant methane production was observed in each bovine sample, despite variability between replicate samples (see Figure 30). Low levels of methane production were observed for wallaroos, with negligible methane produced from cultured digesta of both Red and Eastern Grey kangaroos.

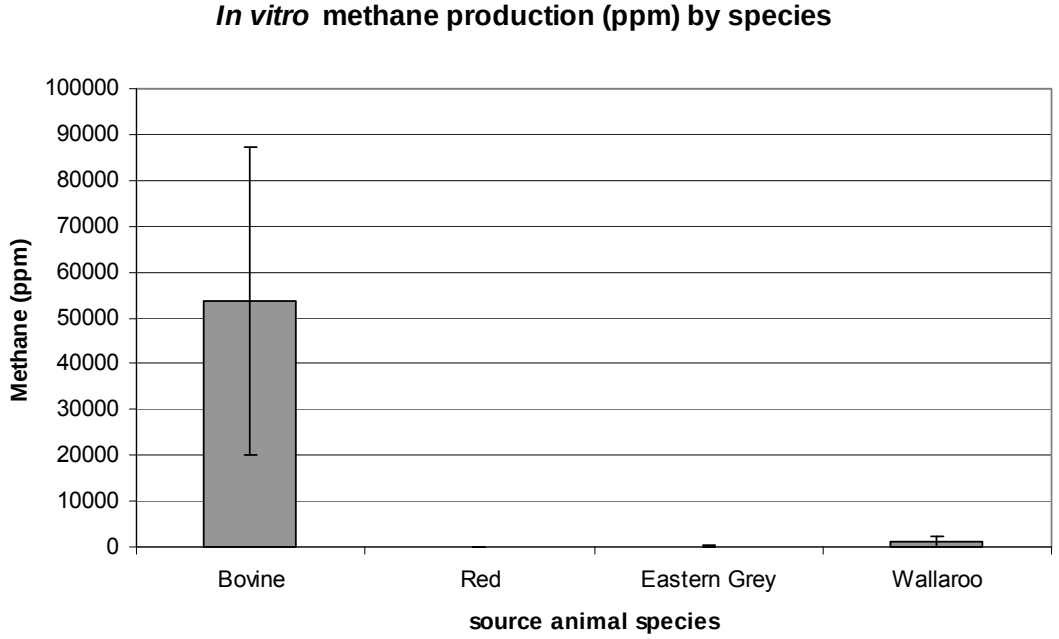

Figure 30: In vitro *methane production over 7 hours. Methane production is presented as ppm (methane) in the 10.7 mL headspace.*

Real time PCR for total archaea and for cells containing the McrA gene are presented in Figure 31. All cattle samples and some of the kangaroo samples had detectable archaea and McrA genes. Numbers in cattle were consistently always high in both assays and at both times whereas in kangaroos, numbers were always lower and fluctuated unpredictably. Similar anomalies were noted earlier between the presence of archaea and cells containing McrA genes. Also, from three kangaroo samples archaea increased over seven hours whereas in five kangaroo foregut cultures, McrA gene numbers decreased (to undetectable levels in two cultures) and two of these kangaroos were the same animals, indicating a lack of congruence between the archaea and the presence of the McrA gene in kangaroos.

This further suggests the dominance of non-methanogenic archaea (when archaea are present) in most kangaroos, with the possible exception of some Wallaroos.

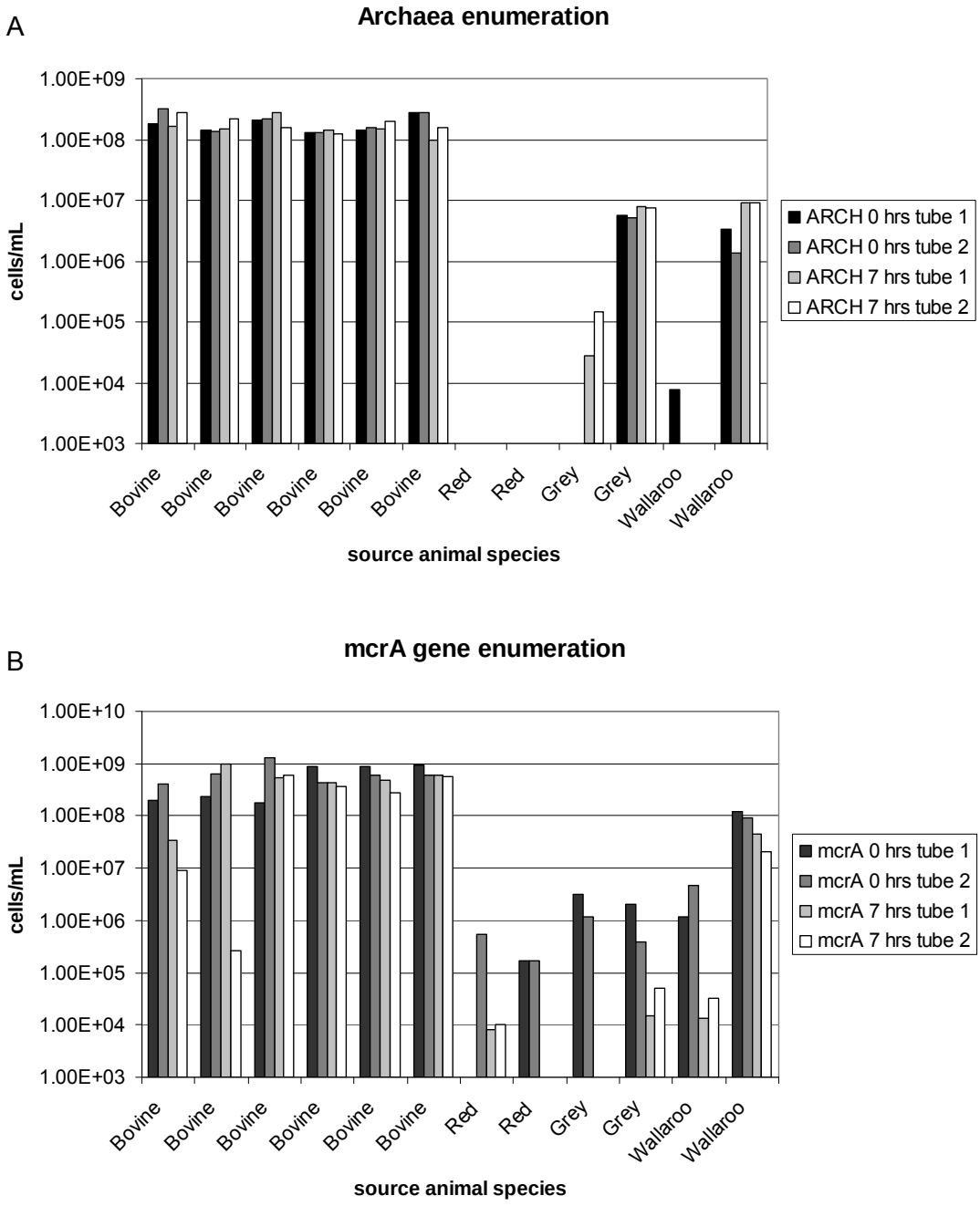

Figure 31: Real time PCR enumerations of in vitro methane production samples for A: total archaea; and B: McrA gene.

5 Success in achieving objectives

5.1 Overall

The objectives of the project were largely achieved. The project did require revision and modification which resulted in one of the original objectives being omitted and replaced with two new objectives. One original objective and the two new objectives were achieved although the research team would have liked to have achieved more, as the project was very challenging and has resulted in a significant number of new questions being posed than had been visualised at the start of this project. To obtain a better idea of why and how reductive acetogenesis outcompetes methanogenesis in kangaroo forestomach contents and to fully define the hydrogen economy and microbial core of hydrogen utilisation in these animals would require further research (see Recommendations).

5.2 Specific objectives

5.2.1 Initial objectives

Objective 1. Evaluating and comparing the ability of reductive acetogens from the kangaroo foregut to colonise and out-compete methanogens in an in vitro *fermentation apparatus and determine the extent of reduction in methane generation.*

Objective 1 was fully achieved. Without inhibition of methanogenesis, the reductive acetogens did not persist in the fermentations and declined to undetectable levels. However, while the reductive acetogens were present methane emissions did appear to be reduced. When methanogenesis was inhibited by the addition of BES, *C. glycolicum* YE255 was again rapidly lost but YE266/YE273 declined at a slower rate and YE257 was able to maintain population density, suggesting that it has promise for future use as an alternative hydrogen utiliser, as long as methanogenesis can be inhibited. The work with BES, while clearly relating to Objective 1, was only added to the project when it was revised.

Objective 2. Evaluating and comparing the ability of selected reductive acetogens to colonise the rumen of cattle, reduce methane generation and improve growth rate.

Following revision of the project it was deemed unlikely, given our lack of knowledge of the hydrogen economy in kangaroo foregut contents that Objective 2 could be met and objectives were modified to increase our knowledge of the processes and microbes involved in hydrogen utilisation in kangaroos.

5.2.2 Revised objectives

New objectives were:

Objective 3. To evaluate the ability of reductive acetogens to out-compete methanogens in the kangaroo foregut through direct measurement on culled wild kangaroos and in vitro fermentations.

Objective 3 has been heavily investigated and a large amount of data collected. In many kangaroos archaea are not present and when they are, they are at lower densities than in ruminants and mostly do not possess methane production genes. The culturable species of reductive acetogens were present in all kangaroos at appreciable densities. Further evidence that these populations produce little, if any, methane (except possibly some Wallaroos) has been generated by observing the comparative emissions when gut contents were incubated *in vitro*. Thus it would appear that in most kangaroos and under most circumstances, reductive acetogenesis has the ability to outcompete methanogenesis and is the dominating mechanism for hydrogen removal.

Objective 4. To investigate factors required to enable kangaroo reductive acetogens to persist in a rumen-like environment.

While it is evident that reductive acetogenesis does outcompete methanogenesis in kangaroos the mechanisms by which it is able to do this remain elusive. The only factor that appears to promote the survival of culturable reductive acetogens in a rumen-like environment *in vitro* is inhibition of methanogenesis. This may be a factor that can be used to help the reductive acetogens to colonise the rumen but it is unlikely to be a factor that would enable them to dominate in their natural environment and it is the latter that is likely to be the important factor(s) if long-term colonisation is to be successful. This objective will require considerably more work to be fully realised.

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. Considerable further strategic research is required to elucidate the core of hydrogen utilising microbes in the kangaroo forestomach and determine the processes required that enable these microbes to outcompete methanogens. These conditions and kangaroo reductive acetogens would need to be established in the rumen of cattle before tangible benefits could flow to the industry. However, if these conditions could be met, not only would the carbon footprint of the red meat industries be markedly reduced but major gains in feed utilisation efficiency, live weight gain and profitability could be realised.

7 Conclusions and recommendations

7.1 Conclusions

1. When methanogenesis is inhibited some kangaroo reductive acetogens can maintain populations in a rumen-like environment. Three species of reductive acetogens (YE266, YE257 and *Clostridium glycolicum* YE255) that had been isolated from kangaroo forestomach contents were evaluated for their ability to colonise in a rumen-like environment with and without the inhibition of methanogenesis by BES. Without inhibition all were rapidly lost from fermentations. With the addition of BES, *C. glycolicum* YE255 declined at a similar rate as it had previously, YE266/YE273 declined at a slower rate but YE257 was able to maintain its' population density throughout the fermentation.
2. BES has a marked archaeacidal impact on methanogen populations but not all species appear affected. BES caused the total cessation of methanogenesis within half an hour of first introduction and total methanogen numbers were reduced by > 90% within 5 days. When BES is added community stability changes markedly, with species with a higher ratio of AT to GC in their DNA becoming relatively more abundant, although total populations are much lower. Interestingly, while these AT rich species appear to survive better than the normally dominant species, they do not appear to need to produce methane to survive.
3. Fermentations initiated with kangaroo foregut contents resulted in very different outcomes that were not predictable. Two fermentations in different fermentors were run, one remained acetogenic but also contained dense populations of apparently non-methanogenic archaea, while in the other, reductive acetogens rapidly disappeared and sometime after they had

become undetectable the fermentation turned methanogenic. It appeared that a single dominant species of methanogen was involved. Why these fermentations were so different was not apparent but could be important to follow up in the future as some of the keys to maintaining an acetogenic versus methanogenic ecosystem could lie in the differences that occurred. What was apparent was that populations of reductive acetogens, archaea and methanogens might be variable between kangaroo species, diets and geographical locations.

4. A study of culturable reductive acetogens, archaea and methanogens in 42 Red and Eastern Grey kangaroos and wallaroos from throughout Queensland was undertaken. In many kangaroos and particularly Red kangaroos, archaea are not present. When present they were at lower densities than in ruminants and mostly did not possess methane production genes. They are also, to a large extent, genetically distinct from known methanogens (16S rRNA genes of different size). The discrepancies found between qualitative and quantitative PCR assays of both total archaea and cells containing McrA genes suggests that the archaeal communities are very diverse and will be novel. Further evidence that these populations produce little if any methane (except possibly some Wallaroos) has been generated by observing the comparative emissions when gut contents were incubated *in vitro*. Culturable reductive acetogens, except *C. glycolicum,* were present at reasonable densities in all kangaroos.
5. Clone libraries of PCR products have shown that the reductive acetogens that are in culture represent only a very small proportion of the diversity of reductive acetogens in kangaroos and the most important species, or consortia of species, in terms of the domination of reductive acetogenesis over methanogenesis, are yet to be cultured. Clones from archaeal 16S rRNA and McrA genes show that of the DNA sequences that are likely to come from methanogens, many are more closely aligned to *Methanosphaera stadmanii* (of human gut origin) than the *Methanobrevibacter sp.* commonly found in cattle. Kangaroos also had archaea that aligned to non-methanogenic archaea (*Thermoplasma sp.*).
6. A number of kangaroos had McrA sequences that clustered with those reported from Tammar wallabies but more importantly were closely related to a group of methane oxidising archaea (anaerobic methanotrophs). Their role in the ecosystem remains to be determined but it is possible that they also contribute to lowering methane emissions.
7. Some 16S rRNA sequences were so unusual as to defy alignment with any previously described sequences.

7.2 Recommendations

This project has generated a lot of data that firstly reaffirms that most kangaroos are unlikely to generate much methane and secondly that the microbiology within the foregut of kangaroos is novel and to fully appreciate the hydrogen economy in these animals and how reductive acetogenesis, and other hydrogen utilising pathways, dominate over methanogenesis will take considerably more work. We recommend that such work be undertaken as this is a microbial ecosystem that functions in a similar fashion to the rumen but produces very little methane. If the pivotal mechanisms and the microbes involved could be determined and understood, it is possible that these principals and microbes, when applied to the rumen of cattle and sheep could markedly reduce methane emissions and increase productivity and profitability. Research in the following directions are recommended to further our knowledge of the hydrogen dynamics within the kangaroo forestomach.

- Continue to further define the functional core of H_2 utilization and determine metabolic activity (RNA studies) as well as defining the species present. FTHFS mRNA would be amplified to give an indication of the most metabolically active acetogens. The identity of these species can then be determined by probing back to large genomic DNA insert fosmid or BAC libraries to identify

clones that contain both the FTHFS and 16S rRNA genes. The 16S rRNA genes can then be sequenced to identify these active acetogens. Identification will assist in targeting for isolation the most active reductive acetogens. Samples of solid digesta could be stored for examination by Fluorescent *in situ* Hybridization (FISH) to determine any spatial interactions (biofilm formations) between the different groups of microbes that may be required to enable acetogenesis to dominate.

- Ascertain whether there are true AMNE (anaerobic methanotrophs) present in kangaroos, as indicated by McrA gene sequence phylogeny and define their role in methane oxidation in the gut.
- Methods that might aid the establishment of reductive acetogens in a rumen-like environment and enhance their ability to persist require further investigation. At present we know that inhibiting methanogenesis does allow some of the cultivable reductive acetogens to persist. This requires further investigation and also a concerted effort to obtain more reductive acetogens (and other hydrogen utilisers) in culture.
- Further studies on the importance of interspecies hydrogen transfer and the inhibition of methanogenesis. For example:-
    - Co-culture reductive acetogens with hydrogen producing syntrophic bacteria, e.g. *R. albus* and other Ruminococci. It may be possible that the kangaroo acetogens are physically closely associated with plant materials and the bacteria that are involved in cellulose breakdown and H_2 production.
    - Investigate inhibitory reactions between acetogens and methanogens and also the Shigellas and other bacteria or archaea.
    - Determine whether inhibitory activity is a result of bacteriocins or other bioactive materials.

These latter studies would have synergies with other current research activities being undertaken in Australia.

8 Bibliography

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**, 143-169.

Barns, S.M., Fundyga, R.E. and Jeffries, M.W. (1994) Remarkable archaeal diversity detected in a Yellowstone-nationalpark hot spring environment. *Proceedings of the National Academy of Science of the United States of America* **91**, 1609-1613.

Breznak, J.A. and Switzer, J.M. (1986) Acetate synthesis from H_2 plus CO_2 by termite gut microbes. *Applied and Environmental Microbiology* **52**, 623-630.

Darland, G., Brock, T.D.,Samsonoff, W., and Conti. S,F, (1970). "A thermophilic, acidophilic mycoplasma isolated from a coal refuse pile". *Science* **170**, 1416–1418.

Denman, S.E., Tomkins, N.W. and McSweeney, C.S. (2007) Quantitation and diversity of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiology Ecology* **62**, 313-322

Evans, P.N., Hinds, L.A., Sly, L.I., McSweeney, C.S., Morrison, M., and Wright, A.G. (2009). Community composition and density of methanogens in the foregut of the Tammar Wallaby (Macropus eugenii) *Applied and Environmental Microbiology* **75**, 2598-2602.

Fricke, W.F., Seedorf, H., Henne, A., Kruer, M., Liesegang, H., Heddrich, R., Gottschalk, G. and Thauer, R.K. (2006). The genome of *Methanosphaera stadmanae* reveals why this human intestinal archaeon is restricted to methanol and H_2 for methane formation and ATP synthesis. *Journal of Bacteriology* **188**, 642-658.

Hallam, S.J., Girguis, P.R., Preston, C.M., Richardson, P.M. and DeLong, E.F. (2003). Identification of methyl coenzyme M reductase A (*mcrA*) genes associated with methane-oxidizing archaea. *Applied and Environmental Microbiology* **69**, 5483-5491.

Hallam, S.J., Putnam, N., Preston, C.M., Detter, J.C., Rokhsar, D., Richardson, P. and DeLong, E.F. (2004). Reverse methanogenesis: testing the hypothesis with environmental genomics. *Science* **305**, 1457-1462.

Hume, I.D. (1982) *Digestive physiology and nutrition of marsupials*. Cambridge: Cambridge University Press.

Joblin, K.N. (1999) Ruminal acetogens and their potential to lower ruminant methane emissions. *Australian Journal of Agricultural Research* **50**, 1307-1313.

Kempton, T.J., Murray, R.M. and Leng, R.A. (1976). Methane production and digestibility measurements in grey kangaroo and sheep. Australian Journal of Biological Science. **29**, 209-214.

Klieve, A.V., Ouwerkerk, D., Turner, A. and Roberton, R. (2002) The production and storage of a fermentor-grown bacterial culture containing *Synergistes jonesii*, for protecting cattle against mimosine and 3-hydroxy-4(1H)-pyridone toxicity from feeding on Leucaena leucocephala. *Australian Journal of Agricultural Research* **53**, 1-5.

Luton, P.E. Wayne, J.M, Sharpe, R.J and Riley P.W. (2002) The *mcrA* gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. *Microbiology* **148**, 3521-3530.

Mackie, R.I. and Bryant, M.P. (1994) *Acetogenesis and the rumen: syntrophic relationships*. In Acetogenesis. Ed: Drake, H.L. Chapman and Hall, New York. pp. 331-364.

Miller, T.L. and Wolin, M.J. (1985) *Methanosphaera stadmaniae* ge. sp. .nov.: a species that forms methane by reducing methanol with hydrogen. *Archives of Microbiology* **141**, 116-122.

Miller, T.L. and Lin, C, (2002). Description of Methanobrevibacter gottschalkii sp. nov., Methanobrevibacter thaueri sp. nov., Methanobrevibacter woesei sp. nov. and Methanobrevibacter wolinii sp. nov *International Journal of Systematic and Evolutionary Microbiology* **52,** 819-822.

NGGIC, 2007. National Greenhouse Gas Inventory 2005 with Methodology Supplement: Environment Australia: Canberra.

Nollet, L., Demeyer, D. and Verstraete, W. (1997) Effect of 2-bromoethanesulfonic acid and *Peptostreptococcus productus* ATCC 35244 addition on stimulation of reductive acetogenesis in the ruminal ecosystem by selective inhibition of methanogenesis. *Applied and Environmental Microbiology*. **63**, 194-200.

Ouwerkerk, D. and Klieve, A.V. (2001) Bacterial diversity within feedlot manure. *Anaerobe* **7**, 59-66.

Ouwerkerk, D., Klieve, A.V. and Forster, R.J. (2002) Enumeration of *Megasphaera elsdenii* in rumen contents by real-time Taq nuclease assay. *Journal of Applied Microbiology* **92**, 753-758.

Ouwerkerk, D., Maguire, A.J., McMillen, L. and Klieve, A.V. (2007) Why kangaroos do not produce methane. *Recent Advances in Animal Nutrition in Australia*. **16**, 101-104.

Ouwerkerk, D., Turner, A.F. and Klieve, A.V. (2008). Diversity of methanogens in ruminants in Queensland. *Australian Journal of Experimental Agriculture.* **48**, 722-725.

Ovreås, L., Forney, L., Daae, F.L. and Torsvik, V. (1997) Distribution of bacterioplankton in meromictic Lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for the 16S rRNA. *Applied and Environmental Microbiology* **63**, 3367-3373.

Posada, D. and Crandall, K.A. (1998), Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817-818.

Raskin, L., Stromley, J.M., Rittmann, B.E. and Stahl, D.A. (1994) Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Applied and Environmental Microbiology* **60**, 1232-1240.

Swofford, D.I. (2002) Phylogenetic Analysis with Parsimony (and Other Methods), Version 4.0b10. Sinauer Associates, Sunderland, M.A.

Takai, K. and Horikoshi K. (2000) Rapid detection and quantification of members of the Archaeal community by quantitative PCR using fluorogenic probes. *Applied and Environmental Microbiology* **66**, 5066-5072.

Weitang, F. (2003) Outlook for China's coal mine methane reduction mitigation, p. 1-5. *In* Proceedings of the 3rd International Methane and Nitrous Oxide Mitigation Conference, Beijing, China, Nov. 16 – 21, 2003.

Whitford, M.F., Forster, R.J., Beard, C.E., Gong, J. and Teather, R.M. (1998) Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. *Anaerobe* **4**, 153-163.

Yu Z and Forster RJ (2005) Nucleic Acid Extraction, Oligonucleotide Probes, and PCR Methods. In 'Methods in Gut Microbial Ecology for Ruminants'. (Eds HPS Makkar, CS McSweeney) pp. 81-104. (Springer Academic Press: Dortrecht).