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Prepared by: Valeria A Torok

South Australian Research & Development Institute

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Locked Bag 991
NORTH SYDNEY NSW 2059

Rumen microbial profiling – a tool to investigate methane mitigation strategies

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1 Abstract

Reducing ruminant methane emissions is an important part of reduction in greenhouse gas emissions. Development of methane mitigation strategies in ruminants need to consider the influence of diet, animal genetics and rumen microbiology, and the degree to which these can be manipulated while maintaining animal performance. Molecular microbial profiling tools have been developed to investigate the overall rumen microbiota. These screening tools have been used in collaborative animal studies investigating dietary and genetic methane mitigation strategies in dairy cows, beef cattle and sheep. Diet was shown to significantly alter rumen microbiota. In some cases dietary manipulation also resulted in successful methane mitigation, which correlated well with changes in rumen microbiota. In the absence of methane mitigation, diet related changes in rumen microbiota could be linked to animal performance traits such as milk fat composition and feed efficiency. Animal genetics was also found to alter rumen microbiota, however successful methane mitigation was dependant on diet. This project has developed rapid high-throughput screening technologies which enable researchers to measure the effects of diverse methane mitigation strategies on rumen microbiota. These technologies can be further developed to provide information to producers on the effectiveness of on-farm methane mitigation strategies.

2 Executive Summary

Reducing ruminant methane emissions is an important objective for ensuring the sustainability of ruminant based agriculture and reducing overall greenhouse gas emissions. However, any methane mitigation strategies need to consider the influence of diet, animal genetics and rumen microbiology and function, and the degree to which these can be manipulated while maintaining animal performance.

The objectives of this project were to: develop and provide high-throughput DNA profiling assays for rumen and faecal microbiota; use the developed profiling assays to characterise the influence of feeding and selected methane abatement strategies on rumen microbiota activity; and to evaluate surrogate assays for rumen function associated with enteric methane emissions suitable for application to large numbers of animals.

The rumen microbiota is composed of a diverse symbiotic population of anaerobic bacteria, archaea (including methanogens), ciliated protozoa and fungi Although, methanogenic archaea are the only known organisms capable of methane production they rely on bacteria, protozoa and fungi to provide digestive products for methanogenesis. Hence, these other organisms also have an indirect influence on methane production. In this project we have developed a high-throughput culture-independent microbial profiling technique for investigating rumen and faecal bacterial, archaeal, fungal and protozoan communities. The microbial profiling techniques developed provide a “snapshot” view of the entire microbial ecosystem present within the gut of the animal. As such, it can be used to investigate the influence of dietary and genetic methane mitigation strategies on rumen and faecal samples. The techniques developed are suitable for large scale studies as the entire process is high-throughput from nucleic acid extraction, molecular profiling to data analysis. Coupled with multivariate statistical analysis it has been shown to be a robust screening tool.

The developed microbial profiling assays were used to investigate rumen and faecal microbiota from eight collaborative trials investigating dietary and genetic methane mitigation strategies in dairy cows, beef cattle and sheep. We were able to show that rumen microbiota was influenced by diet and animal genetics and that these changes could be correlated with methane production data. Diet, duration on allocated feed and environment all were strong drivers of rumen microbiota composition. Where animal genetics was found to influence rumen microbiota and methane production, these changes were also diet dependant. Diet related changes in rumen microbiota did not always translate into significant reductions in methane production. However, these changes in microbial communities may be related to other significant differences observed, such as milk fat production or feed efficiency (animal performance traits). Changes in rumen bacterial communities may have led to changes in the biohydrogenation of unsaturated fatty acids resulting in the changes to the milk fatty acid profiles. Furthermore, gut bacterial communities have been linked to differences in feed efficiency in poultry, as well as metabolic phenotype in humans and animal models.

It was observed that rumen microbial profiles from animals on identical treatments do vary, suggesting genetics has a role to play in rumen ecology. These variations may partly be explained by the variability in methane outputs from these animals. Furthermore, there appears to be a strong interaction between animal genetics and environment on rumen microbiota composition and enteric methane

emissions. This requires further investigation if successful strategies are to be developed for methane mitigation without impacting negatively on animal production.

Although significant changes in archaeal and methanogeic archaeal communities could be linked with significant reductions in enteric methane production, other rumen microbial communities were also shown to be significantly altered and correlated to methane production. For example, significant changes were also detected in bacterial (grape marc supplementation in dairy cows and beef cattle feed efficiency trials) and fungal communities (beef cattle feed efficiency trial) between treatments resulting in altered methane production. Conversely, significant differences in archaeal or methanogenic archaeal communities were not always accompanied by significant reduction in methane production (DHA supplementation trial in dairy cows). Alternatively a lack of any significant differences in archaeal or methanogenic archaeal communities were shown in sheep selected as being divergent in methane production. In these sheep significant differences were instead detected in the rumen bacterial and protozoan communities and these correlated well with methane production. These results emphasise the complex nature of the rumen microbiota, and the need to investigate these communities and their inter relations as a whole if we are to identify reproducible methane mitigation strategies. No studies to date have investigated all communities (bacterial, archaeal, fungal and protozoan) in individual animals and related these changes to enteric methane production. As such, our approach is a “big picture” approach opening the way for more detailed investigations to be used were significant differences are identified.

From our investigation it was shown that the rumen and faecal microbiota were significantly different in abundance of common organisms, as well as each harbouring unique gut specific organisms. Faecal microbial profiling is not seen as a suitable surrogate assay for rumen function. Although diet did influence faecal microbiota, these differences were different to changes observed within the rumen. Furthermore, where significant differences in rumen microbiota linked to methane production were detected, these differences were absent in the faecal microbiota. Although it was not expected that these different gut sections would harbour the same microbial community structure, it may be possible to developed specific assay targeting organisms within the faeces once such organisms (associated with methane production) have been fully characterised and identified within the rumen.

In the current RELRP there have been limited animal trials demonstrating successful methane mitigation strategies, therefore, further work is required to confirm findings of relationships between rumen microbiota and methane production. Identification of organisms associated with methane reduction will enable development of specific and quantitative diagnostic assays.

This project has developed technologies which enable researchers to measure the effects of diverse methane mitigation strategies on rumen microbiota. These technologies can be further developed to provide information to producers on the effectiveness of on-farm methane mitigation strategies.

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3 Background

Enteric methane (CH_4) emissions from ruminant livestock systems contribute 8 to 25% of global greenhouse gas emissions (Beukes et al., 2010; Howden and Reyenga, 1999; Kebreab et al., 2008; Lesschen et al., 2011). Methane produced during anaerobic fermentation in ruminants also represents a feed energy loss of 2-12% (Patra and Saxena, 2009; Reynolds et al., 2011). Reducing ruminant methane emissions is an important objective for ensuring the sustainability of ruminant based agriculture. However, any methane mitigation strategies need to consider the influence of diet, animal genetics and rumen microbiology and function, and the degree to which these can be manipulated while maintaining animal performance.

Methane is formed by methanogenic micro-organisms present within the animal's rumen. These perform the beneficial task of removing hydrogen from the rumen, enhancing the breakdown and fermentation of ingested food and supplying energy for host metabolic functions (Kamra, 2005). The rumen microbiota is composed of a diverse symbiotic population of anaerobic bacteria, archaea (including methanogens), ciliated protozoa and fungi (Kamra, 2005). Although methanogenic archaea are the only known organisms capable of methane production they rely on bacteria, protozoa and fungi to provide digestive products for methanogenesis. Hence, bacteria, protozoa and fungi also have an indirect influence on methane production as they are either involved in hydrogen (H_2) metabolism or because they affect the numbers of methanogens or other members of the microbiota (Bauchop, 1989; Hook et al., 2010; Kamra, 2005).

A large population of methanogens, and other microoganisms, cannot be cultured in the laboratory. The use of molecular techniques, such as microbial profiling, can help characterise shifts in rumen microbial communities associated with feed types, genetic interventions, management and environment. Furthermore, microbial profiling can be used to characterise the rumen microbiota from high and low methane producing animals. Microbial profiling methodologies to be developed in this project will target the rumen and faecal archaeal, methanogenic archaeal, fungal and protozoan communities, as well as, use previously developed bacterial profiling methodology.

The microbial profiling technology which will be developed in this project is based on terminal restriction fragment length polymorphism (T-RFLP). T-RFLP is a culture independent technique for profiling microbial communities based on differences at the nucleic acid or genome level. The advantage of the technique is that it is high-throughput, high resolution and capable of providing a "snap shot" of the entire microbial community at any particular time. Hence, it is an ideal initial screening tool which requires no prior knowledge of the actual microorganism present within the community. T-RFLP has been widely used to investigate gut bacterial communities within poultry. This tool has being used to investigate changes in gut bacterial communities associated with dietary modification, such as addition of feed enzymes, prebiotics and organic acids, as a means of developing alternatives to in-feed antibiotics for the poultry industry (Geier et al., 2009; Geier et al., 2010; Torok et al., 2008). Furthermore, the profiling technique has been used to link changes in gut microbiota with improved bird performance (apparent metabolisible energy and feed conversion efficiency), and to characterise bacterial species associated with performance (Torok et al., 2008; Torok et al., 2011).

This project seeks to develop technologies which will initially enable researchers to measure the effects of diverse strategies to reduce methane emissions from ruminants. In the longer term, the technologies can be further developed to provide information to producers to monitor effectiveness of on-farm methane mitigation strategies.

The project will collaborate with other research projects within the Reducing Methane Emissions from Livestock (RERLP) program evaluating genetic and feeding methane mitigation strategies.

4 Project objectives

The purpose of this project is to develop and provide molecular techniques for use in collaborative research projects to evaluate feeding, breeding and management strategies to reduce methane production in ruminant systems.

This project has the following objectives:

- Develop and provide high-throughput DNA profiling assays for rumen and faecal microbiota associated with methane emissions.

- Use the profiling assays to characterise the influence of feeding and selected methane abatement strategies on rumen microbiota activity.

- Evaluate surrogate assays for rumen function associated with enteric methane emissions suitable for application to large numbers of animals.

5 Molecular profiling methodology development

5.1 Background

Terminal restriction fragment length polymorphism (T-RFLP) is a culture independent technique for profiling microbial communities based on differences at the nucleic acid or genome level (Marsh, 1999). The technique involves obtaining total nucleic acid from a sample which is representative of its microbial community structure. Members of a particular microbial community are amplified *in vitro* using the polymerase chain reaction (PCR) and two short oligonucleotides (termed "primers"), complementary to a segment of the target organisms genetic material, resulting in the generation of desired "amplicons" (DNA formed as a product of amplification). The primers used need to be "universal" in nature (capable of detecting an entire group of microorganisms), while demonstrating specificity to the intended target group of microorganisms at the exclusion of others. The genome region targeted for PCR needs to be somewhat conserved to allow detection of all organisms within a group, while containing internal regions of variability to enable discrimination among members within the microbial population. This variability is identified by restriction enzymes which recognise (usually 4-6 bp) and cut at specific nucleotide sequences within the PCR generated amplicon. As members within a microbial population vary in their genome sequence a unique pattern of cuts will occur for most microbial species. The resulting fragments are separated according to size by capillary electrophoresis on a DNA sequencing machine. Only the terminal fragments which have incorporated a fluorescent dye attached to one of the PCR primers are identified. Results are converted to graphical profiles where peaks can represent taxonomically related groups and/or strains of microorganisms. Using multivariate statistical methods these can be easily compared between samples to identify changes in microbial community composition. The advantage of the technique is that it is high-throughput and can be used to identify changes in microbial community structure in relation to treatment effects, such as, dietary manipulations and host genetic factors. Hence it is an ideal initial screening tool which requires no prior knowledge of the actual microorganism present within the community.

The genome regions we have targeted for T-RFLP development of bacteria, archaea, fungi and protozoa was the ribosomal ribonucleic acid rRNA genes, while for the methanogenic archaea the functional methyl coenzyme M reductase A (*mcrA*) gene was targeted. The rRNA is the RNA component of the ribosome, the enzyme that is the site of protein synthesis in all living cells, while the *mcrA* gene catalyses the terminal step in biogenic methane production. Both prokaryotic and eukaryotic ribosomes can be broken down into two subunits. In prokaryotes the small ribosomal subunit contains the 16S rRNA while the large ribosomal subunit contains two rRNA species (5S and 23S). In most eukaryotes the small ribosomal subunit contains the 18S rRNA, and the large subunit contains three rRNA species (5S, 5.8S and 28S).

5.2 Methodology

5.2.1 Nucleic acid extraction from rumen and faecal samples

Total nucleic acid was extracted from rumen and faecal samples using a modification (Torok et al., 2008) of a SARDI proprietary method (Stirling et al., 2004). This method has previously been used to obtain total nucleic acid extracts from chicken gut digesta and rodent faeces for use in bacterial community analysis. The method involves freeze drying of samples prior to nucleic acid extraction. The SARDI

rumen extraction method was compared with two other methods: QIAmp® DNA Stool Mini Kit (Qiagen); and the repeated bead beating plus column (RBB+C) method (Yu and Morrison, 2004). The latter method has previously been used by other research groups within the RERLP program for denaturing gradient gel electrophoresis (DGGE) analysis of bacterial and archaeal communities.

5.2.2 Development of T-RFLP

5.2.2.1 PCR primer selection

Potential primers for use in the development of T-RFLP to target organisms of interest (archaea, methanogenic archaea, fungi and protozoa) were identified from an extensive search of peer reviewed scientific literature. Primer sequences from studies using various microbial profiling techniques such as T-RFLP, restriction fragment length polymorphism (RFLP), DGGE and automated ribosomal intergenic spacer analysis (ARISA) to investigate rumen and or faecal microbial communities were identified (Appendix 9.1). Furthermore, universal primers targeting organisms of interest were identified from other PCR based investigations (Table 5.1). Primers used for bacterial T-RFLP were those of Torok et al., (2008).

PCR primers identified in Table 5.1 were evaluated *in-silico* using a range of publically available web based tools as listed below:

- Genbank for sourcing genome sequences of interest http://www.ncbi.nlm.nih.gov/Genbank/index.html
- ClustalW 2.0.11 for sequence alignments http://www.clustal.org
- MiCA III PSPA (Primer Sequence Prevalence Analysis) *in silico* PCR amplification of 16S & 18S rRNA gene sequences found in public database. http://mica.ibest.uidaho.edu/primer.php
- Primer BLAST for primer pair specificity checking against all sequences in public genome databases http://www.ncbi.nlm.nih.gov/tools/primer-blast/

5.2.2.2 Specificity of PCR primers to target organism

Specificity of selected primers for T-RFLP development to target groups of organisms were confirmed by cloning and sequencing of resulting amplicons.

5.2.2.3 Restriction enzyme selection

Restriction enzymes which had the maximal resolving power (ability to discriminate between species within a population) were identified *in-silico* using the following resources:

- Cleaver for identifying taxon specific restriction endonuclease recognition sites http://cleaver.sourceforge.net/
- MiCA III ERPA (Enzyme Resolution Power Analysis) analytical tool for the selection and analysis of restriction enzymes http://mica.ibest.uidaho.edu/enzyme.php
- MiCA III Virtual Digest - *in silico* amplification and restriction of 16S and 18S rRNA and *mcrA* sequences http://mica.ibest.uidaho.edu/digest.php

Identified restriction enzymes were then tested *in vitro* to determine those producing the most complex T-RFLP profiling patterns.

Table 5-1 PCR primers identified from previous studies which may be adapted for use in T-RFLP to investigate archaeal, fungal and protozoan communities.

Primer pair	Reference	Methodology	Intended organism
Ar109f/Ar912r	(Lueders and Friedrich, 2003) [1]	T-RFLP	Archaea
Archf364/Archr1386	(Skillman et al., 2004) [2]	Clone library	Archaea
Ar109f/Archr1386	1 and 2	this study	Archaea
Archf364/Ar912r	1 and 2	this study	Archaea
rcAr915f/Arch1386	(Nicholson et al., 2007) and 2	TGGE	Archaea
mcrAf/mcrAr	(Luton et al., 2002)	Clone Libraries	Methanogenic Archaea
GM1/GM2	(Brookman et al., 2000) [3]	Slot blot hybridization	Fungi
ITS1F/ITS4	(Gardes and Bruns, 1993) [4] (Alvarado and Manjon, 2009) [5]	PCR/RFLP in-silico T-RFLP	Fungi
GM1/ ITS4	3 and 5	this study	Fungi
SSU-817F/SSU-1536R	(Borneman and Hartin, 2000)	Clone library	Fungi
NS1/NS2	(Fliegerova et al., 2006)	RFLP	Fungi
NL1/NL4	(Fliegerova et al., 2006)	RFLP	Fungi
CNL12/5SA	(Henrion et al., 1992)	PCR/RFLP	Fungi
HausF/5SA	(Henrion et al., 1992) (Hausner et al., 2000)	PCR/RFLP RFLP	Fungi
HausE/5SArc	(Henrion et al., 1992) (Hausner et al., 2000)	PCR/RFLP RFLP	Fungi
HausE/HausF	(Hausner et al., 2000)	RFLP	Fungi
Neo18SFor/Neo5.8SRev	(Edwards et al., 2008)	ARISA	Fungi
NeocalF/NeocalR	(Lockhart et al., 2006)	PCR	Fungi
CS322F/EU929R	(Puitika et al., 2007) [6]	PCR	Protozoa
PSSU342fmod/ EU929R	(Karnati et al., 2003) and 6	Clone library	Protozoa
316f /539r	(Sylvester et al., 2004)	qPCR	Protozoa
Oph151F/Ento472R	(Skillman et al., 2006)	qPCR	*(Entodinium)*
IsoDas151F/Das472R	(Skillman et al., 2006)	qPCR	*(Dasytricha)*

5.2.3 Analysis of T-RFLP data

PCRs were done in duplicate in 50-µl volumes. Following PCR all amplification products were quantified by fluorometry and duplicate PCRs pooled (Torok et al., 2008). The specificities of the PCR products were analysed by gel electrophoresis on a 2% agarose gel and visualized after staining with GelRed™ (Biotium). Approximately 200ng of PCR product was digested with a desired restriction enzyme in duplicate according to the manufacturer's instructions. The length of fluorescently labelled terminal restriction fragments (T-RF) were determined by comparison with an internal standard (GeneScan 1200 LIZ; Applied Biosystems, Australia) after separation by capillary electrophoresis on a ABI 3700 automated DNA sequencer (Applied Biosystems, Australia) and data were analysed by using GeneMapper v3.7 software (Applied Biosystems, Australia). Data points generated by the GeneMapper software were further analysed by using a custom-built database containing queries to validate data points and generate outputs for statistical analysis

(Torok et al., 2008). T-RFs were defined as peaks with a size of x±2 bp within pseudo replicates of samples and rounded to the nearest even number between samples to produce operational taxonomic units (OTUs).

5.2.4 Multivariate statistical analysis of OTU

OTUs were analysed using multivariate statistical techniques (PRIMER 6 and PERMANOVA+β1, PRIMER-E Ltd., Plymouth, UK). These analyses were used to examine similarities in rumen and faecal microbial communities, identify OTUs accounting for differences observed in microbial communities, and examine correlations between the composition of the microbial community and methane data.

Bray-Curtis measures of similarity (Bray and Curtis, 1957) were calculated to examine similarities between rumen or faecal microbial communities of ruminants from the T-RFLP generated (OTU) data matrices, following standardization and fourth root transformation. The Bray-Curtis similarity co-efficient (Bray and Curtis, 1957) is a reliable measure for biological data on community structure and is not affected by joint absences that are commonly found in microbial data (Clarke, 1993). Analysis of similarity (ANOSIM) (Clarke, 1993) was used to test if rumen/faecal microbial communities were significantly different between treatments (dietary or genetic). The *R*-statistic value describes the extent of similarity between each pair in the ANOSIM analysis, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between the groups.

Similarity percentages (SIMPER) (Clarke, 1993) analyses were done to determine which OTUs contributed most to the dissimilarity between treatments. SIMPER identifies individual species (OTUs) contributing to the overall dissimilarity between treatments. The overall average dissimilarity ($\bar{\delta}$) between microbial communities of ruminants on two treatments shown to significantly differ were calculated and the average contribution of the *i*th OTU ($\bar{\delta}_i$) to the overall dissimilarity determined. Average abundance ($\bar{y}$) of important OTUs in each of the groups were determined. OTUs contributing significantly to the dissimilarity between treatments were calculated ($\bar{\delta}_i/SD(\delta_i)>1$). Percent contribution of individual OTUs ($\bar{\delta}_i\%$) and cumulative percent contribution ($\Sigma\ \bar{\delta}_i\%$) to the top 50% of average dissimilarities were also calculated.

Unconstrained ordinations were done to graphically illustrate relationships between treatments using non-metric multidimensional scaling (nMDS) (Kruskal, 1964; Shepard, 1962). nMDS ordinations attempt to place all samples in an arbitrary two-dimensional space such that their relative distances apart match the corresponding pair-wise similarities. Hence, the closer two samples are in the ordination the more similar are their overall microbial communities. “Stress” values (Kruskal’s formula 1) reflect difficulty involved in compressing the sample relationship into the 2-D ordination.

Constrained canonical analysis of principal coordinates (CAP) biplots (Anderson and Willis, 2003) were constructed to investigate the relationship between OTUs associated with diet or animal genetics and enteric methane emissions. The *a priori* hypothesis that microbial communities were different between diets/genotype were tested in CAP by obtaining a P value using permutation procedures (999 permutations) on the canonical test statistic (squared canonical correlation, δ_1^2). The number of PCO axes (m) was chosen to achieve the maximum proportion of correct allocations (% of Trace (G)) of samples to diet or genetics. Pearson’s correlation (r)

was calculated between the first canonical axis (CAP1) and enteric methane production.

5.3 Results

5.3.1 Nucleic acid extraction

Microbial profiling was done on sub-samples of bovine rumen fluid and faeces which had total nucleic acid extracted by the SARDI, RBB+C and QIAmp® DNA stool methodologies.

5.3.1.1 Rumen fluid

Nucleic acid concentration was measured for each of the resulting extracts and adjusted to an equivalent amount before profiling. PCRs contained ca. 2ng/µl of template DNA. Bacterial profiles generated from the three different extraction templates were comparable (Figure 5.1). The resulting archaeal, methanogenic archaeal and protozoan profiles were also similar from the three differently generated nucleic acid templates, although both the SARDI and QIAmp methods gave more intense profiles (higher peaks) or complex profiles (additional peaks) (Figures 5.2-5.4). The fungal profiles were the most variable when using template from the three different nucleic acid extraction methodologies. Both the RBB+C and QIAmp generated extracts gave unique profiles, with the SARDI extract producing a common profile representative of both the other methods (Figure 5.5). Although the SARDI method did produce a common profile (peak positions) the intensity of generated profiles did differ to the two other extraction methods.

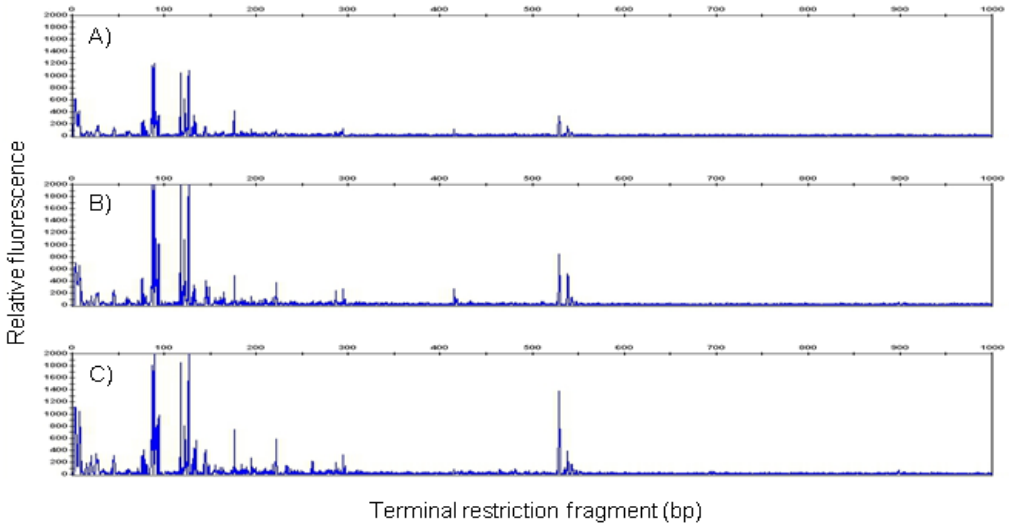


Figure 5-1 Bacterial T-RFLP profiles generated with 27F/907R and *Msp*I from bovine rumen fluid. Sub-samples of rumen fluid were taken from one cow and nucleic acid extracted by one of the following methods A) RBB+C method, B) QIAmp® DNA stool kit and C) SARDI method.

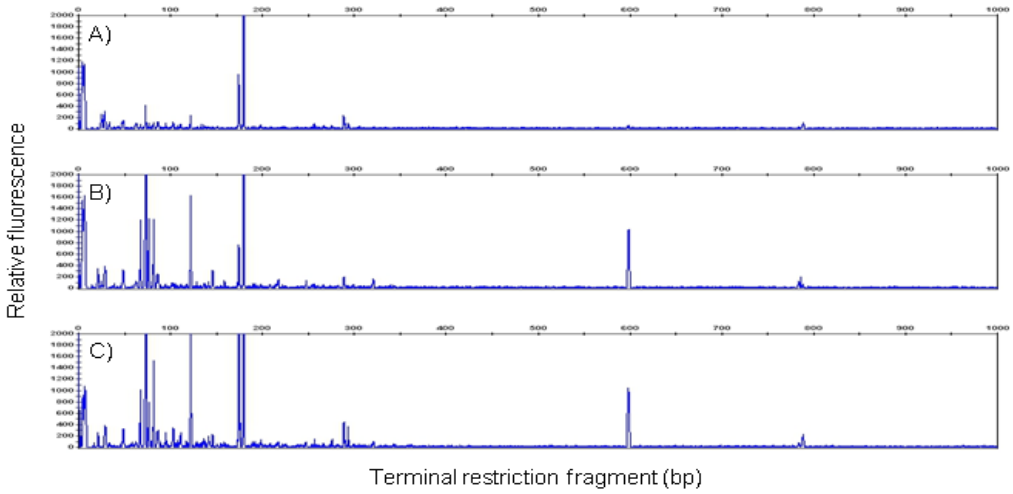


Figure 5-2 Archaeal T-RFLP profiles generated with Ar109fFam/Ar912r and *Mbo*I from bovine rumen fluid. Sub-samples of rumen fluid were taken from one cow and nucleic acid extracted by one of the following methods A) RBB+C method, B) QIAmp® DNA stool kit and C) SARDI method.

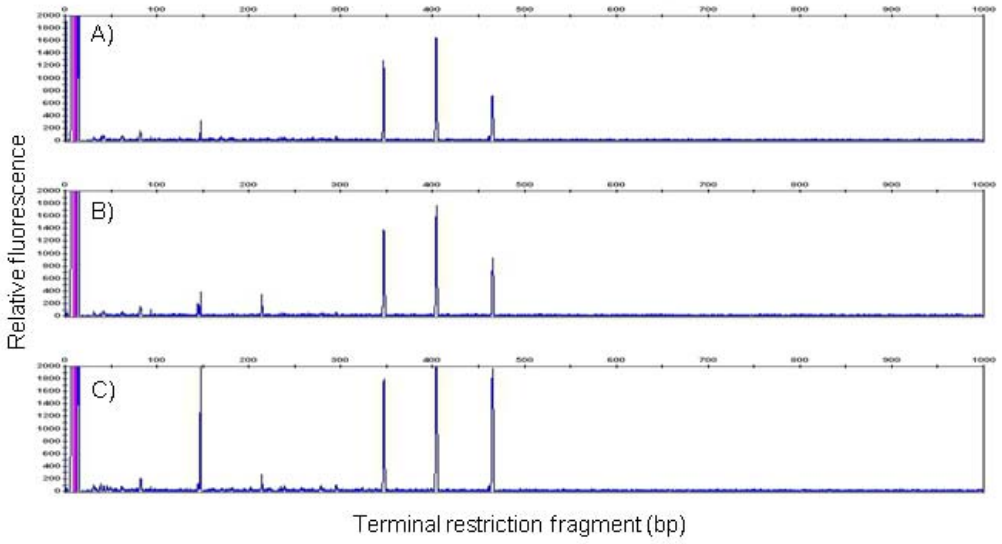


Figure 5-3 Methanogenic archaea T-RFLP profiles generated with mcrAfFam/mcrAr and *Hpy*188I from bovine rumen fluid. Sub-samples of rumen fluid were taken from one cow and nucleic acid extracted by one of the following methods A) RBB+C method, B) QIAmp® DNA stool kit and C) SARDI method.

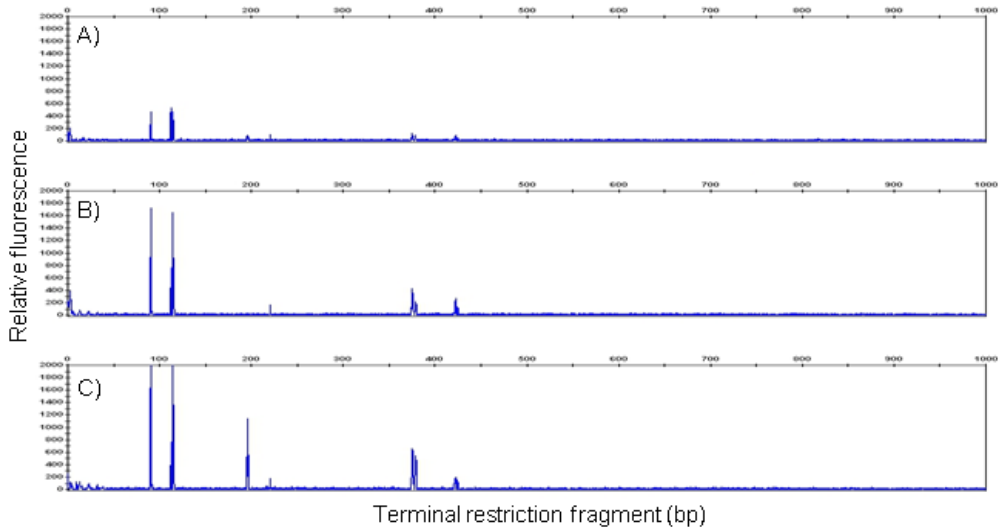


Figure 5-4 Protozoan T-RFLP profiles generated with CS322F/EU929RFam and *Hpy*188III from bovine rumen fluid. Sub-samples of rumen fluid were taken from one cow and nucleic acid extracted by one of the following methods A) RBB+C method, B) QIAmp® DNA stool kit and C) SARDI method.

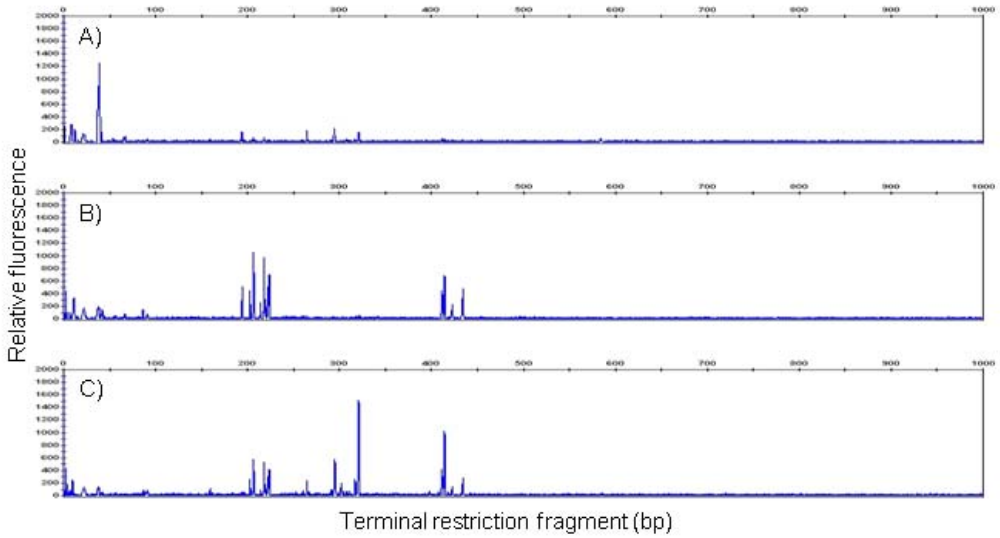


Figure 5-5 Fungal T-RFLP profiles generated with ITS1FFam/ITS4 and *Hin*fI from bovine rumen fluid. Sub-samples of rumen fluid were taken from one cow and nucleic acid extracted by one of the following methods A) RBB+C method, B) QIAmp® DNA stool kit and C) SARDI method.

5.3.1.2 Faecal samples

Nucleic acid was extracted from a sub-sample of freeze dried bovine faecal matter from four individual cows by one of the following extraction methods: RBB+C, QIAmp® DNA stool and SARDI. In our hands the RBB+C and QIAmp® DNA stool kit generated extracts which contained inhibitors to PCR. Presence of inhibitors in the nucleic acid extracts were confirmed by spiking reactions with a positive plasmid control (Figure 5.6). The concentration of DNA which was used in the PCR was 6.3-9.4 ng/µl for the SARDI extracts, 5.8-16 ng/µl for the RBB+C extracts and 1.8-2.7 ng/µl for the QIAmp® DNA stool extracts.

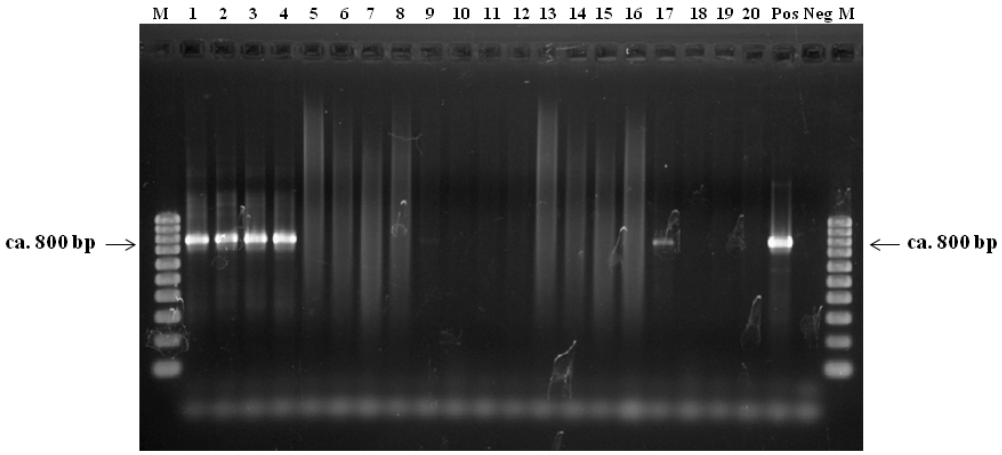


Figure 5-6 Archaeal PCR (Ar109f/Ar912r) on nucleic acid extracted from bovine faecal material using the SARDI, RBB+C and QIAmp® DNA stool kit methods. M) 100bp DNA ladder. 1-4) PCR on SARDI nucleic extracts, cow 1-4 respectively. 5-8) PCR on RBB+C nucleic extracts, cows 1-4 respectively. 9-12) PCR on QIAmp nucleic extracts, cow 1-4 respectively. 13-16) PCR on RBB+C nucleic extracts spiked with positive control template. 17-20) PCR on QIAmp nucleic extracts spiked with positive control template. Pos) Positive control PCR. Neg) No template control PCR.

5.3.2 Primer selection

In selecting primers for T-RFLP development several criteria needed to be met. These included:

- specificity to target group of organisms
- universality to all organisms within the target group
- generation of an amplicon large enough (500-1200bp) to allow discrimination based on variations between genome sequence
- generation of a single amplicon.

Primers identified from previous profiling studies (T-RFLP and DGGE) in ruminants were predominantly used to investigate bacterial community structure (Appendix 9.1). Furthermore, those primers used for DGGE analysis of archaeal, fungal and protozoan communities were not appropriate for T-RFLP analysis due to the short amplions generated (ca. 200 bp). Therefore, additional primers targeting organisms of interest including archaea, methanogenic archaea, fungi and protozoa were identified for development of T-RFLP (Table 5-1). Primer combinations were excluded from use in T-RFLP if they generated multiple non-specific amplicons, failed to produce an amplicon, generated target amplicons smaller that 500 bp or generated a single amplicon which was shown to be non-specific to the target organism following sequencing (Table 5-2).

Primers investigated for fungal profiling targeted the 18S rRNA, 28S rRNA and well as the internal transcribed spacer (ITS) and intergenic spacer (IGS) regions. In fungi the 18S and 28S rRNA regions were found to be highly conserved hence preventing good discrimination among members within the population. Therefore, the ITS regions were selected for T-RFLP analysis due to the higher sequence variability in this region. Primers selected for bacterial, archaeal and protozoan profiling targeted the small ribosomal subunits (16S or 18S rRNA). Primers selected for methanogenic archaeal profiling targeted the functional *mcrA* gene.

Table 5-2 Identification of PCR primers for use in T-RFLP which met the criteria of generating a specific amplicon greater than 500bp within a genome region showing inter-species variability.

Primer pair	Organism targeted[a]	Specificity (in-silico)[a]	Amplicon size (bp)	Genome target	T-RFLP potential
Ar109f/Ar912r	Ar	Ar	~800	16S rRNA	Yes
Archf364/Archr1386	Ar	Ar	~1000	16S rRNA	No. Non-specific amplicons
Ar109f/Archr1386	Ar	Ar	~1300	16S rRNA	No. Non-specific amplicons
Archf364/Ar912r	Ar	Ar & Bac	~550	16S rRNA	No. Amplicon too small
rcAr915f/Arch1386	Ar	Ar	~470	16S rRNA	No. Non-specific amplicons
mcrAf/mcrAr	Meth	Meth	~470	mcrA	Yes
GM1/GM2	Fu	Fu & Pl	410-470	18S rRNA, ITS1 & 5.8S rRNA	No. Non-specific amplicons
ITS1F/ITS4	Fu	Fu	590-730	18S rRNA, ITS1, 5.8S rRNA & ITS2	Yes
GM1/ ITS4	Fu	Fu	590-730	18S rRNA, ITS1, 5.8S rRNA & ITS2	No. Protozoa detected
SSU-817F/SSU-1536R	Fu	Fu & Pl	~700	18S rRNA	No. Region too conserved. Only plant fungi detected
NS1/NS2	Fu	Fu & Pl	~500	18S rRNA	No. Region too conserved. Protozoa detected
NL1/NL4	Fu	Fu & Pl	~750	28S rRNA	No. Region too conserved
CNL12/5SA	Fu	Fu & Pl	~1000	IGS1	No. Non-specific amplicons
HausF/5SA	Fu	Fu & Pl	~1000	IGS1	No. No amplicon
HausE/5SArc	Fu	Fu & Pl	~2000	IGS2	No. No amplicon
HausE/HausF	Fu	Fu & Pl	~3000	IGS & 5S rRNA	No. No amplicon
Neo18SFor/Neo5.8SRev	Fu	Fu	~350-400	18S rRNA, ITS1 & 5.8S rRNA	No. Non-specific amplicons
NeocalF/NeocalR	Fu	Fu	~800	18S rRNA	No. Region too conserved
CS322F/EU929R	Pr	Pr	~550	18S rRNA	Yes
PSSU342fmod/EU929R	Pr	Pr, Fu & Pl	~600	18S rRNA	Yes
316f /539r	Pr	Pr	~200	18S rRNA	No. Amplicon too small
Oph151F/Ento472R	*Entodinium*	*Entodinium*	~320	18S rRNA	No. Too specific and amplicon too small
IsoDas151F/Das472R	*Dasytricha*	*Dasytricha*	~320	18S rRNA	No. Too specific and amplicon too small

[a] Ar=archaea, Bac=bacteria, Fu=fungi, Meth=methanogenic archaea, Pr=protozoa, Pl=plant.

Primer pairs showing the greatest potential for T-RFLP development were used to generate amplicons from bovine rumen DNA (Table 5.2). These amplicons were cloned and sequenced to confirm their specificity to the intended target group (Appendix 9.2). Primers investigated were: CS322F/EU929R and P-SSU342mod/EU929R (protozoan); GM1/ITS4, ITS1F/ITS4, SSU-817F/SSU-1536R, NeocalF/NeocalR and NS1/NS2 (fungi); and Ar109f/Ar912r and mcrAf/mcrAr (archaea).

Both protozoan primer pairs (CS322F/EU929R and P-SSU342mod/EU929R) detected rumen protozoa (*Isotricha sp*, *Eudiplodinium sp* and *Entodinium sp*), however, CS322F/EU929R was selected for further T-RFLP development as it did

not generate primer dimer nor did it show the potential to detect fungal and plant sequences *in-silico*.

Fungal PCR primers GM1/ITS4 and NS1/NS2 were discounted from T-RFLP application as they both were shown to be non-specific, detecting protozoan sequences (Appendix 9.2). Fungal primer pair SSU-817f/SSU-1536r was also discounted as it was found to be biased to the detection of plant fungi which probably entered the rumen via the feed. Fungal primer pair ITS1F/ITS4 gave two bands of ca. 800 and 650 bp. The 800 bp band was confirmed to be rumen fungi, while the 650 bp band was confirmed to be plant fungi. *In-silico* restriction of the plant fungi generated OTU 260-360, while rumen fungi fell outside this range making it possible to separate the plant fungi from the rumen fungi. Of the 12 fungal PCR primers tested (Table 5.2) the ITS1F/ITS4 was the best candidate. Although, it did amplify non-rumen fungi it did target a region within the rRNA with sufficient sequence variation to allow discrimination between fungal groups within the rumen. NeocalF/NeocalR only detected rumen fungi but covered a highly conserved region making subsequent discrimination with restriction enzymes difficult. NeocalF/NeocalR also did not detect any additional rumen fungal species which had not already been identified by ITS1F/IST4 (Appendix 9.2).

Primers selected for final T-RFLP development were: bacterial (27F/907R); Archaeal (Ar109f/Ar912r and mcrAf/mcrAr); fungal (ITS1F/ITS4); and protozoal (CS322F/EU929R). The archaeal communities were covered more extensively as these contain the methanogens. Hence, both the phylogenetic (16S rRNA) and functional (*mcrA*) groups were covered.

5.3.3 Restriction enzyme selection

Restriction enzymes tested *in-silico* and *in-vitro* are shown in Table 5.3.

Table 5-3 Restriction enzymes evaluated for T-RFLP against each primer pair *in-silico* and *in-vitro*

Primer Name	Enzymes used for T-RFLP
Ar109fFam/Ar912r	*Alu*I, *Cfo*I, *Hae*III, *Mbo*I
Ar109f/Ar912rHex	*Alu*I, *Hae*III, *Hpy188*III, *Mbo*I
mcrAfFam/mcrAr	*Hpy188*III, *Hpy188*I, *HpyCH4*V, *Taq*I
ITS1FFam/ITS4	*Hinf*1, *Hpy188*III, *HpyCH4*V, *Taq*I
CS322F/EU929RFam	*Dde*I, Hpy188III, Hpy*CH4*V, TaqI
P-SSU342mod/EU929RFam	*Dde*I, *Hpy188*III *HpyCH4*V *Taq*I
27FFam/907R	*Msp*I, *Cfo*I

Complexity (numbers of peaks) and discriminatory potential (variability of profiles among animals) were determined by comparing T-RFLP profiles among four cows (one cow from local abattoir and three dairy cows from DPI Ellinbank Research Centre). The restriction enzymes which resulted in the most complex T-RFLP profiles and discriminated most among the four analysed rumen samples were: *Hae*III and *Mbo*I (Ar109fFam/Ar912r); *Hpy188*III (Ar109f/Ar912rHex); *Hpy188*I and *HpyCH4*V (mcrAfFam/mcrAr); *Hinf*I (ITS1FFam/ITS4); *Hpy188*III (CS322F/EU929RFam) and *Msp*I (27FFam/907R).

5.3.4 Rumen and faecal sample collection SOP

An SOP was developed and provided to collaborators within the RELRP for rumen and faecal samples collection (Appendix 9.3).

5.4 Discussion

We have shown that the SARDI extraction methodology is appropriate for the DNA isolation of a range of rumen and faecal microorganisms including bacteria, archaea, fungi and protozoa. Rumen microbial profiles generated from SARDI nucleic acid extracts were similar to those generated from nucleic acids obtained using either the RBB+C and QIAmp® DNA stool kit methodologies with the exception to the fungal communities. Fungal profiles generated from the RBB+C and QIAmp nucleic acid extracts vastly different. The SARDI extracts produced a fungal profile which encompassed profiles observed by both the other two methodologies, hence making it more appropriate for fungal community structure analysis. We were unable to make the same comparison with the faecal microbial communities using nucleic acids extracted by the SARDI, RBB+C and QIAmp® DNA stool kit methodologies as extracts obtained using the RBB+C and QIAmp® DNA stool kit contained inhibitors to PCR. We have previously encountered this problem when extracting nucleic acids from rodent faecal samples using the QIAmp® DNA stool kit. In addition to producing nucleic acids free on inhibitors the SARDI method has other advantages over both the RBB+C and QIAmp® DNA stool kit in that it is truly a high-throughput method, able to extract 100-200 samples per day. We also believe that the SARDI extraction methodology produces nucleic acids which may be more representative of the microbial community structure. Our method extracts nucleic acid from 10 ml unfiltered rumen fluid which would include microbes attached to particulate plant or feed material within the rumen sample. Both the RBB+C and QIAmp methods extract DNA from a pellet obtained from 1ml of rumen fluid.

Microbial profiling methodologies were developed and refined for archaea, methanogenic archaea, fungi and protozoa by investigating previous studies in this field (ruminants) and broader investigations of microbial community structure in various ecological systems. The T-RFLP methodologies developed in this study have been shown to be specific to their target organism and have a high level of discriminating power between members within a population. These methods have advantages over other profiling techniques (DGGE and TGGE) in that they have a higher resolving power, are truly high-throughput and cost effective making T-RFLP an ideal initial screening tool to investigate impact of dietary and host genetic factors on rumen and faecal microbiota. Once treatment differences are detected it would be possible to identify particular microorganisms driving differences using pyrosequencing technologies coupled with strong bioinformatics capabilities.

6 Collaborations with animal trials investigating methane mitigation strategies

6.1 Enteric methane abatement strategies for ruminant production systems in south eastern Australia (B.CCH.1009)

6.1.1 Experiment 1: The relationship between dietary tannin & methane production in early lactation dairy cows fed on a high protein diet in spring

6.1.1.1 Introduction

The experiment was performed by Peter Moate and done at DPI Ellinbank Research Centre, Victoria between September-November 2009. The aim of the experiment was to investigate if feeding condensed tannin to dairy cows influenced methane and milk production. For specific details please refer to B.CCH.1009 final report. Rumen and faecal samples were collected from this experiment and microbial profiling was done to investigate changes in the microbiota with respect to diet and methane production.

6.1.1.2 Methodology

Thirty-two dairy cows were fed one of four dietary treatments (n=8/treatment). All diets were pellet based and consisted of a control diet (no tannin) or one of three diets with varying levels of condensed tannin (80 g/cow/d, 160g/cow/d or 240 g/cow/d). Rumen fluid (stomach tubes), rumen solids and faecal samples were collected from all individual cows at two time points corresponding to periods following methane measurement (SF_6 methodology). The first collection was done at the end of the first week (covariate period) prior to cows being placed on their dietary treatments. The second collection was done once cows had been on their respective dietary treatments for two weeks.

Microbial profiling was only done on rumen fluid and faecal samples from the dietary experimental period. Microbial profiling was done to examine the bacterial (27FFam/907R *Msp*I), archaeal (Ar109fFam/Ar912r *Hae*III, Ar109fFam/Ar912r *Mbo*I, Ar109f/Ar912rHex *Hpy188*III, mcrAfFam/mcrAr *Hpy188*I and mcrAfFam/mcrAr *HpyCH4*V), fungal (ITS1FFam/ITS4 *Hinf*I) and protozoal (CS322F/EU929RFam *Hpy188*III) communities. Data generated by T-RFLP was validated (section 5.2.3) and statistically analysed for dietary differences (section 5.2.4).

6.1.1.3 Results

The resolving power of the various PCR primer and restriction enzyme combinations varied. Archaeal 16S rRNA profiling was all done with the primer pair Ar109fFam/Ar912r, however, the restriction enzyme *Mbo*I resulted in the best discrimination among individuals with n=44 OTU identified. This was followed by *Hpy188*III (n=33 OTU), while *Hae*III resulted in the least discrimination between archaeal communities with n=28 OTU. Archaeal profiling targeting the *mcrA* gene (mcrAfFam/mcrAr) generally gave higher discrimination than the 16S rRNA profiling on analysed samples (n=41 OTU with *Hpy188*I and n=68 OTU with *HpyCH4*V). Bacterial (27FFam/907R *Msp*I), fungal (ITS1FFam/ITS4 *Hinf*I) and protozoan (CS322F/EU929RFam *Hpy188*III) profiling generated 121 OTU, 87 OTU and 22 OTU respectively.

Significant differences (P=0.001) in bacterial, archaeal, methanogenic archaeal and fungal communities were observed between the rumen and faecal samples irrespective of dietary treatment (Table 6.1). No significant differences in microbial community composition with respect to dietary treatment were detected for any of the microbial profiling assays investigated (Table 6.1). This is not surprising as preliminary analysis of experimental feed failed to identify differing levels of tannin in the diets (Peter Moate, personal communication). Furthermore, urinary and faecal analysis of nitrogen from these cows failed to identify differences associated with diet. These observations support the absence of actual differences in dietary tannin levels among the treatments.

Two things were obvious from the microbial profiling data from these cows: firstly there was variation in microbial community composition among animals; and secondly, that the faecal and rumen microbial communities were different in both abundance of common OTU and presence/absence of unique gut specific OTU. This is graphically demonstrated for the archaeal communities in Figure 6.1. Fig 6.1 shows the individual microbial profiles as abundance of operational taxonomical units (OTU) for each animal. OTU represent individual microbial species or taxonomically related groups of organisms. Generally the rumen microbial communities were less complex than the faecal communities with the exception of the fungal communities. In this experiment we found that the faecal protozoan communities were not PCR amplified from all samples and when an amplicon was generated its size varied compared to that identified within the rumen community. Absence of faecal protozoan amplicons were not a result of PCR inhibition as other faecal microbial communities were amplified from these same nucleic acid extracts. It is not expected that rumen protozoa would survive in other parts of the gut or make their way into the faeces. For these reasons the faeces may not be a suitable surrogate assay for rumen function. Our rumen protozoan profiling results would predict the possible presence of *Entodinium caudatum* (OTU 424), *Epidinium caudatum* or *Ophryoscolex purkynjei* (OTU 376) and *Eupilodinium maggii* (OTU 198). These predictions are based on *in-silico* analysis of obtained rumen protozoan genome sequence. Several other OTU were also detected which are currently unidentifiable.

Table 6-1 Analysis of similarity (ANOSIM) of microbial communities by gut location (rumen versus faeces) and/or diet.

Profiling assay (Two-way ANOSIM)	**Diet (Global *R*, P value)**	**Gut location (Global *R*, P value)**
27FFam/907R *Msp*I	0.012, 0.370	1.000, 0.001
Ar109fFam/Ar912r *Hae*III	0.029, 0.189	0.653, 0.001
Ar109fFam/Ar912r *Mbo*I	0.003, 0.441	0.841, 0.001
Ar109f/Ar912rHex *Hpy188*III	-0.015, 0.635	0.836, 0.001
mcrAfFam/mcrAr *Hpy188*I	0.021, 0.264	0.967, 0.001
mcrAfFam/mcrAr *HpyCH4*V	0.004, 0.441	0.707, 0.001
ITS1FFam/ITS4 *Hinf*I	-0.008, 0.535	0.922, 0.001
Profiling assay (One-way ANOSIM)	**Diet (Global R, P value)**	
CS322F/EU929RFam *Hpy188*III	0.004, 0.380	

The Global *R* statistic value describes the extent of similarity between each pair in the ANOSIM, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between the groups. A significance level (P value) of less than 0.050 is considered significant.

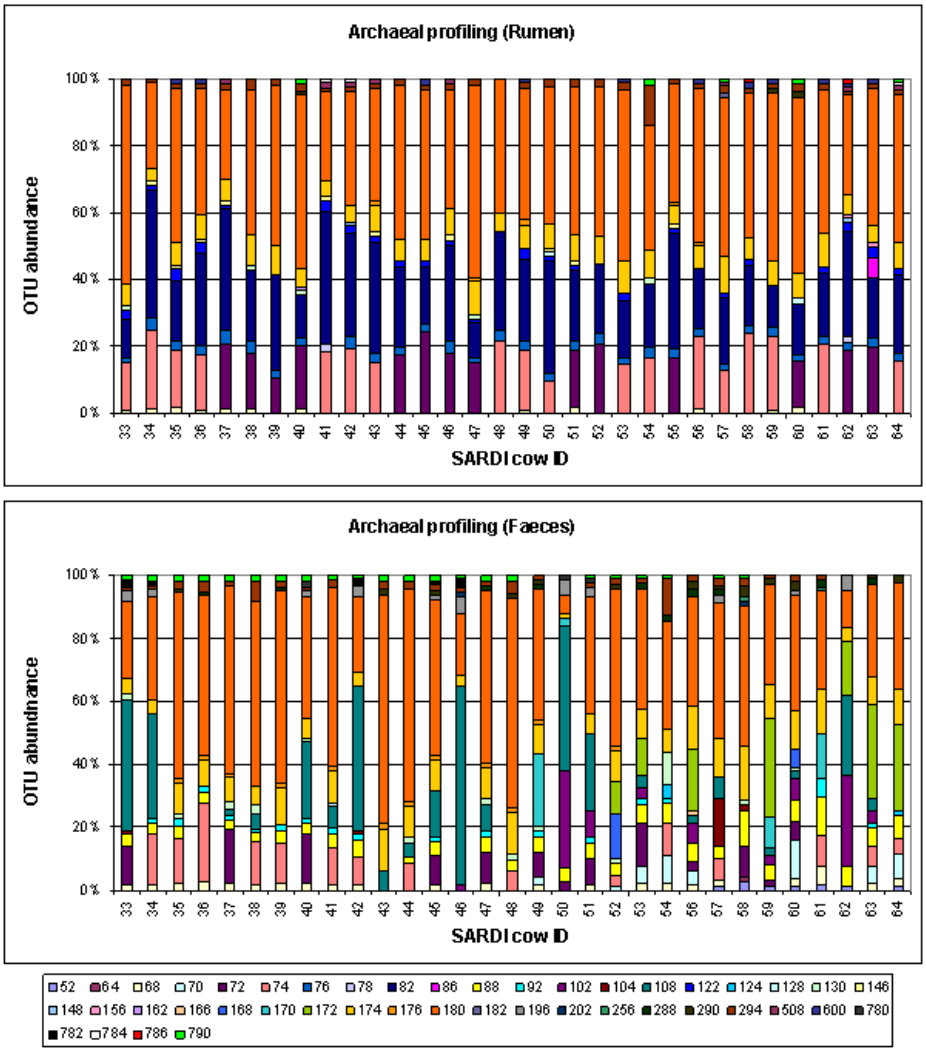


Figure 6-1 Archaeal profiles from the rumen and faeces of individual dairy cows from the Ellinbank tannin experiment. Archaeal profiles were generated with Ar109fFam/Ar912r *Mbo*I. n=44 OTU were detected across both the rumen and faeces. OTU are ordered largest to smallest (top to bottom) within the bar graphs.

6.1.1.4 Discussion

Although this experiment aimed to investigate the influence of increasing levels of dietary condensed tannins on methane emission in dairy cattle, for technical reasons the desired differences in dietary composition were not achieved (P. Moate, personal communication). Furthermore no significant differences in methane production associated with dietary treatment were detected. Despite this the 32 rumen and faecal samples from these cows were invaluable in fine tuning our profiling methodologies, enabling investigation of inter-animal variability and demonstrating that rumen and faecal microbial communities differ significantly. The results of our microbial profiling were consistent with there being no actual dietary differences.

6.1.2 Experiment 2: The relationship between dietary docosahexanoic acid (DHA) & methane production in mid-lactation dairy cows

6.1.2.1 Introduction

This experiment was run by Peter Moate at DPI Ellinbank Research Centre, Victoria between January-May 2010. The aim of the experiment was to investigate the influence dietary supplementation with DHA (docosahexanoic acid or C22:6) had on methane and milk fat production in dairy cows. For specific details please refer to B.CCH.1009 final report. Rumen and faecal samples were collected from this experiment and microbial profiling was done to investigate changes in the microbiota with diet and enteric methane production.

6.1.2.2 Methodology

Thirty-two dairy cows (n=8/treatment) were assigned to one of four dietary treatments: control (no DHA); DHA1 (25 gm DHA/day); DHA2 (50 gm DHA/day); and DHA3 (75 gm DHA/day). Rumen (stomach tube) and faecal samples were collected from individual cows. Microbial communities (bacteria, archaea, methanogenic archaea, fungi and protozoa) were profiled within the rumen. Faecal microbial communities were only investigated where significant differences were detected within the rumen. Data generated by T-RFLP was validated (section 5.2.3) and ANOSIM (section 5.2.4) was used to test whether rumen and faecal microbial communities were significantly different among dietary treatments.

6.1.2.3 Results

Significant differences in milk fat production associated with DHA treatment were detected, although no significant differences in methane production were detected among dietary treatments, (P Moate, personal communication).

Microbial profiling of rumen samples showed there were significant differences associated with diet within the bacterial, archaeal and methanogen populations (Table 6.2). No significant differences were detected in relation to DHA treatment within the rumen fungal or protozoan populations (Table 6.2). Rumen bacterial communities (27FFam/907R *Msp*I) were significantly different between dietary treatments ($R=0.106$, $P=0.038$) with significant pairwise differences ($P<0.05$) detected between the control and DHA2 treatment groups. This is graphically demonstrated in a nMDS ordination (Figure 6.2). nMDS ordinations attempt to place all samples in an arbitrary two-dimensional space such that their relative distances apart match the corresponding pairwise similarities. Where significant differences were detected in the archaeal communities (Ar109fFam/Ar912r *Mbo*I) associated with dietary treatment, significant pairwise differences were detected between the control and each of the DHA supplemented groups, as well as between the lowest and highest DHA supplemented groups (Figure 6.3a). Likewise where significant differences were detected in the methanogen communities (mcrAfFam/mcrAr *Hpy188*I) associated with dietary treatment, there were significant pairwise differences between the control group and either of the two higher DHA supplemented groups, as well as between the lowest and highest DHA supplemented groups (Figure 6.3b).

Where significant differences relating to DHA treatment were detected within the rumen microbial communities, the same microbial communities (bacteria, archaea and methanogens) were investigated in the faeces. No significant changes

in faecal microbial communities associated with DHA treatment were detected (Table 6.3).

Table 6-2 Rumen microbial profiling of 32 dairy cows from Ellinbank DHA experiment

Micro-organism	Microbial profiling assay	One-way ANOSIM*
Bacteria	27FFam/907R *Msp*I	R=0.106, P=0.038
Archaea	Ar109fFam/Ar912r *Hae*III	R=-0.019, P=0.740
	Ar109fFam/Ar912r *Mbo*I	R=0.357, P=0.001
	Ar109f/Ar912rHex *Hpy188*III	R=0.008, P=0.372
Methanogens	mcrAfFam/mcrAr *Hpy188*I	R=0.128, P=0.009
	mcrAfFam/mcrAr *HpyCH4*V	R=0.033, P=0.255
Fungi	ITS1FFam/ITS4 *Hinf*I	R=-0.030, P=0.675
Protozoa	CS322F/EU929RFam *Hpy188*III	R=-0.018, P=0.619
	CS322F/EU929RFam *Taq*I	R=-0.011, P=0.575
	CS322F/EU929RFam *Dde*I	R=-0.046, P=0.863

* The R value describes the extent of similarity between each pair in the ANOSIM, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between the groups. P < 0.05 is significant.

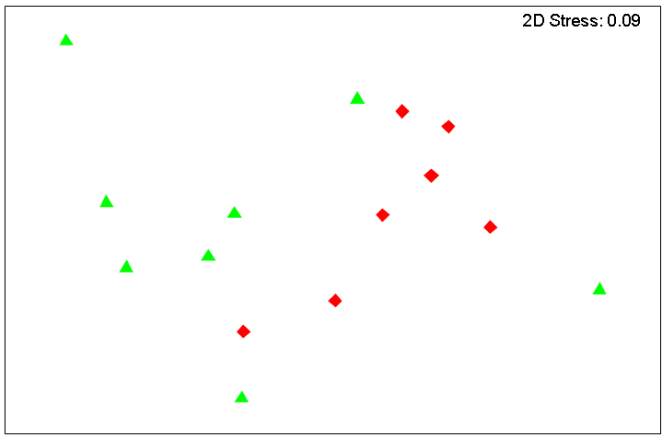


Figure 6-2 nMDS of bacterial communities associated with diet. Bacterial profiles were generated with 27FFam/907R *Msp*I. Dietary treatments are ♦=no DHA control and ▲=DHA2 (50 gm DHA/day). Each point in the ordination shows the overall microbial profile of an individual animal. The closer two points are in the ordination the more similar are their profiles.

Table 6-3 Faecal microbial profiling of 32 dairy cows from Ellinbank DHA experiment.

Micro-organism	Microbial profiling assay	One-way ANOSIM*
Bacteria	27FFam/907R *Msp*I	R=-0.010, P=0.565
Archaea	Ar109fFam/Ar912r *Mbo*I	R=0.014, P=0.371
Methanogen	mcrAfFam/mcrAr *Hpy188*I	R=-0.102, P=0.978

* The R value describes the extent of similarity between each pair in the ANOSIM, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between the groups. P < 0.05 is significant.

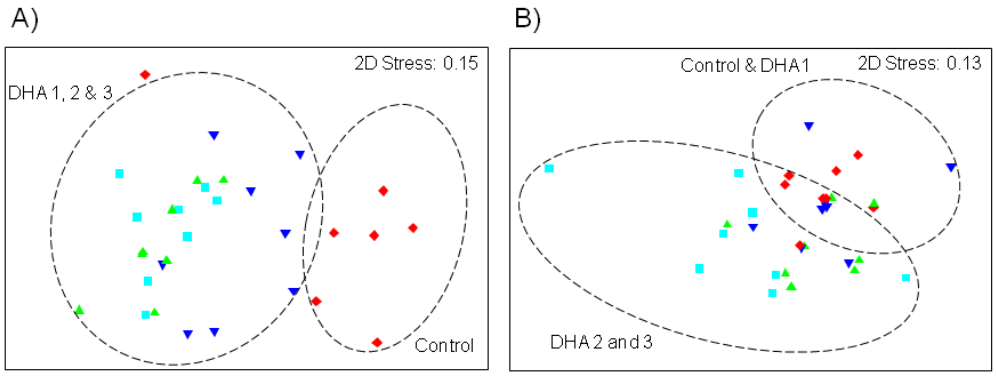


Figure 6-3 nMDS of archaeal and methanogenic archaeal communities associated with diet. A) Archaeal profiles were obtained with Ar109fFam/Ar912r *Mbo*I. B) Methanogenic archaeal profiles were obtained with mcrAfFam/mcrAr *Hpy188*I. Dietary treatments are ♦=no DHA control, ▼=DHA1 (25 gm DHA/day), ▲=DHA2 (50 gm DHA/day) and ■=DHA3 (75 gm DHA/day). Each point in the ordination shows the overall microbial profile of an individual animal. The closer two points are in the ordination the more similar are their profiles.

The DHA feeding experiment was done on the same dairy cows as were used in the previous feeding experiment entitled "The relationship between dietary tannin and methane production in early lactation dairy cows fed on a high protein diet in spring" (section 6.1.1). Although neither the tannin nor DHA experiments showed significant differences in methane production, the ranking of individual cows were investigated between experiments. Cows did not appear to maintain their ranking in methane production between experiments (P. Moate, personal communication). Interestingly, the methanogenic archaeal rumen profiles of the 32 cows were significantly different between the two experiments (Figure 6.4) suggesting diet and possibly environment have a stronger influence on the rumen microbiota than the host.

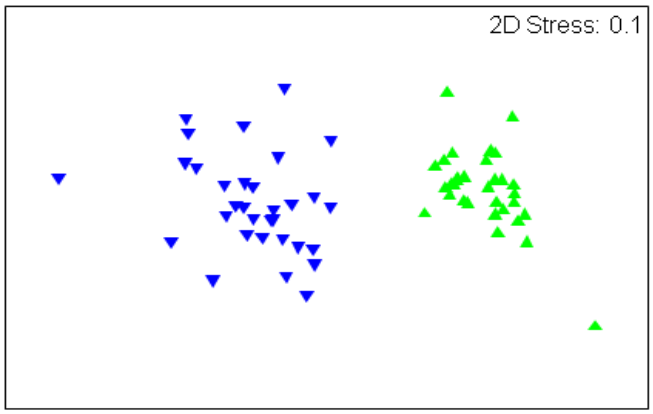


Figure 6-4 nMDS of rumen methanogenic archaeal profiles (mcrAfFam/mcrAr *Hpy188*I) associated with experiment. Profiles from 32 dairy cows from Ellinbank tannin experiment (▼) and profiles from the same cows during the Ellinbank DHA experiment (▲).

6.1.2.4 Discussion

Although no significant methane differences were detected in response to DHA supplementation within this trial there were significant changes in milk fat composition associated with feeding DHA (P. Moate, personal communication). Our microbial profiling assays have successfully shown that dietary supplementation with DHA does alter some microbial communities including the bacteria, archaea and methanogens within the rumen. Some of the organisms may be involved in biohydrogenation and hence be responsible for the changes observed in milk fat composition associated with DHA supplementation. Significant DHA related changes in the rumen microbiota were not reflected within the faecal microbial profiles of these animals. Furthermore, diet appears to have a greater influence on rumen micobiota than the host itself as rumen profiles of individual cows were shown to significantly differ between two independent feeding trials.

6.1.3 Experiment 3: Influence of the combination of dietary fat & tannin on methane emissions by dairy cows

6.1.3.1 Introduction

This experiment was led by Peter Moate at DPI Ellinbank Research Centre, Victoria between September-December 2010. Unlike the other experiments within B.CCH.1009 this was the only experiment done on fistulated dairy cows. The aim of the experiment was to investigate influence of tannin and fats, both individually and in combination, on methane and milk production. For more detail refer to B.CCH.1009 final report.

6.1.3.2 Methodology

The experiment used ten fistualated dairy cows all of which underwent an initial covariate period. Following the covariate period eight cows were rotated through four treatments: control (800 ml/cow/day of water); fat (800 ml/cow/day of cottonseed oil); tannin (400 g/cow/day of tannin from *Acacia mearnsii*); and tannin/fat (400 g/cow/day of tannin from *Acacia mearnsii* and 800 ml/cow/day of cottonseed oil). All treatments were infused into the rumen via the rumen fistula. The two remaining cows were maintained on the control treatment and acted as donor cows to refaunate experimental cows with 5 kg rumen material following each experimental treatment. The experimental cows remained on designated treatments for 22 days each. Methane measurements were taken in respiration chambers over a two day period (day 20 -21) and rumen samples were taken on day 22. On day 22 the entire rumen content was bailed out, sub-sampled, replaced, refaunated with 5 kg donor cow rumen content and the subsequent experimental treatment commenced.

Rumen fluid from the fistula and faecal samples were collected from each of the ten cows during the covariate period and the eight cows which were rotated through the four experimental dietary treatments. Microbial communities were profiled within the rumen using the assays listed in Table 6.4. Data generated by T-RFLP was validated (section 5.2.3) and ANOSIM (section 5.2.4) was used to test whether rumen microbial communities were significantly different among dietary treatments and between covariate and experimental periods.

Table 6-4 List of micro-organisms investigated by microbial profiling and details of primers and restriction enzyme used to characterise each microbial community.

Micro-organism	Microbial profiling assay
Bacteria	27FFam/907R *Msp*I
Archaea	Ar109fFam/Ar912r *Mbo*I
Methanogens	mcrAfFam/mcrAr *Hpy188*I
Fungi	ITS1FFam/ITS4 *Hinf*I
Protozoa	CS322F/EU929RFam *Hpy188*III

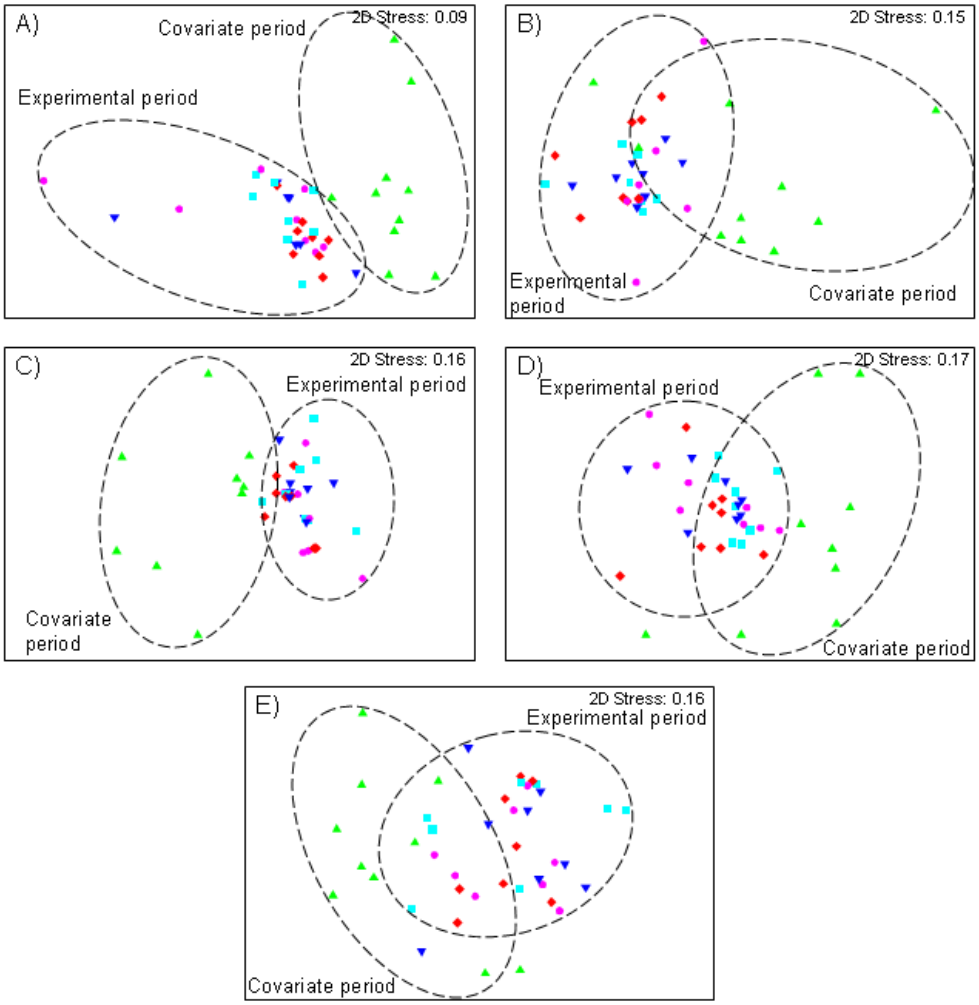


Figure 6-5 nMDS ordination of rumen microbial communities from fistulated dairy cows on covariate (▲), control (■), tannin (♦), fat (▼) and fat/tannin (●) treatments. A) Bacterial communities. B) Archaeal communities. C) Methanogenic archaeal communities. D) Fungal communities. D) Protozoan communities.

6.1.3.3 Results

Methane production was reduced by 12% in cows on the fat only treatment, 3% on the tannin only treatment and 4% on the combined fat/tannin treatment as compared to the control treatment. However, these reductions were not statistically significant (P. Moate, personal communication).

For all five microbial communities investigated (Table 6.4) the rumen microbiota of the cows on the covariate period were significantly ($P<0.05$) different to the rumen microbiota of cows on each of the experimental treatments. No significant differences in rumen microbiota associated with the experimental dietary treatments

were observed (Table 6.5). Figure 6.5 gives a graphical representation of differences observed between the covariate and experimental samples and the lack of differences observed among experimental samples for each of the five microbial communities investigated.

Table 6-5 Rumen microbial profiling of fistulated dairy cows on experimental treatments (control, fat, tannin and fat/tannin) infused into the rumen fistula.

Micro-organism	One-way ANOSIM*
Bacteria	R=0.003, P=0.431
Archaea	R=-0.008, P=0.586
Methanogens	R=-0.027, P=0.742
Fungi	R=-0.075, P=0.984
Protozoa	R=-0.024, P=0.636

* $P < 0.05$ is significant.

6.1.3.4 Discussion

The lack of significant differences in microbial communities between the four dietary treatments (experimental period) may have been due to the low numbers of replicate cows (n=8) used in this study, or due to the artificial rumen manipulations undertaken in these animals. It would have been interesting to investigate the rumen microbiota of the two cows which acted as rumen donors for refuanating the experimental cows; however, these samples were not collected. Interestingly, the rumen microbiota of cows during the covariate period was significantly different to those of the same cows during the experimental period. This was in spite of the fact that one of the experimental dietary treatments (control) was the same as the covariate period diet. The covariate and control diets were confirmed to be the same (P. Moate, personal communication). In light of this, the significant differences observed in the rumen microbiota between cows on the covariate and experimental periods could truly be a result of the artificial rumen manipulations used during the experimental period.

Although, some insignificant reductions in methane production were observed when comparing the fat, tannin and fat/tannin treatments with the control group (P. Moate, personal communication), no methane measurements were taken during the covariate period. It would have been interesting to investigate influence of the rumen manipulation on methane production. Furthermore, it would have been interesting to investigate changes in methane production during the covariate and experimental periods in light of the significantly differences observed in rumen microbiota during these two periods.

6.1.4 Experiment 4: Influence of two forms of grape marc on methane emissions by dairy cows

6.1.4.1 Introduction

The experiment was led by Peter Moate and done at DPI Ellinbank Research Centre, Victoria between March-June 2011. The aim of the experiment was to investigate the influence dietary supplementation with grape marc on methane, milk production, rumen volatile fatty acid (VFA) profiles and rumen protozoan counts in dairy cows. Thirty two Holstein-Friesian cows received one of three dietary treatments: control; dried grape marc (DGM); or ensiled grape marc (EGM). For specific details relating to the experiment refer to B.CCH.1009 final report. Rumen and faecal samples were collected from this experiment and microbial profiling was

done to investigate diet related changes in rumen and faecal microbiota which could be linked with enteric methane production.

6.1.4.2 Methodology

Thirty-two intact, lactating, Holstein-Friesian cows were allocated to one of three treatments:

- Control (CON) treatment (n=12). Cows received a daily diet comprising ca. 6.0 kg DM of crushed wheat, 0.2 kg DM of molasses, 0.1 kg DM of minerals and *ad libitum* lucerne hay.
- Dried grape marc (DGM) treatment (n=10). Cows received a daily diet comprising 5 kg DM of DGM, 1.0 kg DM of crushed wheat, 0.2 kg DM of molasses, 0.1 kg DM of minerals and *ad libitum* lucerne hay.
- Ensiled grape marc (EGM) treatment (n=10). Cows received 5.0 kg DM of EGM, 1.0 kg DM of crushed wheat, 0.2 kg DM of molasses, 0.1 kg DM of minerals and *ad libitum* lucerne hay.

Cows were on their allocated treatments for at least 18 days prior to methane measurement using the SF_6 methodology. Rumen (stomach tube) and faecal samples were collected to correspond with the completion of the methane measurement period. Rumen and faecal microbial communities were analysed using microbial profiling assays outlined in Table 6.4. Data generated by T-RFLP was validated (section 5.2.3) and multivariate statistical tests (section 5.2.4) were used to investigate changes in microbial community structure linked to diet, gut location (rumen vs faeces) and methane production. Where significant differences were detected in microbial communities unconstrained ordinations (nMDS) were done to graphically illustrate relationships between treatments and constrained canonical analysis of principal coordinates (CAP) biplots were constructed to investigate the relationship between OTUs associated with diet and enteric methane emissions (g CH_4/kg DMI).

6.1.4.3 Results

Dietary supplementation with either EGM or DGM was shown to reduce methane emissions in cows by approximately 20% compared with the control group (P. Moate, personal communication). Dietary supplementation with grape marc also influenced milk production, milk fat composition and rumen VFA profiles (P. Moate, personal communication). No significant differences were detected in rumen protozaon communities among treatments as assed by microscopic investigation (P.Moate, personal communication).

For all the microbial communities investigated (Table 6.4) there were significant (P=0.001) difference between the rumen and faecal microbiota regardless of dietary treatment (Table 6.6). This separation between rumen and faecal microbiota is graphically demonstrated for each of the five microbial profiling assays (Figure 6.6).

Table 6-6 Influence of diet and gut location (rumen versus faeces) on microbial community composition. Two-way ANOSIM*

Micro-organism	Diet	Gut
Bacteria	Global R=0.136, P=0.001	Global R=1.000, P=0.001

Archaea	Global R=0.130, P=0.004	Global R=0.827, P=0.001
Methanogens	Global R=0.074, P=0.032	Global R=0.949, P=0.001
Fungi	Global R=0.204, P=0.001	Global R=0.863, P=0.001
Protozoa	Global R=0.006, P=0.360	Global R=1.000, P=0.001

* The R value describes the extent of similarity between each pair in the ANOSIM, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between the groups. P < 0.05 is significant.

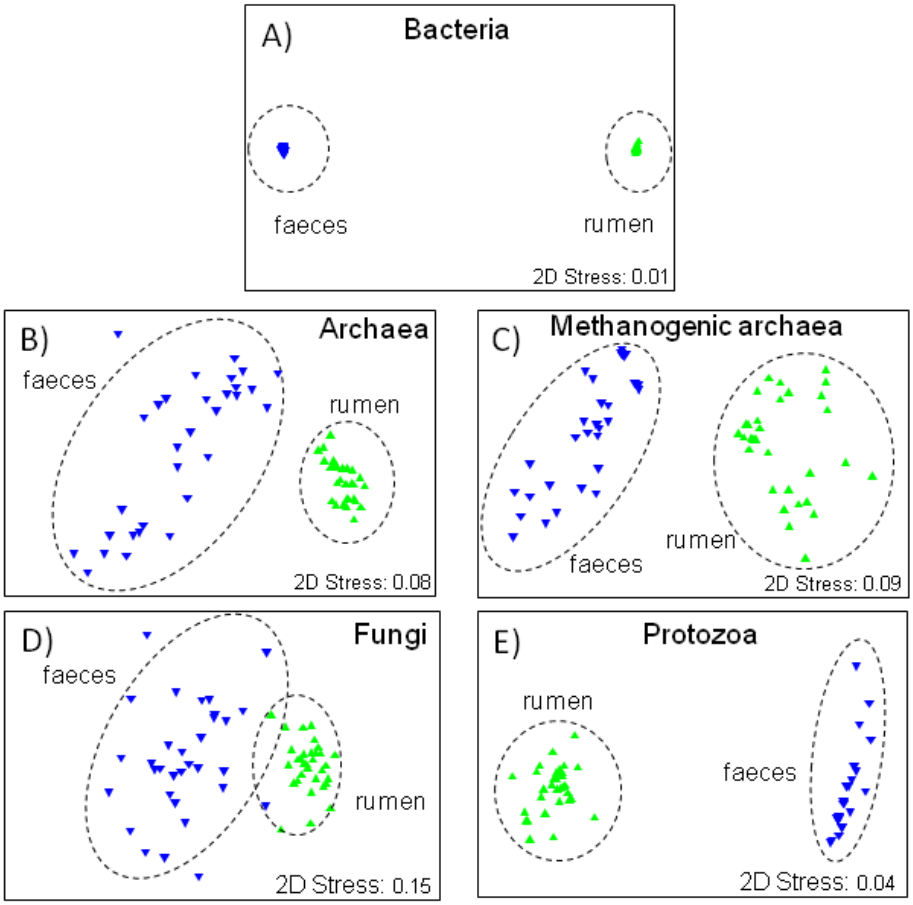


Figure 6-6 nMDS ordination of microbial communities from the rumen and faeces of 32 dairy cows from the Ellinbank grape marc experiment. A) Bacterial communities. B) Archaeal communities. C) Methanogenic archaeal communities. D) Fungal communities. E) Protozoan communities. Faecal microbial communities (▼). Rumen microbial communities (▲).

Furthermore, significant differences associated with dietary treatment were also observed regardless of gut location (Table 6.6). Therefore, the influence of dietary treatment on microbial community structure was investigated separately within both the rumen and faeces (Table 6.7). Bacterial communities were significantly altered by diet within the rumen and faeces. Within the rumen dietary supplementation with either EGM or DGM altered bacterial communities in relation to the control group. However, within the faeces no significant pairwise comparisons were detected, possibly due to the low animal replication and high inter-animal variability. The methanogenic archaeal communities (based on the analysis of the functional *mcrA* gene) were not altered by any of the dietary treatments within either the rumen or faeces. Interestingly, the archaeal communities which include the methanogens (based on phylogenetic analysis rather than function) were altered by dietary treatment within the rumen but not within the faeces. The rumen archaeal

communities were altered by addition of DGM but not EGM. Fungal communities were altered by diet within the faeces only. Both forms of grape marc altered the fungal community in comparison with the control group. Furthermore, the two forms of grape marc appeared to be influencing the fungal communities differently. The protozoan communities were unaltered by dietary treatment. Significant differences are graphically demonstrated in Figure 6.7.

Table 6-7 One-way ANOSIM of rumen and faecal microbial communities associated with dietary treatment. For each microbial group the influence of dietary supplementation with grape marc was investigated. Where significant differences in rumen and faecal microbiota were detected, the pairwise* differences between dietary treatments were investigated further.

Rumen microbial profiles				Faecal microbial profiles			
Bacteria (Global R=0.117, P=0.001)				Bacteria (Global R=0.094. P=0.028)			
	Control	EGM	DGM		Control	EGM	DGM
Control		**0.277**	**0.225**	Control		**0.096**	**0.066**
EGM	*0.001*		**0.031**	EGM	*0.064*		**0.127**
DGM	*0.002*	*0.223*		DGM	*0.129*	*0.052*	
Archaea (Global R=0.176, P=0.003)				Archaea (Global R=0.091, P=0.064)			
	Control	EGM	DGM				
Control		**0.034**	**0.343**				
EGM	*0.271*		**0.176**				
DGM	*0.004*	*0.025*					
Methanogens (Global R=0.046, P=0.170)				Methanogens (Global R=0.102, P=0.068)			
Fungi (Global R= 0.038, P=0.140)				Fungi (Global R=0.370, P=0.001)			
					Control	EGM	DGM
				Control		**0.471**	**0.312**
				EGM	*0.001*		**0.312**
				DGM	*0.002*	*0.001*	
Protozoa (Global R=0.024, P=0.243)				Protozoa (Global R=-0.019, P=0.605)			

* For each pairwise comparison the R value (bold) and P value (italics) are indicated. P < 0.05 is significant.

The individual microbial profiles (bacterial, archaeal, methanogenic archaeal, fungal and protozoan) obtained from both the rumen and faecal samples of each cow (n=32) are shown in Appendix 9.4. Two things are evident from this data: firstly, that there is great inter-animal variation in microbial community composition within a treatment group; and secondly, that the rumen and faecal microbiota differ in both presence/absence of unique gut associated microbes and abundance of common microbes. The inter-animal variation within a treatment group indicates a need for robust statistical analysis.

CAP analysis was used to investigate the correlation between significant diet related shifts in rumen microbiota and methane production. Constrained CAP analysis, done on rumen bacterial (Figure 6.8) and archaeal (Figure 6.9) communities, produced biplots from the first canonical analysis of principal coordinates axis (CAP1) against methane production (g CH_4/kg DMI) of individual cows. A good correlation was seen between rumen bacterial community composition and methane production when the control diet was compared with the EGM diet (r=0.69) (Figure 6.8a) or DGM diet (r=0.60) (Figure 6.8b). Furthermore, a good correlation was seen between rumen archaeal community composition and methane production when the control diet was compared with DGM (r=0.68) (Figure 6.9a). Significant changes in archaeal community composition between the EGM and DGM diets were not well correlated with methane production (r=0.31) (Figure 6.9b).

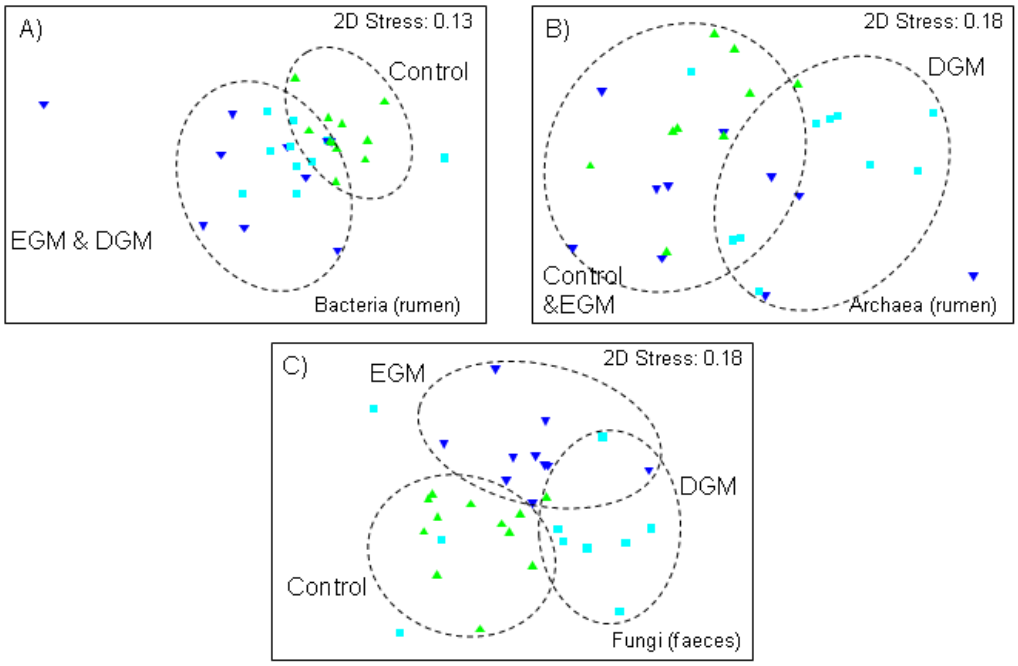


Figure 6-7 nMDS ordination of microbial communities from 32 dairy cows from the Ellinbank grape marc experiment. A) Bacterial communities within the rumen. B) Archaeal communities within the rumen. C) Fungal communities within the faeces. Control diet (▲), EGM (▼) and DGM (■).

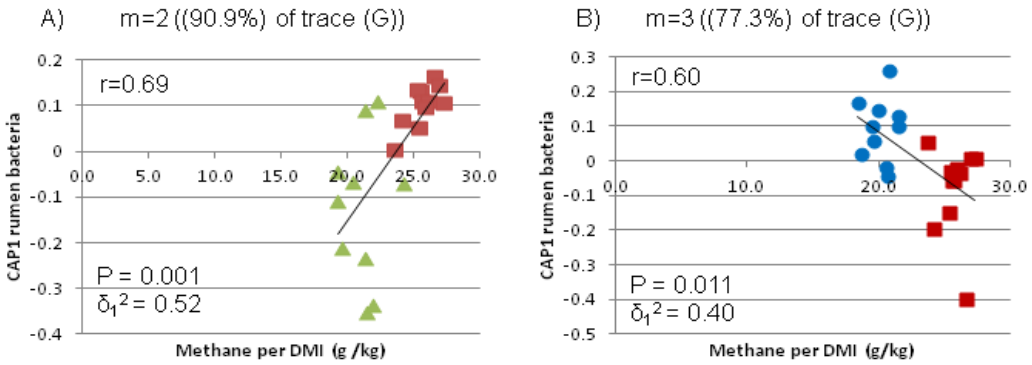


Figure 6-8 CAP of diet-associated rumen bacterial communities related to methane production per DMI. A) CAP-versus-methane biplots for cows on the control and EGM diets. B) CAP-versus-methane biplots for cows on the control and DGM diets. CAP analysis was based on Bray-Curtis similarities calculated from fourth-root transformed species abundances. "m" achieves the maximum proportion of correct allocations (% of trace [G]) of samples to diet. (■) control diet, (▲) EGM and (●) DGM.

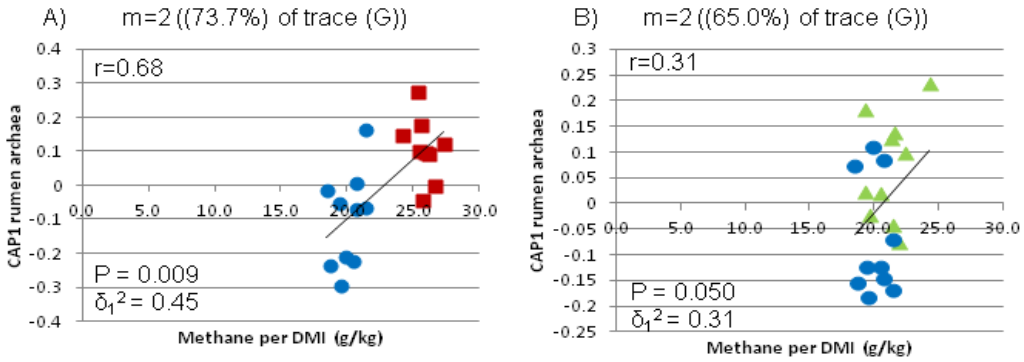


Figure 6-9 CAP of diet-associated rumen archaeal communities related to methane production per DMI. A) CAP-versus-methane biplots for cows on the control and DGM diets. B) CAP-versus-methane biplots for cows on the EGM and DGM diets. CAP analysis was based on Bray-Curtis similarities calculated from fourth-root transformed species abundances. "m" achieves the maximum proportion of correct allocations (% of trace [G]) of samples to diet. (■) control diet, (▲) EGM and (●) DGM.

6.1.4.4 Discussion

The grape marc feeding experiment was not only successful in demonstrating significant changes in rumen and faecal microbiota linked with dietary treatment, but also showed that changes in rumen bacterial and archaeal communities were correlated with methane production. As such, this is the first report that directly correlates diet-associated changes in rumen microbial community with reductions in enteric methane production. This was done by using CAP analysis to correlate individual methane variables (g CH_4/kg DMI) with patterns in rumen microbial community composition on canonical biplots. Both the archaea and bacterial communities are known to be involved in methane production through either hydrogen utilization or production. The diet related changes we observed in rumen bacterial communities, support the differences observed in rumen VFA production (a product of bacterial metabolism) and milk fat composition (influenced by microbial biohydrogenation) by P. Moate.

We have also shown that rumen and faecal microbiota differ significantly regardless of dietary treatment. Hence, faecal samples may not be the most appropriate surrogate assay for rumen function. Furthermore, diet associated changes observed within the rumen bacterial and archaeal communities were not reflected within the faecal communities. Interestingly significant changes related to diet were observed within the faecal fungal communities which were not observed within the rumen. In this study neither the rumen nor faecal protozoan communities were influenced by diet. This former is in support of P. Moate's microscopy findings suggesting rumen protozoa did not differ among dietary treatments.

Although, microbial profiling of faecal samples may not be an adequate surrogate for rumen function, if presence/absence of specific organisms associated with methane reduction could be identified within the rumen then quantitative assays could be developed to investigate these organisms within the faeces. This would provide a more targeted approach for development of a surrogate assay for rumen function.

6.2 Breeding low methane sheep & understanding the biology behind how they do it (B.CCH.1015)

6.2.1 University of Western Australia study (Samantha Bickell & Phil Vercoe)

6.2.1.1 Introduction

Rumen microbiota was investigated from 30 anticipated "high" and 30 anticipated "low" methane producing sheep from an experiment "Seeking confirmation of high and low methane emitting sire groups" done at UWA between March–August 2010. For more information on animal experimentation please refer to B.CCH.1015 final report. The aim of our investigation was to determine if rumen microbiota was influenced by animal genetics and enteric methane production.

6.2.1.2 Methodology

Sixty wethers originating from the information nucleus flock (INF), anticipated to be "high" (n=30) and "low" (n=30) methane producers, were measured for methane production in respiration chambers to establish methane production status at two independent time points. Rumen and faecal samples were collected from each animal corresponding with their chamber measurement on both occasions. Microbial profiling (bacterial, archaeal, methanogens, fungal and protozoan) was done on rumen samples from all sheep corresponding with the initial sampling period. Microbial profiling assays for micro-organisms referred to in this report are shown in Table 6.4. Data generated by T-RFLP was validated (section 5.2.3) and microbial communities were statistically (section 5.2.4) investigated against the following factors: anticipated "high/low" methane producing groups (n=30/group); actual top and bottom (n=10/group) methane producing sheep (g CH_4/23hr); top and bottom methane producing sheep (n=10/group) adjusted for feed intake (g CH_4/kg FI); and top and bottom (n=10/group) methane producing sheep adjusted for feed intake and metabolic weight (g CH_4/(g FI/LW^0.75).

6.2.1.3 Results

No significant differences were detected in any of the microbial communities investigated between the anticipated "high" and "low" methane producing sheep (Table 6.8). The anticipated "high" and "low" methane producing sheep were also found not to differ in their methane production (Samantha Bickell, personal communication). Furthermore, no correlation was found in methane production in sheep between their first and second methane chamber measurement (Samantha Bickell, personal communication).

Table 6-8 Rumen microbial profiling of sheep anticipated to be "high" and "low" methane producers.

Micro-organism	One-way ANOSIM*
Bacteria	R=-0.009, P=0.660
Archaea	R=-0.024, P=0.902
Methanogens	R=-0.009, P=0.621
Fungi	R=0.001, P=0.373
Protozoa	R=-0.028, P=0.941

* The R value describes the extent of similarity between each pair in the ANOSIM, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between the groups. $P < 0.05$ is significant.

Graphical representation of the methanogenic communities from all 60 sheep are shown in Figure 6-10. It is apparent from this representation that there is between sheep variation in rumen microbiota composition, despite being fed similar feeds and being reared in the same environment. In light of the absence of differences in rumen microbiota relating to anticipated "high" and "low" methane grouping, we proceeded to analyse the top and bottom 10 sheep from the cohort for actual methane, methane adjusted for feed intake and methane adjusted for feed intake and metabolic weight. Table 6.9 shows average ± SD for the top and bottom methane producing sheep identified by each of the above methods.

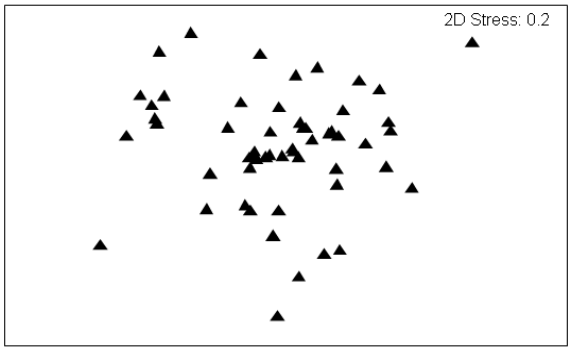


Figure 6-10 nMDS ordination of rumen methanogens from 60 sheep fed the same diet. Each point in the ordination shows the overall microbial profile of an individual animal. The closer two points are in the ordination the more similar are their profiles.

Table 6-9 Identifying extreme methane producers from a cohort of 60 sheep.

Methane measure[a]	Low (n=10) (mean±SD)	High (n=10) (mean±SD)
Actual ($g\ CH_4$/23hr)	4.86 ± 0.54	12.82 ± 3.16
Adjusted for feed intake ($g\ CH_4$/kg FI)	3.58 ± 0.39	10.86 ± 3.28
Adjusted for FI & metabolic weight ($g\ CH_4$/(g FI/kg LW^$^{0.75}$))	0.07 ± 0.01	0.22 ± 0.07

[a] Raw data provided by S. Bickell

Identified top and bottom methane producing sheep did not change greatly with the three different methods used to rank sheep (actual methane, methane adjusted for feed intake or methane adjusted for feed intake and metabolic weight). In fact, seven out of the ten animals from the high and low methane producing groups were consistently the sample regardless of method used to determine grouping. Changes in microbial community structure where investigated against methane groups as determined by each of the three methods mentioned above (Table 6-10). No significantly differences were detected in any of the five rumen microbial communities investigated in relation to extremes in methane production from these sheep.

Table 6-10 One-way ANOSIM of rumen microbial communities from top and bottoms methane producing sheep as determined by three different methods.

Profiling assay	Methods of methane ranking*		
	g CH_4/23h	g CH_4/kg FI	g CH_4/(g FI/kg LW^$^{0.75}$)
Bacteria	R=-0.072, P=0.891	R=0.006, P=0.368	R=-0.010, P=0.536
Archaea	R=-0.058, P=0.818	R=-0.022, P=0.666	R=-0.037, P=0.688
Methanogenic archaea	R=0.042, P=0.212	R=0.055, P=0.126	R=0.054, P=0.166
Fungi	R=0.040, P=0.234	R=0.017, P=0.352	R=0.062, P=0.189
Protozoa	R=-0.026, P=0.514	R=-0.020, P=0.547	R=-0.088, P=0.971

* $P < 0.05$ are significant.

6.2.1.4 Discussion

As no significant differences in either methane production or rumen microbial community structure were detected between anticipated “high” and “low” methane producing sheep based on genetic selection, we proceeded to investigate the rumen microbiota from extreme methane producing sheep from the cohort of 60. Three approaches were used to determine the extreme (n=10/group) methane producers (actual methane production, methane adjusted for feed intake and methane adjusted for feed intake and metabolic weight). The three ranking approaches used largely resulted in the identification of the same high and low methane producing sheep. Rumen profiling of the extreme methane producing sheep, as determined by each of the three methods, failed to identify significant differences in any of the microbial communities associated with methane classification. These results may be due to the low animal replication per extreme methane group (n=10) or the small cohort of animal investigated (n=60) in identifying extreme methane producers. In this study we were unable to link rumen microbiota to animal phenotype.

6.2.2 University of New England study (John Goopy, Hutton Oddy & Roger Hegarty)

6.2.2.1 Introduction

This sheep trial was run by John Goopy at UNE, Armidale. Methane production was measured in respiration chambers during 2010/2011 from one hundred and sixty undifferentiated ewes originating from the Australian Wool Innovation (AWI) flock. The top and bottom ten methane producing sheep, as determined g CH_4/kg DMI/day, were remeasured for a second time in chambers (1 – 9 months apart) to determine if they maintained their ranking over time. For more information please refer to B.CCH.1015 final report. Rumen microbial communities were investigated from the extreme methane producing sheep as identified by the initial screening process, and the same sheep when they had methane production re-measured. The aim of our investigation was to determine if rumen microbiota was influenced by animal genetics and enteric methane production.

6.2.2.2 Methodology

Rumen microbial communities were investigated from 20 divergent (n=10 high and n=10 low) methane producing sheep as determined by an initial screening process involving 160 ewes, and the same 20 sheep when they were remeasured for methane production 1-9 month(s) later. The microbial profiling assays used to investigate the bacterial, archaeal, methanogenic archaeal, fungal and protozoan

communities are detailed in Table 6.4. Data generated by T-RFLP was validated (section 5.2.3) and multivariate statistical tests (section 5.2.4) were used to investigate changes in microbial community structure linked with methane grouping and sampling period. Where significant differences were detected in microbial communities unconstrained ordinations (nMDS) were done to graphically illustrate relationships between treatments. Constrained canonical analyses of principal coordinates (CAP) biplots were also constructed to investigate the relationship between OTU associated with animal genetics and actual enteric methane emissions (g CH_4/kg DMI/day).

6.2.2.3 Results

Methane production from the extreme high and low methane producing sheep, as determined during the initial screening period, was 24.79±1.09 g CH_4/kg DMI (mean±SD) and 19.27±0.85 g CH_4/kg DMI, respectively. When these designated high and low methane sheep were re-measured they were found to produce 22.86±0.96 g CH_4/kg DMI and 21.3±2.43 g CH_4/kg DMI, respectively (John Goopy, personal communication).

Microbial profiling showed that both the sample collection timing (initial screening versus re-measurement) and attributed methane phenotype (high versus low) had significant effects on rumen microbiota composition (Table 6-11). Bacterial, archaeal, methanogenic archaeal and protozoan communities differed significantly within individual sheep between the first and second rumen collection. Only the fungal communities did not change with progression of time (first versus second sampling). Furthermore, significant differences were detected in the bacterial and protozoan communities between the high and low methane producing sheep for both the initial rank determination and the subsequent re-measurement. Differences in rumen bacterial populations associated with the two collection periods are graphically shown in Figure 6-11, while differences in rumen bacterial populations between the designated high and low methane producing sheep are shown for the initial ranking (Figure 6-12a) and re-measurement (Figure 6-12b) periods.

Table 6-11 Influence of methane grouping of sheep and sampling time on the microbial community structure. Two-way ANOSIM*

Micro-organism	methane phenotype	Sampling time
Bacteria	Global R=0.119, P=0.020	Global R=0.252, P=0.001
Archaea	Global R=0.043, P=0.148	Global R=0.092, P=0.029
Methanogens	Global R=0.043, P=0.131	Global R=0.092, P=0.036
Fungi	Global R=0.080, P=0.076	Global R=0.052, P=0.157
Protozoa	Global R=0.273, P=0.001	Global R=0.091, P=0.038

* $P < 0.05$ is significant.

CAP analysis was used to investigate the correlation between significant phenotypic related shifts in rumen microbiota and methane production. Constrained CAP analysis, done on rumen bacterial (Figure 6.13) and protozoan (Figure 6.14) communities, produced biplots from the first canonical analysis of principal coordinates axis (CAP1) against methane production (g CH_4/kg DMI/day) of individual sheep. A good correlation was seen between rumen bacterial communities and methane production based on designated phenotype (r=0.70) (Figure 6.12a) from the initial screening (rank determination) but not during the subsequence reassessment period (r=0.33) (Figure 6.13b). However, when the rumen protozoan communities were investigated a good correction was found between rumen protozoa and methane production based on designated phenotype for both the initial rank determination (r=0.78) and reassessment periods (r=0.70) (Figure 6.14).

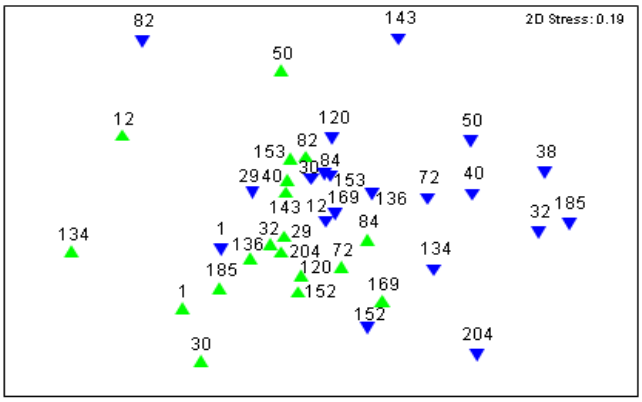


Figure 6-11 nMDS ordination of rumen bacterial profiles from sheep identified by first (▲) and second (▼) rumen collection. Numbers represent ewe identification number. Each ewe should be represented twice.

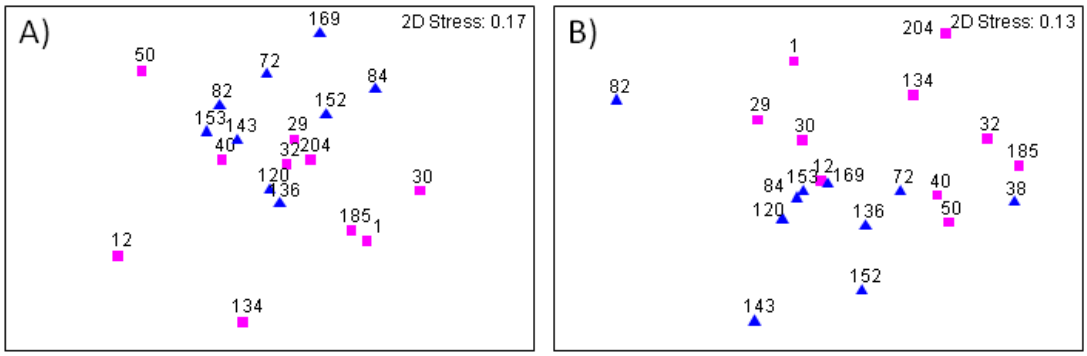


Figure 6-12 nMDS ordinations of rumen bacterial profiles from sheep identified as divergent in methane production. A) rumen bacteria profiles from first sample collection (rank determination). B) rumen bacterial profiles from sheep on second sample collection. High (■) and low (▲) methane producers. Numbers represent ewe identification number.

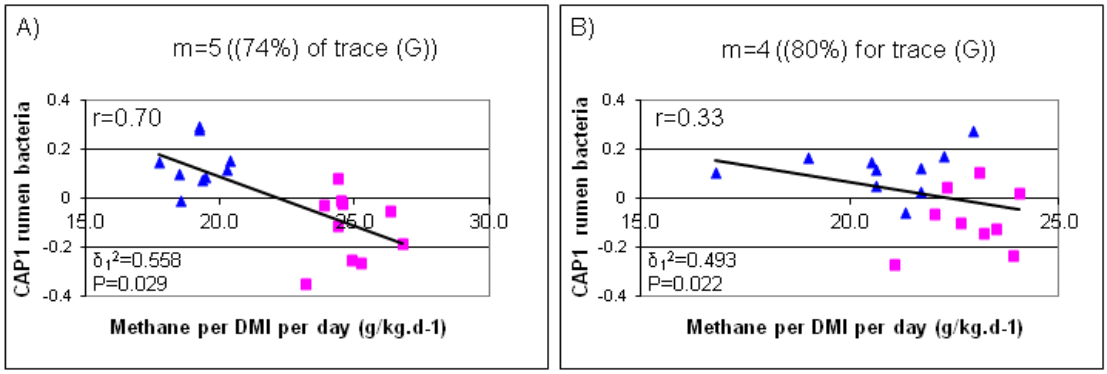


Figure 6-13 CAP of host phenotypic rumen bacterial communities related to methane production per DMI per day. A) CAP-versus-methane biplot for sheep from initial screening for methane extremes. B) CAP-versus-methane biplot for sheep following re-measurement for methane status. CAP analysis was based on Bray-Curtis similarities calculated from fourth-root transformed species abundances. "m" achieves the maximum proportion of correct allocations (% of trace (G)) of samples to phenotype. (■) high methane sheep. (▲) low methane sheep.

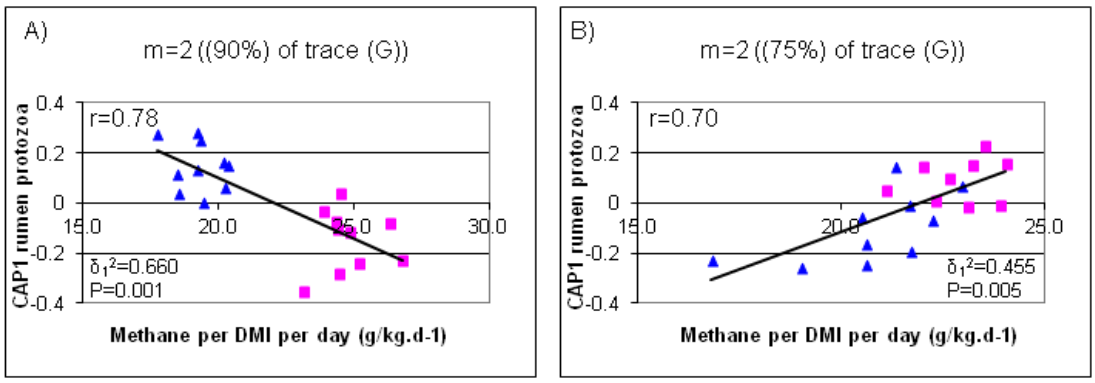


Figure 6-14 CAP of host phenotypic rumen protozoan communities related to methane production per DMI per day. A) CAP-versus-methane biplot for sheep from initial screening for methane extremes. B) CAP-versus-methane biplot for sheep following re-measurement for methane status. CAP analysis was based on Bray-Curtis similarities calculated from fourth-root transformed species abundances. "m" achieves the maximum proportion of correct allocations (% of trace (G)) of samples to phenotype. (■) high methane sheep. (▲) low methane sheep.

6.2.2.4 Discussion

We have shown that the rumen microbiota does change within an animal over time, regardless of the host's underlying genetic background. These changes may be related to numerous factors such as environment, age and subtle changes in diet. Despite this, we have also shown the certain changes in rumen microbiota linked with phenotype (methane production) can be maintained over time. In this study the rumen bacterial and protozoan communities different significantly between the designated high and low methane producing sheep over time (initial screening and reanalysis). These results are promising although more work is required to characterise animals which are truly divergent for methane production at a level that is heritable.

We have shown that rumen bacterial and protozoan communities were correlated with methane production. This was done by using CAP analysis to correlate individual methane variables (g CH_4/kg DMI/day) with patterns in rumen microbial community composition on canonical biplots. As such, this is the first report that directly correlates phenotype-associated changes in rumen microbial community with reductions in enteric methane production. Both the bacteria and protozoan communities are known to be indirectly involved in methane production through hydrogen utilization, hydrogen production or symbiotic relationships with archaea.

6.3 Investigating influence of diet on enteric methane production in sheep

6.3.1 Introduction

A sheep feeding trial was done by Zoey Durmic at the University of Western Australia in 2009. This experiment was part of a Sheep CRC experiment investigating the effect of different diets on methane outputs in individual sheep. Rumen samples had been collected from these sheep corresponding to respiration chamber measurements in methane production at two time intervals. The aim of our work was to investigate the influence of diet type and duration on particular diet on rumen microbial communities and investigate linkage with methane production.

6.3.2 Methodology

The experiment included 48 sheep which were placed on one of four dietary treatments (n=12/treatment):

- DP - Milne deluxe pellet
- SP - Milne standard pellet
- FM - loose farmer mix
- CL - chaff/lupin CSIRO animal house ration

Methane measurements (chamber and Tedlar bags) and rumen samples were collected from these animals after they had been on their allocated diets for two (phase I) and eight (phase II) weeks. Rumen microbial communities (bacterial, archaeal, methanogenic archaeal, fungal and protozoan) were investigated from phase I and phase II using assays outlined in Table 6.4. Data generated by T-RFLP was validated (section 5.2.3) and multivariate statistical tests (section 5.2.4) were used to investigate changes in microbial community structure linked to diet and duration of feeding. Where significant differences were detected in microbial communities, unconstrained ordinations (nMDS) were done to graphically illustrate relationships among treatments.

6.3.3 Results

Both diet type and duration on allocated feed (phase) significantly influenced each of the five microbial communities investigated (Table 6.12). The graphical representation of significant differences associated with dietary phase and dietary treatment in the methanogenic archaeal communities are shown in Figure 6.15.

6-12 Rumen microbial profiling of sheep fed four diets for two and eight weeks (phase I and II) respectively.

Micro-organism	Two-way crossed ANOSIM (Diet)	Two-way crossed ANOSIM (Phase)
Bacteria	R=0.240, P=0.001	R=0.376, P=0.001
Archaea	R=0.361, P=0.001	R=0.354, P=0.001
Methanogens	R=0.156, P=0.001	R=0.476, P=0.001
Fungi	R=0.444, P=0.001	R=0.178, P=0.001
Protozoa	R=0.296, P=0.001	R=0.213, P=0.001

P < 0.05 is significant.

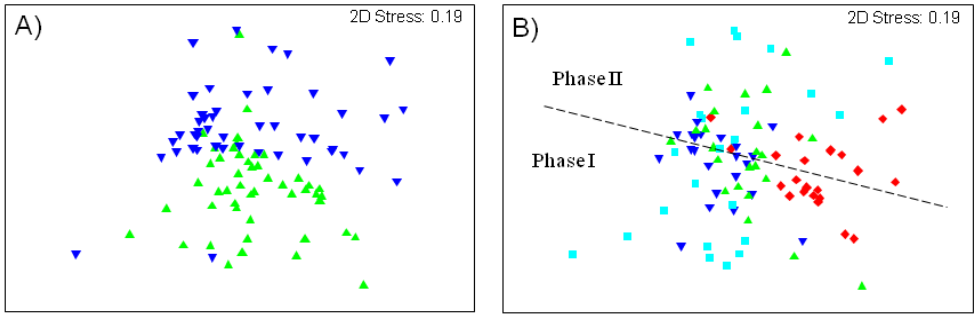


Figure 6-15 nMDS ordination of rumen methanogenic archaeal communities from sheep in the Sheep CRC feeding trial. A) nMDS ordination of methanogenic communities identified by feeding phase. (▲) phase I and (▼) phase II. B) Same ordination as in A), however, rumen methanogens are identified by diet: (▲) DP, (▼) SP, (■) FM and (♦) CL. Each point in the ordination shows the overall methanognic profile of an individual animal. The closer two points are in the ordination the more similar are their profiles.

Influence of diet was further investigated for each of the feeding phases (Table 6.13). In summary, for the bacterial and fungal communities there were significant differences between each of the dietary treatments investigated during both phases. The archaeal communities differed significantly between all diets with the exception of between DP and SP (phase I) and DP and FM (phase II). The methanogenic archaea were significantly different between SP versus FM, SP versus CL and FM versus CL diets in both phases. In addition, during the phase II period methanogenic archaea differed significantly between the DP and CL diets. The protozoan communities significantly differed between all dietary treatments with the exception of between DP and CL during phase I. During phase II protozoan communities differed significantly for sheep on the DP versus FM, SP versus FM and FM versus CL diets.

Table 6-13 One-way ANOSIM of rumen microbial communities associated with diet for each of the five microbial assays investigated at phase I and phase II. The R-statistic (above the diagonal) and significance level (below the diagonal; italics) are shown between pair wise comparisons. Significance levels shown in bold were considered significant (P<0.05).

	Phase I				Phase II			
Assay	Bacteria (Global R=0.309, P=0.001)				Bacteria (Global R=0.170, P=0.001			
Diet	DP	SP	FM	CL	DP	SP	FM	CL
DP		0.174	0.357	0.399		0.172	0.201	0.100
SP	***0.008***		0.166	0.353	***0.002***		0.084	0.222
FM	***0.001***	***0.006***		0.530	***0.001***	***0.032***		0.286
CL	***0.001***	***0.001***	***0.001***		***0.009***	***0.001***	***0.001***	
Assay	Archaea (Global R=0.360, P=0.001)				Archaea (Global R=0.363, P=0.001)			
Diet	DP	SP	FM	CL	DP	SP	FM	CL
DP		0.075	0.226	0.437		0.137	0.095	0.647
SP	*0.066*		0.165	0.611	***0.045***		0.164	0.787
FM	***0.001***	***0.004***		0.661	*0.052*	***0.005***		0.411
CL	***0.001***	***0.001***	***0.001***		***0.001***	***0.001***	***0.001***	
Assay	Methanogen (Global R=0.177, P=0.003)				Methanogen (Global R=0.165, P=0.005)			
Diet	DP	SP	FM	CL	DP	SP	FM	CL
DP		0.06	0.121	0.107		0.113	-0.007	0.151
SP	*0.142*		0.349	0.137	*0.058*		0.221	0.320
FM	*0.053*	***0.003***		0.311	*0.417*	***0.016***		0.178
CL	*0.074*	***0.014***	***0.005***		***0.049***	***0.002***	***0.020***	
Assay	Fungi (Global R=0.459, P=0.001)				Fungi (Global R=0.488, P=0.001)			
Diet	DP	SP	FM	CL	DP	SP	FM	CL
DP		0.203	0.211	0.468		0.154	0.590	0.330
SP	***0.022***		0.150	0.798	***0.015***		0.315	0.644
FM	***0.018***	***0.044***		0.698	***0.001***	***0.001***		0.892
CL	***0.001***	***0.001***	***0.001***		***0.001***	***0.001***	***0.001***	
Assay	Protozoa (Global R=0.199, P=0.001)				Protozoa (Global R=0.388, P=0.001)			
Diet	DP	SP	FM	CL	DP	SP	FM	CL
DP		0.281	0.242	-0.056		-0.016	0.752	-0.027
SP	***0.001***		0.366	0.164	*0.544*		0.557	0.027
FM	***0.002***	***0.003***		0.240	***0.001***	***0.001***		0.736
CL	*0.942*	***0.027***	***0.002***		*0.609*	*0.248*	***0.001***	

Preliminary data for phase I methane output from these sheep is available (Zoey Durmic, personal communication). This data is based on Tedlar bags and indicates that sheep on the SP and FM diets produced significantly less methane than sheep on the DP and CL diets. This data is summarised against the differences observed in microbial community composition in these sheep during phase I (Table 6.14). The VFA profiles also varied significantly among dietary treatments in phase I (Zoey Durmic, personal communication). Acetate concentrations were significantly

higher in sheep fed the DP diet than the SP diet and the acetate:propionate ratio was significantly lower in sheep of the DP diet than either SP or FM diets (Zoey Durmic, personal communication).

Table 6-14 Summary of significant differences observed among dietary treatments in microbial communities and methane production (phase I).

	Diet*			
Profiling assay	DP	SP	FM	CL
Bacteria	a	b	c	d
Archaea	a	a	b	c
Methanogens	abc	a	b	c
Fungi	a	b	c	d
Protozoa	a	b	c	a
Methane† (ppm)	91.9 [a]	62.5 [b]	51.9 [b]	87.3 [a]

* DP (Milne deluxe pellet), SP (Milne standard pellet), FM (loose farmer mix), CL (chaff/lupin CSIRO animal house ration).

† Methane measured over a 24hr period using Tedlar bags. Methane data was supplied by Zoey Durmic. Dietary treatments with a common letter within rows did not differ significantly (P>0.05).

6.3.4 Discussion

We have shown that both diet type and duration maintained on a particular diet both influenced overall rumen microbial composition in these sheep. The microbial differences observed may be responsible for the methane differences indicated in these sheep during phase I feeding based on Tedlar bag data. However, it will be possible to investigate these relationships in more detail for both phase I and phase II feeding once individual respiration chamber data from these animals is fully analysed and available. The differences observed in acetate and acetate:propionate ratio (phase I) would support preliminary differences observed in methane production between deluxe pellet and standard pellet fed sheep. The production of both acetate and propionate can influence methane production as acetate production generates hydrogen while propionate generation utilizes hydrogen, hence both have the potential to increase and decrease methane production, respectively. As VFA production is a product of bacterial fermentation these results would also support our findings that the bacterial populations were significantly different between sheep on the deluxe pellet and standard pellet diets (phase I).

6.4 Influence of diet and genotype on methane emissions from beef cattle.

6.4.1 Introduction

A trial investigating the influence of residual feed intake (RFI) in beef cattle on methane emissions was done in 2009. This trial was done by Fiona Jones of the Department of Agriculture and Food (DAF), Bunbury, Western Australia and jointly funded by DAF and the Cattle Industry Compensation Act. The cows used in this study were part of the Beef CRC Maternal Productivity Project. RFI in beef cattle was shown to be linked with reductions in methane emissions when animals are grazed on high quality pasture but not when grazed on a poor quality pasture (Jones et al., 2011). Full details of this experiment are outlined in Jones et al. (2011). High RFI and low RFI animals are low and high feed efficiency animals, respectively.

6.4.2 Methodology

Rumen samples were investigated from 47 Angus heifers shown to be divergent for RFI: 22 low RFI and 25 high RFI cows. All animals were part of a previously described feeding trial investigating methane emissions in the field using the open path Fourier Transform infrared spectrophotometer (OP-FTIR) technique (Jones et al., 2011). Rumen samples from each animal were collected twice, while animals were either receiving a high quality winter pasture (810 g/kg DMD) or low quality summer pasture (550g/kg DMD) (Jones et al., 2011). Faecal samples from these animals were not available. Rumen microbial communities (bacterial, archaeal, methanogenic archaeal, fungal and protozoan) were investigated from all cows on both diets using assays outlined in Table 6.4. Data generated by T-RFLP was validated (section 5.2.3) and multivariate statistical tests (section 5.2.4) were used to investigate changes in microbial community structure linked to diet and RFI pheneotype. Where significant differences were detected in microbial communities unconstrained ordinations (nMDS) were done to graphically illustrate relationships among treatments and SIMPER analysis was done to identify OTU (microbial groups) driving these differences.

6.4.3 Results

Table 6-15 Rumen microbial differences between high and low RFI cows when fed a high and low quality pasture.

Micro-organism	ANOSIM (Low quality pasture)	ANOSIM (High quality pasture)
Bacteria	R=0.160, P=0.001	R=0.065, P=0.034
Archaea	R=0.058, P=0.053	R=0.208, P=0.001
Methanogens	R=0.053, P=0.060	R=0.105, P=0.009
Fungi	R=0.190, P=0.001	R=0.313, P=0.001
Protozoa	R=0.034, P=0.124	R=-0.029, P=0.871

P < 0.05 is significant.

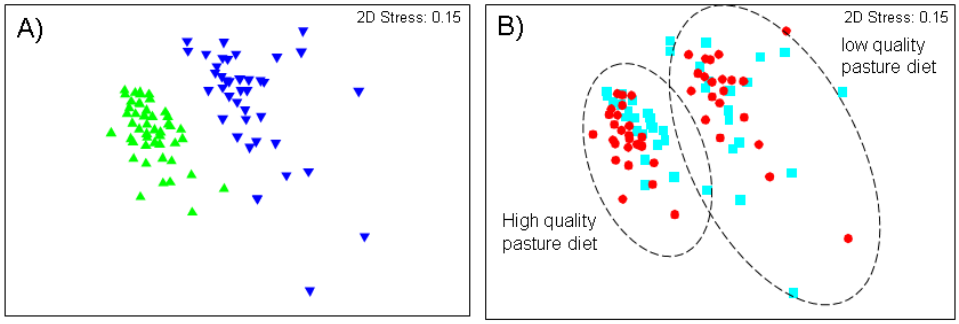


Figure 6-16 nMDS ordination of rumen archaeal communities from divergent RFI cow lines fed high and low quality pasture. A) nMDS ordination of archaeal communities identified by high (▲) and low (▼) quality pasture. S) Same ordination as in A), however, rumen archaeal communities are identified by high (●) and low (■) RFI lines.

Microbial profiling of rumen samples showed that diet significantly altered all microbial communities investigated in this study, regardless of RFI grouping: bacteria (Global R=0.68, P=0.001); archaea (Global R=0.716, P=0.001); methanogenic archaea (Global R=0.741, P=0.001); fungi (Global R=0.862, P=0.001) and protozoa (Global R=0.323, P=0.001). RFI phenotype also significantly (P<0.05) influenced bacterial and fungal communities, irrespective of dietary treatment (Table 6.15). Furthermore, archaeal and methanogenic communities only differed significantly between high and low RFI cows when they were fed the high quality pasture. The strong diet associated differences in rumen archaeal communities is graphically

shown in Figure 6.16a, while Figure 6.16b shows the diet dependant RFI associated difference in rumen archaeal communities. No significant influence of RFI line on rumen protozoan community structure was detected (Table 6.15).

Where significant differences between RFI phenotype were detected on a particular diet, SIMPER analysis was used to identify OTU strongly associated with phenotype (Table 6.16). For example, OTU 134 (archaea) was found to be more abundant in the rumen of high RFI (low feed efficiency) cows while OTU 102 (archaea) was more abundant in the rumen of low RFI (high feed efficiency) cows. This is graphically demonstrated in Figure 6.17.

Table 6-16 OTU contributing significantly to differences in microbial communities between high and low RFI cows.

Microbial community	Pasture quality	OTU with a strong phenotype association	
		High RFI (low efficiency)	Low RFI (high efficiency)
Archaeal	high	134, 292 & 788	72, 82 & 102
Methanogen	high	148 & 460	466
Bacterial	high	128, 222 & 166	134 & 232
	low	132, 148, 166 & 544	118, 134 & 542
Fungal	high	68 & 302	194, 222, 294, 392, 412 & 416
	low	116	88, 158, 162, 248, 252, 262, 266, 292 & 294

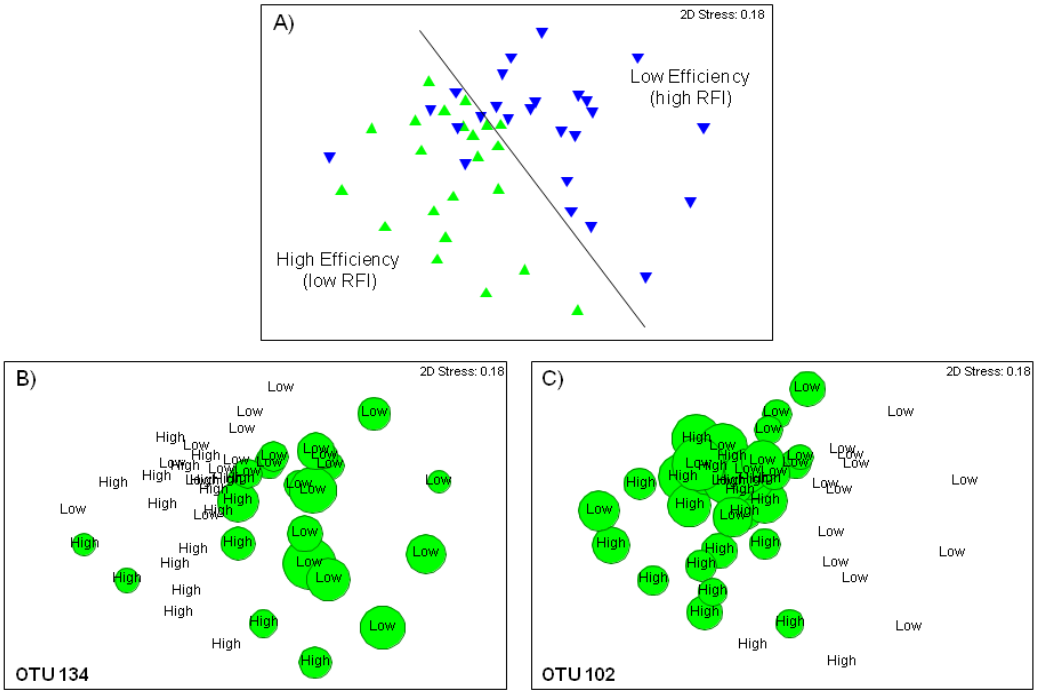


Figure 6-17 Identifying OTU driving differences in rumen archaeal profiles between high and low feed efficiency cows on high quality pasture. A) nMDS ordination of rumen archaeal communities from high and low RFI cows. B) same ordination as in A), however association of OTU 134 with cow phenotype is indicated. (Low)=high RFI and (High)=low RFI. C) Same ordination as in A), however association of OTU 102 with cow phenotype is indicated. (Low)=high RFI and (High)=low RFI. Area of bubble is proportional to quantity of OTU identified.

6.4.4 Discussion

Jones et al. (2011) showed that low and high RFI cows differed significantly ($P<0.05$) in methane production (0.34 ± 1.017 g CH_4/kg LW and 0.46 ± 0.023 g CH_4/kg LW respectively) when fed a high quality pasture but not when fed a low quality pasture (0.26 ± 0.013 g CH_4/kg LW and 0.26 ± 0.018 g CH_4/kg LW respectively). We have currently shown that rumen microbial communities from these animals were both influenced by diet and RFI genetic background. However, diet was shown to have a greater influence in rumen microbiota. Despite this, it was observed that when cows were fed a high quality pasture, specific shifts in archaeal and methanogenic archaeal communities were detected between high and low RFI cows. Changes in these particular microbial communities (known to be directly involved in methane production) were not apparent when the same animals were fed the low quality pasture. Together these data are supportive of the significant methane differences observed between high and low RFI cows when fed high quality pasture, but not when fed the low quality pasture.

Interestingly, cows on the low quality pasture generally produced less methane than when they were on the high quality pasture. The gross differences we observed in microbial community composition relating to diet may be linked with this observation. Although diet was a stronger driver of rumen microbial community composition, the animal's genetic RFI background seemed to have a consistent influence on the rumen bacterial and fungal communities, regardless of diet. As methane measurement from this trial was not based on individual animals, we were not able to investigate the relationships between individual cow microbial community structure and methane output. However, we were able to identify OTU (microbial groups) which significantly varied in abundance between the high and low RFI animals, and which were linked with group methane difference. Further work will allow these organisms to be identified. This could lead to the development of specific, quantitative and inexpensive diagnostic tests for animal phenotype or methane potential.

7 Conclusion

In this project we have refined a uniform extraction methodology for use in rumen and faecal samples to successfully monitor a variety of microbial communities (bacteria, archaea, fungi and protozoa). We have also developed high-throughput, high resolution, culture-independent microbial profiling assays for investigating these microbial communities. Furthermore, we have collaborated with numerous projects within RERLP, using DNA profiling to investigate dietary and genetic methane mitigation strategies in sheep, dairy cows and beef cattle. In cases where mitigation strategies were successful we have identified correlations between the rumen microbial communities and methane emissions.

We have refined nucleic extraction methodology (based on a SARDI proprietary method previously modified to investigate gut bacterial communities in poultry) for use in investigating rumen and faecal microbiota in ruminant livestock. Despite concerns that a single extraction methodology would not efficiently isolate nucleic acid from all microbial communities of interest (bacteria, archaea, fungi and protozoa) we have shown that a single methodology is appropriate for all these organisms. Nucleic acid sequencing of microorganism group specific PCR amplicons has confirmed the presence of all these organisms within a single nucleic acid extract. The SARDI extraction methodology has two definite benefits over other techniques in that it is truly high-throughput (100-200 samples can be extracted per day) and that the extraction method is not restricted to a particular phase of the sample (i.e. filtered rumen fluid or solid material), hence capturing a more representative proportion of the microbial community composition. Both the SARDI and QIAmp® DNA stool kit extraction methods were shown to generate more complex profiles for the rumen bacterial, archaeal, methanogenic archaeal and protozoan communities than the RBB+C method, although all three methods produced comparable profiles. The three extraction methodologies did, however, significantly differ in their rumen fungal community representation. The RBB+C and QIAmp® DNA stool kit produced fungal profiles which were very different to each other with the SARDI extract generating a common profile representative of the other two methods. These same comparisons could not be made based on the faecal microbial communities as in our hands both the RBB+C and QIAmp® DNA stool kit produced extracts which contained inhibitors to PCR. A sample collection protocol for rumen and faecal material (Appendix 9.3) for SARDI microbial profiling has been developed and provided to collaborators.

Profiling assays were developed and evaluated for archaeal, methganogenic archaea, fungi and protozoa. These assays were shown to be specific to their target group and optimized to detect maximal variation within a community. High inter animal variation was demonstrated in rumen and faecal microbial communities even when animals were on the same dietary treatment, although differences associated with treatments were greater. We have previously reported high inter-animal variation in poultry gut microbiota and have successfully used multivariate statistical methods for analysing samples associated with treatments (Torok et al., 2008). Many T-RFLP studies have used cluster analysis to depict grouping of related samples (Dunbar et al., 2000; Gomez et al., 2004; Kuske et al., 2002; Lan et al., 2004; Moeseneder et al., 1999; Perez-Jimenez and Kerkhof, 2005). However, a disadvantage of this method is that it groups samples into discrete clusters, and does not display their inter-relations on a continual scale (Clarke and Warwick, 2001). Other studies have used principal component analysis (PCA) to examine community structure resulting from T-RFLP data (Kuske et al., 2002; Park et al., 2006; Wang et al., 2004). However, PCA

analysis is not appropriate where data contain many “zeros” or where observations (species) exceed total number of samples (Clarke and Warwick, 2001), as is usually the case for T-RFLP data. We have currently shown that T-RFLP in conjunction with several multivariate statistical techniques, such as, unconstrained (nMDS) and constrained (CAP) ordinations, statistical tests of the hypothesis (ANOSIM) and characterisation of species responsible for the pattern differences (SIMPER), are all useful tools for investigating the composition of the rumen/faecal microbial communities.

The developed microbial profiling assays were used to investigated rumen and faecal microbiota from collaborative experiments analysing dietary and genetic methane mitigation strategies in dairy cow, beef cattle and sheep. All trials measured methane from animals using varying technologies (SF_6, respiration chamber or OP-FTIR). In total, eight trials were investigated and of these three demonstrated significant methane reductions for which relationship of rumen and faecal microbiota could be investigated in more detail. Of the four dairy cow experiments (B.CCH.1009) two of the dietary experiments (DHA and grape marc trials) showed significant changes in rumen and faecal microbiota associated with diet. However, only the grape marc trial also showed significant reductions in enteric methane associated with dietry treatment. Constrained CAP was used to correlate individual methane data (g CH_4/kg DMI) with patterns in rumen bacterial and fungal community composition on canonical biplots. Of the two breeding mitigation strategy trials investigated in sheep (B.CCH.1015), we were able to demonstrate significant differences in rumen bacterial and protozoan communities in sheep divergent in methane production from the UNE trial. CAP was used to correlate individual methane data (g CH_4/kg DMI) with patterns in rumen bacterial and protozoan community composition. Finally, investigation of microbial communities from the DAF beef cattle trial showed that both feed efficiency and diet significantly influenced rumen microbial communities. We demonstrated that high and low RFI cows varied in their archaeal and methanogenic archaeal communities only when fed high quality pasture, supporting the methane difference reported by Jones et al. (2011). Furthermore, we were able to identify OTU (microbial groups) closely associated with RFI phenotypes which may be responsible for the methane difference observed.

Diet associated changes in rumen microbiota were detected in four of the eight trials investigated. In some of these trials (DHA, grape marc and Sheep CRC feeding trial) differences in milk fat and/or rumen VFA were also detected. Such changes would be consistent with differences we observed in rumen microbial communities. Bacterial communities are known to be involved in biohydrogenation, hence may influence milk fat, and VFA production as a by product of metabolism. Furthermore, we have demonstrated that not only does diet influence rumen microbiota composition, but that duration on diet (Sheep CRC feeding trial) or other environmental/temporal factors (UNE sheep genetics trial) also influence rumen microbiota indicating that the rumen microbiota is a continually changing community.

One of the objectives of this project was to investigate the potential of faecal microbial profiling as a surrogate for rumen function. In three trials we showed that rumen and faecal microbiota were significantly different, regardless of treatment. These differences were a result of both differences in abundance of common OTU and differences in unique gut specific OTU. Diet was found to influence both rumen and faecal microbiota, but often not in the same way i.e. significant differences identified in one gut compartment were often not reflected in the other. For the limited trials demonstrating significant differences in methane production and availability of faecal samples for analysis, the significant diet related differences in rumen microbiota were not detected within the faecal samples. Hence, faecal microbial

profiling does not appear to be an appropriate surrogate assay for rumen function. This is not to say that faeces may not act as a surrogate based on other analytical methodologies. For example, if specific microbial organisms shown to be consistently linked with methane production could be identified within the rumen then specific and quantitative assays could be developed for detecting them as markers within the faeces.

In conclusion, microbial profiling used in collaborative animal trials investigating dietary and genetic methane mitigation strategies have shown changes in rumen microbiota to be correlated with reduction in enteric methane. However, further work is required to elucidate the organisms involved and to validate findings across a number of trials. Furthermore, diet and environmental factors were shown to have a much stronger influence on rumen microbiota than animal genetics. Where animal genetics was shown to influence changes in rumen microbiota and be linked to methane emission these were dependent on diet, indicating a strong host by environment interaction. Rumen microbiota was also shown to influence animal performance as determined by changes in milk fat production and feed efficiency.

8 Recommendations

- It is important to monitor rumen microbiota as part of research on genetic and dietary influence on methane emission as this underpins our ability to understand changes in enteric methane production.
- Further work needs to be done to investigate rumen microbiota in animal trials demonstrating successful and significant methane mitigation strategies
- In trials where rumen microbiota is linked with significantly differences in methane production, the organisms driving these differences should be identified via further sequencing
- Specific and quantitative diagnostic assays should be developed to microoganisms linked with significant reductions in enteric methane production.
- Together, these technologies (profiling and quantitative diagnostics) will provide information to producers wishing to monitor effectiveness of on-farm methane mitigation strategies and enable progress to be made towards the Australian government's goal of reducing greenhouse emissions from agriculture.

9 Appendices

9.1 Primers identified from microbial profiling studies on rumen, faecal and fermenter culture samples.

Profiling method	Organism targeted*	Primer pair†	Reference
DGGE	Bac Fibrobacter	357f-519r Fib400f-Fib713r	(Larue et al., 2005)
ARISA	Bac	ITS1F-ITSReub	(Welkie et al., 2010)
DGGE	Bac/Ar	F968-R1401, 357F-518R	(Huws et al., 2007)
DGGE DGGE	Bac Fu	F968GC-R1401 MN100-MNGM2C	(Huws et al., 2011) (Khejornasrt and Wanapat, 2011)
DGGE	Ar	m915a-1386r	(Knight et al., 2011)
DGGE	Ar Meth Bac	341F-534r mcrAf-mcrAr 520f-799R2	(Popova et al., 2011)
DGGE	Ar	Arc344-519r	(Mohammed et al., 2011)
DGGE	Bac	New ipoDGGE(F)-New ipoDGGE(R)	(Perumbakkam and Craig, 2011)
DGGE	Treponema	gTrepoF-BAC926R	(Bekele et al., 2011)
DGGE	Prot	Reg1062F-Reg1302R, RP841F-RP1416R, RP841F-Reg1302R	(Kittelman and Janssen, 2011)
DGGE	Ar	Arc344f-519r	(Kongmun et al., 2011)
DGGE	Bac Prot	342f-534r 1055F-1400R	(Belanche et al., 2010)
DGGE	Ar	Arc344f-Arc519r, uniMet1F-uniMet1R	(Zhou et al., 2010)
DGGE	Bac Prot	357f-519r PSSU316f-PSSU539r PSSU1320f-PSSU1617r	(Karnati et al., 2007)
DGGE	Pro	316f-EUK516r	(Boeckaert et al., 2007)
DGGE	Bac	F968-R1401, 799F2-R1401	(Edwards et al., 2007)
DGGE	Bac	U968-L1401	(Mao et al., 2008)
DGGE	Ar	A2Fa-A348r, A24f-A329r, A24f-A348r, Arc344f-519r, A357f-A693r, Arc344f-Arc915r, Arch915-Uni-b-rev, A1040f-UA1204r	(Yu et al., 2008)
DGGE	Bact	HDA1-HDA2	(Guan et al., 2008)
DGGE	Prot	PSSU316f-PSSU539r PSSU1320f-PSSU1617r	(Sylvester et al., 2005)
DGGE	Prot	PSSU316f-PSSU539r	(Regensbogenova et al., 2004)
DGGE	Ar	Arch344F-Univ522R	(Ouwerkerk et al., 2008)
DGGE	Bact	357f-519r	(Kim et al., 2011)
Profiling method	**Organism targeted***	**Primer pair†**	**Restriction enzymes**	**Reference**
T-RFLP	Bac	27F‡-342r	*Hha*I	(Khafipour et al., 2009)
T-RFLP	Bac	8F‡-805R	*Msp*I	(Fernando et al., 2010)
T-RFLP	Bac Butyrivibo	27f‡-1389r B395f‡-B812r	*Hha*I, *Msp*I *Hha*I	(Belenguer et al., 2010a)
T-RFLP	Bac Meth	515f‡-1391R Met86F‡-Met1340R	*Hae*III, *Mse*I *Aci*I, *Alu*I, *Hha*I, *Mse*I	(Frey et al., 2010)
T-RFLP	Bac Meth	27F‡-1389R MLF‡-MLR	*Hae*III, *Hha*I, *Msp*I *Taq*I	(Yanez-Ruiz et al., 2010)
T-RFLP	Bac	27F‡-1492r	*Hha*I, *Msp*I	(Ozutsumi et al., 2008)
T-RFLP	Bac	8F‡-1492R	*Rsa*I, *Msp*I, *Hha*I	(Johnson et al., 2009)
T-RFLP	Bac	46F‡-1080R	*Hae*III, *Hha*I, *Msp*I	(Ridwan et al., 2009)
T-RFLP	Bac	27F‡-907r	*Hha*I, *Msp*I, *Afa*I	(Miyagawa et al., 2007)
T-RFLP	Bac	27F‡-1389r	*Hha*I, *Hae*III, *Msp*I	(Belenguer et al., 2010b)
T-RFLP	Bac	27F‡-1389r	*Hha*I	(Lopez-Campos et al., 2010)
T-RFLP	Bac	27F‡-1100R	*Hha*I	(Romero-Perez et al., 2011)
T-RFLP	Bac	27F‡-1389r	*Alu*I, *Hae*III, *Msp*I	(Huws et al., 2007)
T-RFLP	Bac	27F‡-1389r	*Hha*I	(Vasta et al., 2010)
RFLP	Fu	ITS1F-EminITS4	*Dra*I	(Griffith et al., 2009)

* Bac=Bacteria, Meth=Methanogen, Ar=Archaea, Fu=Fungi, Pr=Protozoa

† Notes on primers. 27F and 8F are near identical. 1389r and 1391r cover overlapping regions with1391r containing degenerate bases. MNGM2C, GM2 and ITS2 cover the same region but vary in length. 341f and 344f have a 14bp overlap. 519r and 534r overlap.

‡ Indicates primer which is fluorescently labelled.

9.2 Genome sequence information on organisms detected by PCR primers evaluated for T-RFLP development.

Primer Combination	Organism	Classification
CS322F/EU929R	*Isotricha prostoma*	Protozoa (rumen)
	Eudiplodinium maggii	Protozoa (rumen)
	Entodinium furca	Protozoa (rumen)
P-SSU342-f mod/EU929R	*Isotricha prostoma*	Protozoa (rumen)
	Eudiplodinium maggii	Protozoa (rumen)
	Entodinium furca	Protozoa (rumen)
GM1/ITS4	*Isotricha prostoma*	Protozoa (rumen)
ITS1F/ITS4	*Neocallimastix frontalis*	Fungi (rumen)
	Cyllamyces sp	Fungi (rumen)
	Aspergillus penicillioides	Fungi (plant)
	Sordariomycete sp	Fungi (plant)
	Leptosphaerulina chartarum	Fungi (plant)
	Cryptococcus saitoi	Fungi (plant)
	Alternaria alternata	Fungi (plant)
	Fusarium sp	Fungi (plant)
	Wallemia sebi	Fungi (plant)
SSU-817F/SSU-1536R	*Glomus mosseae*	Fungi (plant)
	Wallemia sebi	Fungi (plant)
	Scopulariopsis brevicaulis	Fungi (plant)
	Edyuillia athecia	Fungi (plant)
	Alternaria cheiranthi	Fungi (plant)
	Plectosphaerella cucumerina	Fungi (plant)
	Aspergillus versicolor	Fungi (plant)
NeocalF/NeocalR	*Neocallimastix frontalis*	Fungi (rumen)
	Cyllamyces aberensis	Fungi (rumen)
NS1/NS2	*Isotricha prostoma*	Protozoa (rumen)
	Eudiplodinium maggii	Protozoa (rumen)
Ar109f/Ar912r	*Methanobrevibacter thaueri*	Methanogen
	Methanobrevibacter ruminantium	Methanogen
	Methanogenic archaeon CH1270	Methanogen
mcrAf /mcrAr	*Methanobrevibacter ruminantium*	Methanogen
	Methanobrevibacter millerae	Methanogen
	Methanobrevibacter gottschalkii	Methanogen
	Methanomicrobiales sp	Methanogen
	Methanosarcina sp	Methanogen
	Methanococcoides sp	Methanogen
	Methanobacterium sp	Methanogen

9.3 Collection of rumen fluid & faecal samples for microbial profiling

Rumen fluid collection

1. Pre-label all vials clearly with permanent marker; label both the tube and lid.
   - **Use 25 mL polypropylene tubes**
     (Sarstedt cat # 60.9922.270PP or 60.9922.271PP; ph (08) 8349 6555)

2. Collect ca. 10 ml of **unfiltered** rumen fluid per animal into 25 mL polypropylene tube. **NB: 10 mL corresponds to a half filled tube.**

- Fill tube half way as indicated in the photo
- **NEVER** fill the tube completely
- Keep samples cold once collected & freeze as soon as possible.

3. Once samples are collected store at -80°C if possible. If no -80°C facilities are available store samples at -20°C. **Do not allow samples to thaw once they are frozen**.

Faecal samples collection

1. Pre-label all vials clearly with permanent marker; label both the tube and lid.
   - **Use 25 mL polypropylene tubes**
     (Sarstedt cat # 60.9922.270PP or 60.9922.271PP; ph (08) 8349 6555)

2. Faecal samples should be taken from the same animals as rumen fluid has been taken.

3. Collect faecal matter from an individual animal into supplied 25 mL polypropylene tube.

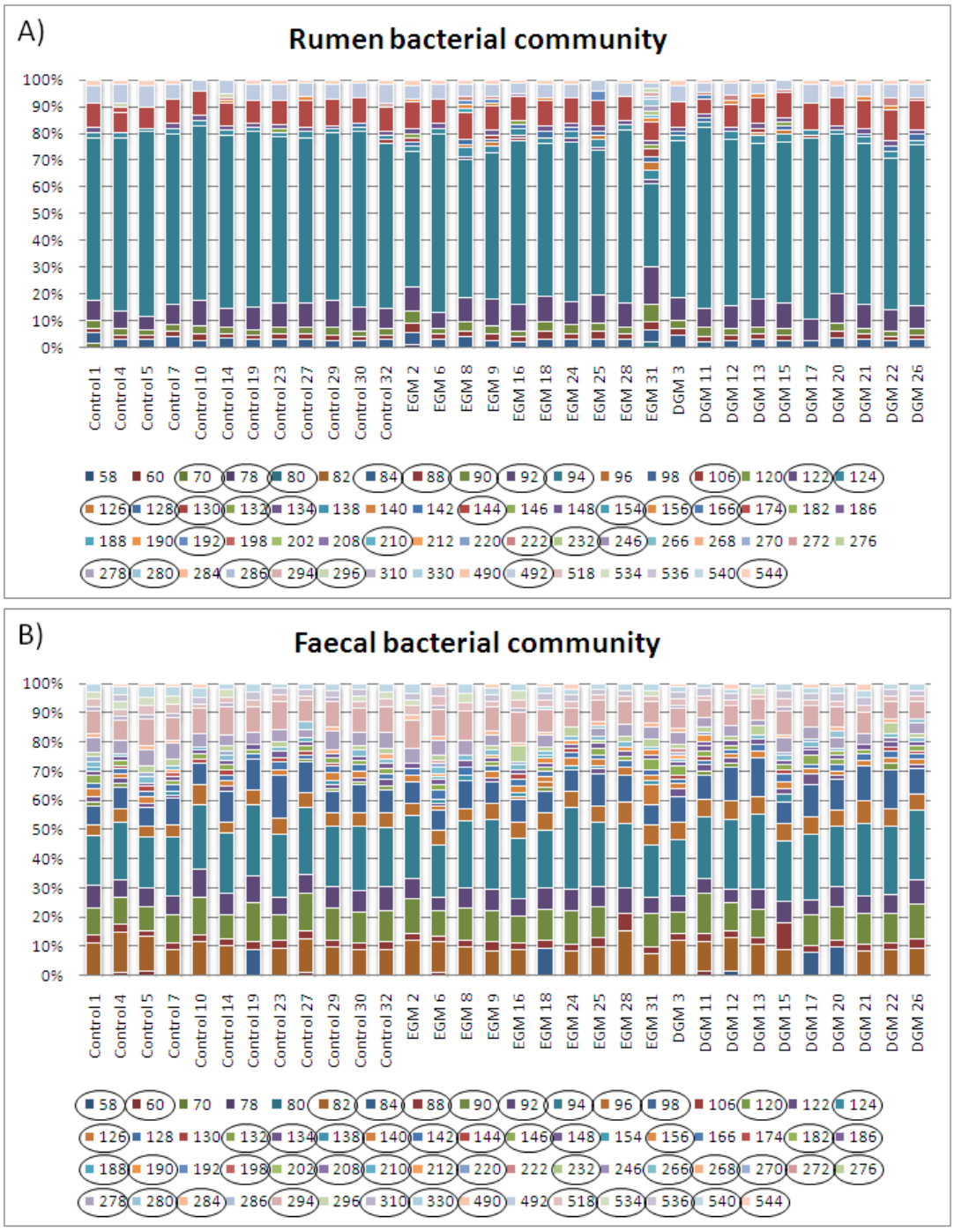
   - Fill tubes ¾ full
   - **NEVER** fill the tube completely
   - Keep samples cold once collected & freeze as soon as possible.

4. Once samples are collected store at -80°C if possible. If no -80°C facilities are available store samples at -20°C. **Do not allow samples to thaw once they are frozen**.

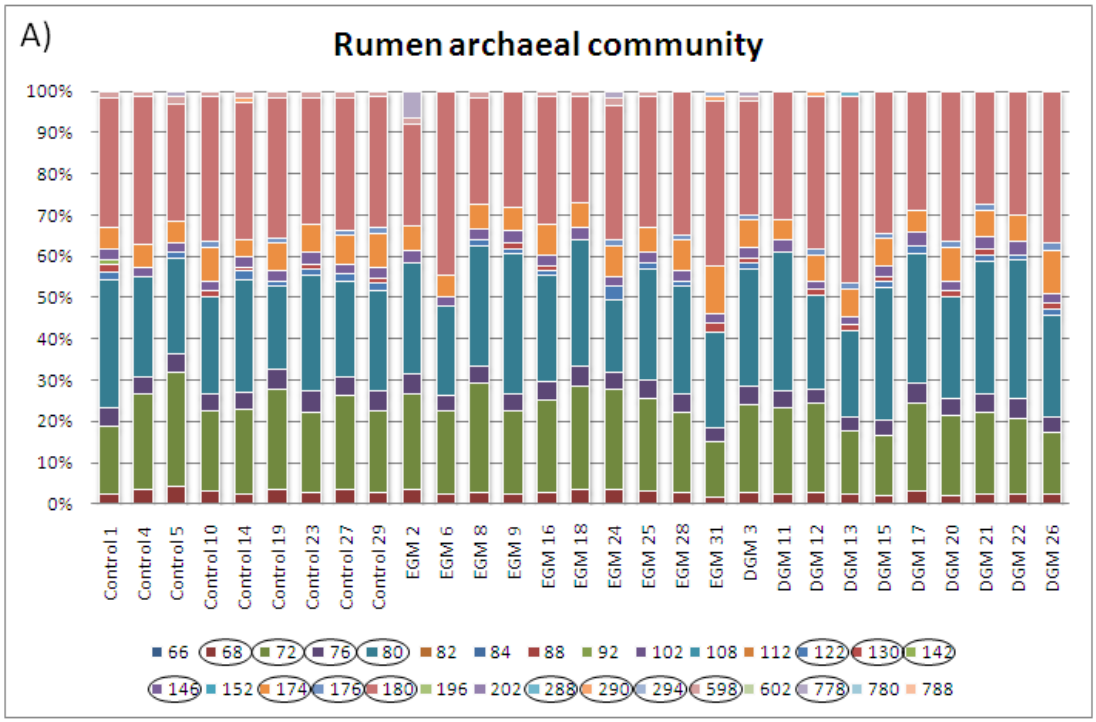
GENERAL INFORMATION

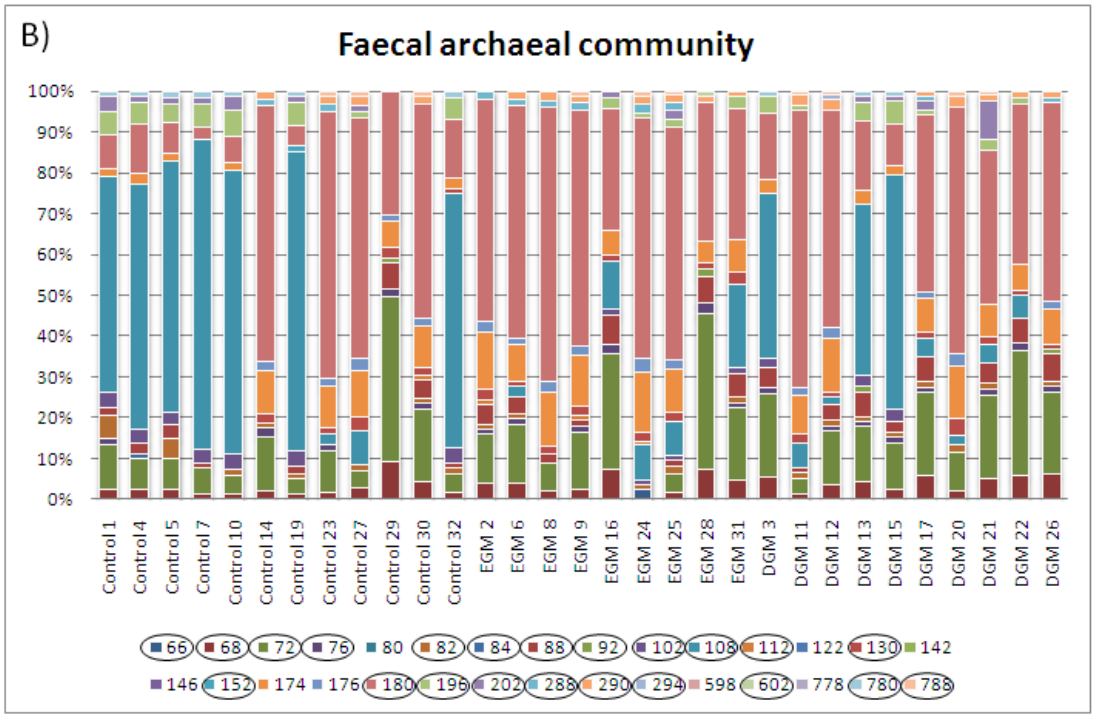
- Valeria Torok (valeria.torok@sa.gov.au) will organise shipping of samples to SARDI. Please contact her to organise this.
- Samples must be accompanied with an **electronic** & **hardcopy** data sheet with the following information: Sample ID, date collected, ruminant species, treatment, sample type (rumen fluid or faecal), fistulated or rumen tube sampling, etc.
- Samples should only be collected from animals for which corresponding methane measurements have been made.

9.4 Rumen and faecal profiling of 32 dairy cows from grape marc experiment (B.CCH.1009)

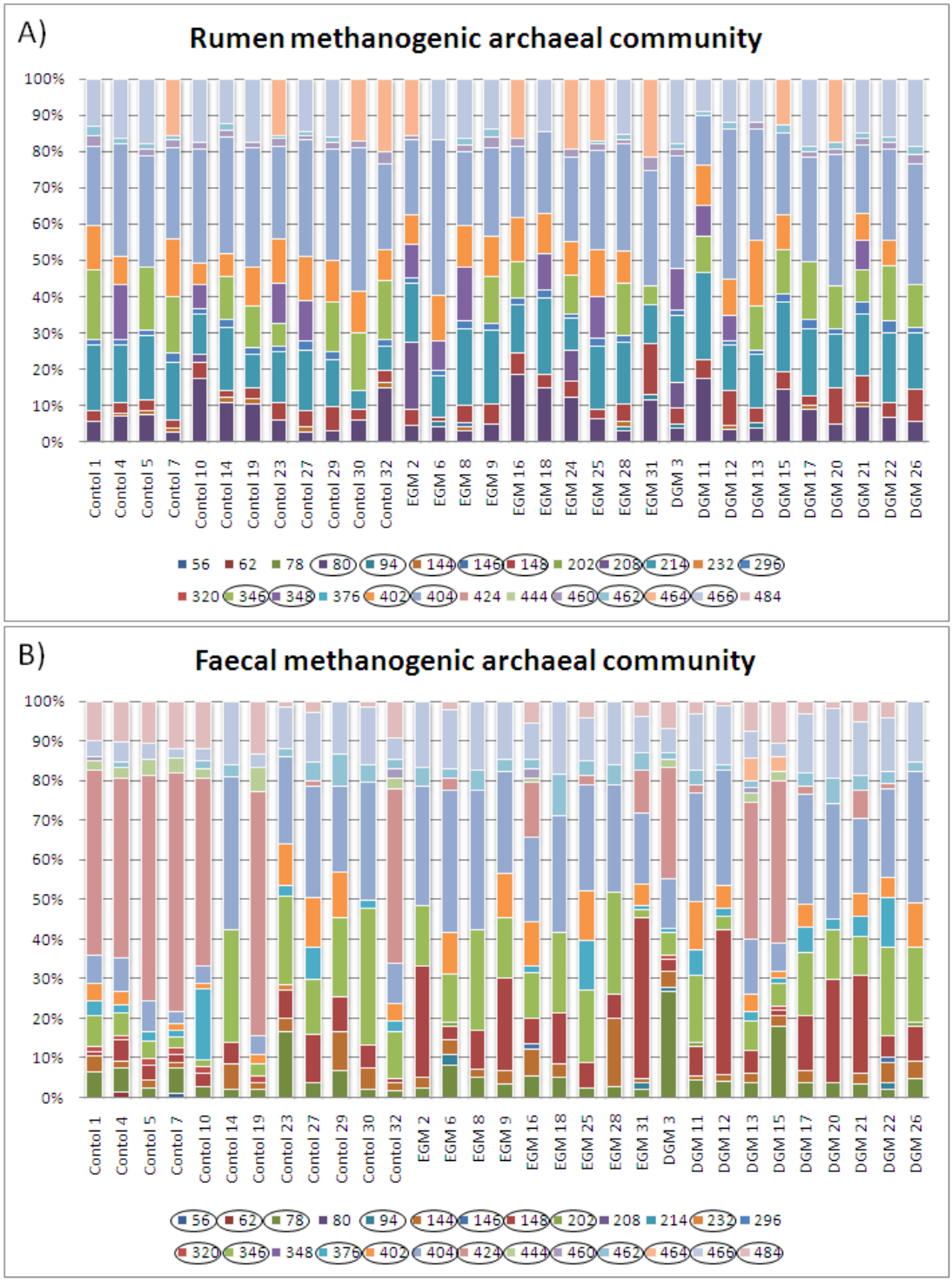


Bacterial profiles from the rumen and faeces of individual dairy cows from the Ellinbank grape marc experiment. n=66 OTU (operational taxonomic units) were detected across both the rumen and faeces. n=33 OTU (A; circled) and 50 OTU (B; circled) were detected within the rumen and faeces respectively. OTU are ordered from largest to smallest (top to bottom) within the bar graphs. Dietary treatment and animal number are indicated below bars. Dietary treatments were control (control), ensiled grape marc (EGM) and dried grape marc (DGM).

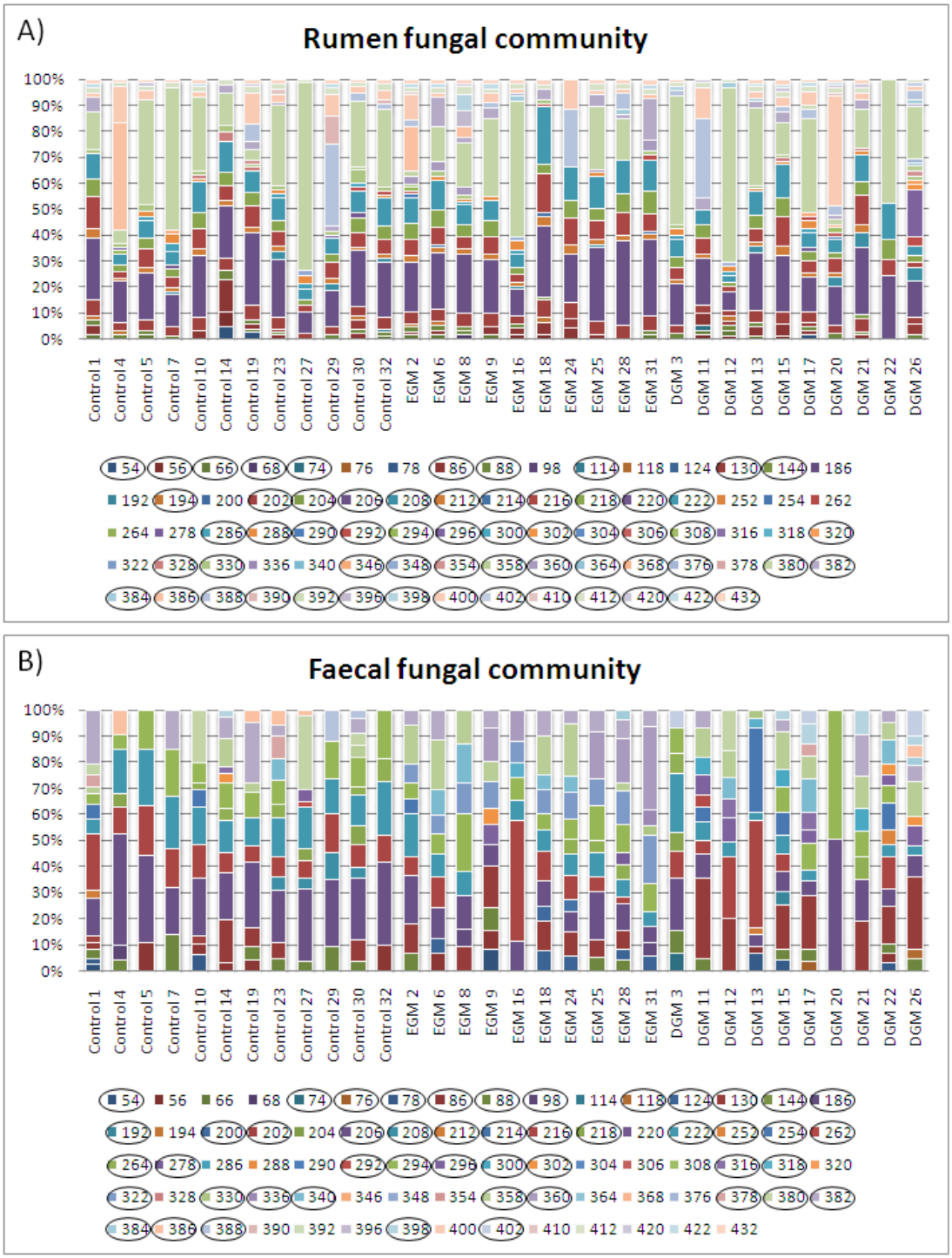




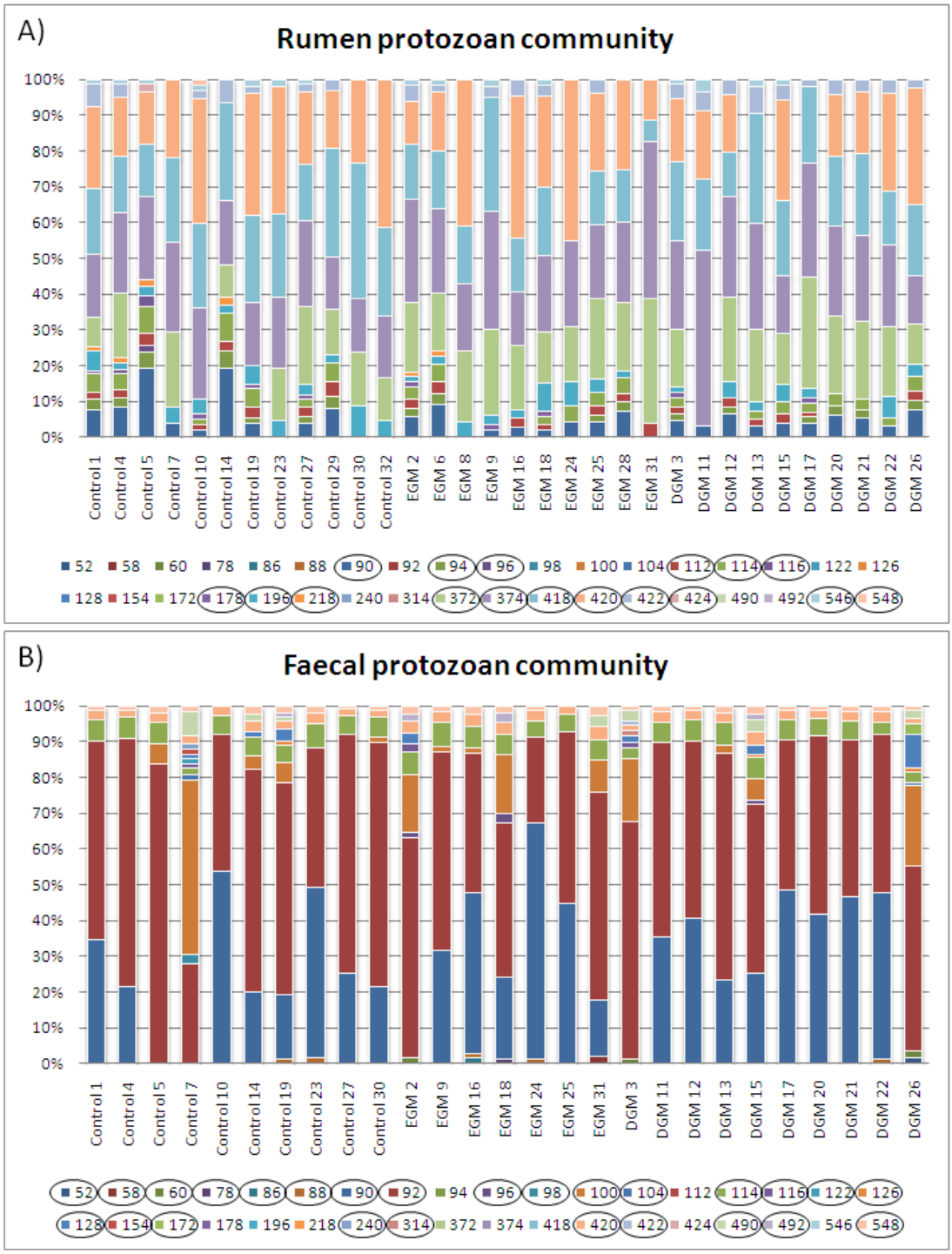
Archaeal profiles from the rumen and faeces of individual dairy cows from the Ellinbank grape marc experiment. n=30 OTU (operational taxonomic units) were detected across both the rumen and faeces. n=16 OTU (A; circled) and 24 OTU (B; circled) were detected within the rumen and faeces respectively. OTU are ordered from largest to smallest (top to bottom) within the bar graphs. Dietary treatment and animal number are indicated below bars. Dietary treatments were control (control), ensiled grape marc (EGM) and dried grape marc (DGM).



Methanogenic archaeal profiles from the rumen and faeces of individual dairy cows from the Ellinbank grape marc experiment. n=26 OTU (operational taxonomic units) were detected across both the rumen and faeces. n=16 OTU (A; circled) and 21 OTU (B; circled) were detected within the rumen and faeces respectively. OTU are ordered from largest to smallest (top to bottom) within the bar graphs. Dietary treatment and animal number are indicated below bars. Dietary treatments were control (control), ensiled grape marc (EGM) and dried grape marc (DGM).



Fungal profiles from the rumen and faeces of individual dairy cows from the Ellinbank grape marc experiment. n=78 OTU (operational taxonomic units) were detected across both the rumen and faeces. n=59 OTU (A; circled) and 48 OTU (B; circled) were detected within the rumen and faeces respectively. OTU are ordered from largest to smallest (top to bottom) within the bar graphs. Dietary treatment and animal number are indicated below bars. Dietary treatments were control (control), ensiled grape marc (EGM) and dried grape marc (DGM).



Protozoan profiles from the rumen and faeces of individual dairy cows from the Ellinbank grape marc experiment. n=36 OTU (operational taxonomic units) were detected across both the rumen and faeces. n=17 OTU (A; circled) and 26 OTU (B; circled) were detected within the rumen and faeces respectively. OTU are ordered from largest to smallest (top to bottom) within the bar graphs. Dietary treatment and animal number are indicated below bars. Dietary treatments were control (control), ensiled grape marc (EGM) and dried grape marc (DGM).

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