final report

Project code: B.CCH.1027

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Date published: April 2012

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

Phylogenetic identification of rumen microbial organisms linked with significant differences in methane emissions

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

1 Abstract

Feed efficiency in cattle has been linked to degree of enteric methane production. Cows which are more efficient in utilizing feed produce less methane, although this response is diet related. The micro-organisms present within the rumen are responsible for the digestion of feed and production of methane. These micro-organisms have been shown to be influenced by both the animal's genotype and diet, with the latter having a greater effect. The aim of this study was to characterise the rumen bacterial, archaeal and fungal communities by deep sequencing technology and to identify micro-organisms linked with high feed efficiency cows producing less enteric methane. We found that cows producing less enteric methane contained a more diverse bacterial community at the level of phyla. The rumen methanogenic archaeal communities from high feed efficient cows were more abundant in methanogenic archaeon related species and less abundant in *Methanosphaera stadtmanae*. Rumen fungal communities also differed significantly between high and low feed efficient cows. Although, many organisms identified have not yet been grown in the laboratory, the genome sequences generated will enable the development of diagnostic tools to investigate relationships of rumen micro-organisms with methane production, which will be of use in evaluating methane mitigation methodologies.

2 Executive Summary

Feed efficiency in beef cattle has been linked to degree of enteric methane production. Jones et al. (2011) showed that low residual feed intake (RFI), or high feed efficiency, cows produced less methane than high RFI (low feed efficiency) cows when fed a high quality winter pasture but not when fed a low quality summer pasture. Using microbial profiling technology we showed in B.CCH.1008 that the rumen microbial communities from these animals were both influenced by diet and RFI genetic background. Rumen bacterial, archaeal, methanogenic archaeal, fungal and protozoan communities were all significantly influenced by diet irrespective of RFI genotype. Rumen bacterial and fungal communities were influenced by RFI genotype regardless of diet. Archaeal and methanogenic archaeal communities were also influenced by RFI genotype, but were diet dependant. Only cows on the winter pasture demonstrated significant changes in the archaeal (including methanogenic archaeal) communities in relation to RFI genotype, consistent with these animals producing less methane.

The aim of this study was to characterise the rumen bacterial, archaeal and fungal communities by pyrosequencing technology and to identify micro-organisms linked with high feed efficiency cows producing less enteric methane. Pyrosequencing is a deep sequencing technology which can be used to identify microbial genome sequences and assign phylogenetic information to the population.

Pyrosequencing of bacterial communities showed the majority of bacteria detected within the rumen belonged to the phyla Bacteroidetes (48.3%) and Firmicutes (33%). The majority of Bacteroidetes were classified as unclassified Bacteroidales (13.4%) and *Prevotella* (29.6%), while the majority of Firmicutes identified belonged to unclassified Clostridiales (9.2%), *Lachnospiraceaea* (6.6%) and *Ruminococcaceae* (6.6%). Rumen bacterial communities were significantly different between cows fed the summer and winter pastures regardless of feed efficiency genotype. However, feed efficiency genotype also had a significant influence on rumen bacterial communities irrespective of pasture type. We found that a broader range of phyla were associated with the high feed efficiency cows which produce less methane on the winter diet. These included Firmicutes, Bacteroidetes, TM7, Fibrobacteres, Lentisphaerae and Armatimonadetes. Firmicutes and Bacteroidetes were primarily associated with the low feed efficiency cows on the winter diet and included Bacteroidales, Clostridiales, *Ruminococcaceae*, *Butyrivibrio* and *Prevotella*.

Although no differences in methane production were observed between high and low RFI on the summer diet, in general cows on the summer pasture produced less methane than cows on the winter pasture (Jones et al., 2011). Some of the significant diet related changes identified in bacterial communities may be linked with this observation. OTU more abundant in the rumen of cows fed the low quality summer pasture were related to Alphaproteobacteria, Bacteroidales, *Ruminococcaceae*, *Prevotellaceae*, Chlamydiae, *TP21* and *YS2*. The majority of OTU more abundant in the rumen of cows fed the high quality winter pasture belonged to the Firmicutes and could be classified further to *Lachnospiraceae*, *Ruminococcaceae*, *Clostridiales XIII Insertae Sedis*, *Butyrivibrio*, *Coprococcus*, *Ruminococcus*, *Lachnospira pectinoschiza* and *Eubacterium cellulosolvens*. Bacteroidetes related to *Prevotella* and Bacteroidales were also more abundant in the rumen of cows fed the winter pasture. Despite dietary difference there were significant differences observed in bacterial communities linked with RFI genotype, although these were not generally common between diets suggesting that diet had a greater effect than genotype on bacterial community structure. Nevertheless, in our

study we found *Fibrobacter succinogenes* and *Ruminococcus albus* were more abundant in the high feed efficiency cows regardless of pasture type. These cellulolytic organisms, which aid digestion, may be the reason these cows are partially more feed efficient.

Pyrosequencing of archaeal communities showed that the majority of rumen archaea were classified as Methanobacteria (93%), all belonging to the family *Methanobacteriaceae*. Thermoplasmata (6%) and Methanomicrobia (<1%) were also detected. Rumen archaeal communities were significantly different between high and low feed efficiency cows on the winter pasture. We found that *Methanosphaera stadtmanae* was less abundant in low RFI animals which produced less methane. *M. stadtmanae* has been reported to be less frequently associated with low RFI cows (Zhou et al., 2009), although relationship to methane production was not determined. We also found methanogenic archaeon related to CH1270 to be more abundant in the low methane producing high feed efficiency cows on the winter pasture. Methanogenic archaeon related to CH1270 have been previously shown to be negatively correlated with methane yield (Zhou et al., 2011).

Pyrosequencing of fungal communities showed that there were significant differences in the rumen fungi between the low methane producing low RFI cows and the high RFI cows when fed the winter pasture. Rumen fungi significantly associated with low RFI animals grouped closest to the genus *Anaeromyces* (ca 80% similarity), although these organisms are not likely to be *Anaeromyces* but rather an unclassified group of rumen fungi. It was also observed that several sequences related to other uncultured rumen fungi (>92% similarity) were more abundant in high RFI cows. Despite the poor current classification of rumen fungi, these communities may play a greater than previously thought role in enteric methane production. We found the rumen fungal and bacterial communities to be more variable among the divergent methane producing RFI cows than the archaeal communities.

Deep sequencing of rumen microbial communities from cows divergent in feed efficiency have identified organisms related to genotype and diet, but more importantly linked to significant reductions in enteric methane production. Together these data provide a detailed picture of the symbiotic relationships among rumen micro-organism rather than a targeted and isolated investigation of a particular group of organisms. Although, it is the methanogenic archaea which are generally considered most important in enteric methane production these micro-organisms should not be studied in isolation as they rely on bacteria, protozoa and fungi to provide digestive products for methanogenesis. The data generated in this project could be used to develop targeted quantitative assays to particular organisms deemed of interest. As most micro-organisms have not been cultured to date the importance of unclassified micro-organisms in methane mitigation should not be overlooked. The significant difference detected in rumen microbial communities using deep sequencing are in agreement with the broader shifts detected in community structure by microbial profiling (B.CCH.1008). This indicates that the two technologies, which differ in both their resolution and throughput capabilities, can be used complimentarily in evaluating and developing methane mitigation strategies in ruminant livestock.

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3 Background

Enteric methane (CH_4) released from ruminant livestock systems contribute to global greenhouse gas emissions. The methane produced during anaerobic fermentation in ruminants also represents a feed energy loss. 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 metabolism or because they affect the numbers of methanogens or other members of the microbiota (Bauchop, 1989; Hook et al., 2010; Kamra, 2005).

In B.CCH.1008 (Rumen microbial profiling – a tool to investigate methane mitigation strategies) we developed high through-put microbial profiling tools to investigate rumen microbiota (bacteria, archaea, fungi and protozoa) in ruminant livestock. Furthermore, we used these tools to demonstrate a linkage between rumen microbial communities and feed efficiency, as well as, enteric methane production in beef cattle. Jones et al (2011) showed that high and low residual feed intake (RFI) beef cattle significantly differed in methane emissions when grazed on high quality winter pasture but not when grazed on low quality summer pasture. By microbial profiling the rumen samples from these cows we showed that diet (high versus low quality pasture) significantly ($P<0.05$) altered the bacterial, archaeal (including methanogens), fungal and protozoan communities, regardless of RFI grouping. Furthermore, RFI genotype was also shown to significantly ($P<0.05$) influenced bacterial and fungal communities, irrespective of dietary treatment. Archaeal and methanogenic communities were significantly different between high and low RFI cows, but only when cows were fed the high quality winter pasture, consistent with methane differences only being observed between these cows. No significant influence of RFI was observed on rumen protozoan community structure. These results confirmed that both animal genetics and dietary changes alter the rumen microbiota and that these are linked to degree of methane emissions. Furthermore, there appears to be a deeper underlying symbiotic relationship between rumen organisms which relate to both animal production and methane emissions.

Microbial profiling has been shown to be an appropriate screening tool for identifying dietary and genetic differences in rumen microbiota which are potentially linked with enteric methane production. The aim of this project was to identify particular organisms, at the genome sequence level, driving the previously identified differences linked to feed efficiency and enteric methane emission. This would be achieved by deep sequencing of the rumen microbial communities with 454 pyrosequencing technology. This would provide phylogenetic information on

organisms based on the ribosomal rRNA and internal transcribed spacer (ITS) regions. The information generated in this study will be unique, as limited 454 sequencing information is available for rumen microbial communities as a whole (bacterial, archaeal and fungal). Where information is available it is based on metagenomic analysis of specific organisms (not community structure) or to microbial communities investigated *in vitro*. There are no phylogenetic studies available which have investigated rumen microbial communities in relation to enteric methane production or presented a complete symbiotic picture of the entire rumen microbiota. These data will be useful in developing specific and quantitative assay (qPCR) which could be used to screen efficacy of methane abatement strategies in ruminants.

4 Project objectives

Identify key organisms driving differences in communities which significantly differ between high and low methane producing cows under specific genetic and dietary treatments.

5 Methodology

5.1 Animal trial and microbial profiling outcomes

Rumen microbial communities (bacterial, archaeal, methanogenic archaeal, fungal and protozoan) were investigated from 47 Angus heifers shown to be divergent for RFI: 22 low RFI and 25 high RFI cows. Results from this investigation have been reported in detail in B.CCH.1008 final report section 6.4. In brief, 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 cow 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). Jones et al. (2011) showed that low and high RFI cows differed significantly ($P<0.05$) in methane production (0.34 $\pm$ 1.017 g CH_4/kg LW and 0.46 ± 0.023 g CH_4/kg LW respectively) when fed a high quality winter pasture but not when fed a low quality summer pasture (0.26 ± 0.013 g CH_4/kg LW and 0.26 ± 0.018 g CH_4/kg LW respectively). We have 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 on rumen microbiota. Rumen bacterial, archaeal, methanogenic archaeal, fungal and protozoan communities were all significantly influenced by diets irrespective of RFI genotype. Rumen bacterial and fungal communities were influenced by RFI genotype regardless of diet. Archaeal and methanogenic archaeal communities were also influenced by RFI genotype, but were dependant on diet. Only cows on the winter pasture demonstrated significant changes in the archaeal (including methanogenic archaeal) communities in relation to RFI genotype.

To gain a better insight into the differences observed in relation to feed efficiency and methane production it was the aim of this study to investigate the bacterial communities of these cows on both the summer and winter pastures, and the archaeal and fungal communities of these cows on the winter pasture.

5.2 Pyrosequencing background

Pyrosequencing is a method of DNA sequencing (determining the order of nucleotides in DNA) based on the "sequencing by synthesis" principle. It differs from Sanger sequencing, in that it relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides. Pyrosequencing is a deep sequencing technology which can be used to identify microbial genome sequences and assign phylogenetic information to the population. Although 454 pyrosequencing technology was first established in 2005, it is only recently that the technology has been taken from large genome facilities into research laboratories and has become more affordable.

5.3 Nucleic acid extract

Total nucleic acid was extracted from rumen samples using a modification (Torok et al., 2008) of a SARDI proprietary method (Stirling et al., 2004). These same nucleic acid extracts were used for microbial profiling analysis of bacterial, archaeal (including methanogenic archaea), fungal and protozoan communities in B.CCH.1008.

5.4 Development of pyrosequencing for rumen microbial communities

All PCR amplicons generated for microbial profiling (see B.CCH.1008 for details) were too long for pyrosequencing analysis. Therefore, alternative primer pairs specific to organisms of interest needed to be evaluated. All primer pairs evaluated for bacterial, archaeal and fungal pyrosequencing were chosen to amplify similar genome regions as previously investigated by microbial profiling, and in each case contained one of the same primers used for each of the profiling assays.

5.4.1 Bacteria

Bacterial 454 pyrosequencing was done with primers 27F and 534R (Nossa et al., 2010) (Table 5-1). This primer combination has been used by many groups and is located in the most variable region of the 16S rRNA (V2-V3). This primer pair produced an amplicon of 598 bp.

5.4.2 Archaea

Several primers were evaluated for archaeal 454 pyrosequencing to be used in conjunction with either Ar109F or Ar912r (Table 5-1). Primers Parch519 (Ovreås et al., 1997) and 691R (Watanabe et al., 2004), were examined for use with Ar109F *in-silico*. However, neither primer combinations were found to be specific for archaea. Primer Ar109F was investigated with Arch518r (Sorensen and Teske, 2006) *in-vitro*. This primer pair generated multiple amplicons from rumen samples which precluded it from further investigation. Other primers tested with the Ar109F were EK510R (Baker et al., 2003) and A533b (Großkopf et al., 1998). However, both Ar109F/EK510R and Ar109F/A533b generated non-specific amplicons *in-vitro* and were discounted from use in 454 pyrosequencing. Specificity of our T-RFLP primers to archaea were predominately due to the Ar912r (Egert et al., 2004) primer. Therefore, primer pair Ar912r and Ar344f (Casamayor et al., 2002) was investigated and found to generate a single PCR amplicon of 648 bp which was suitable for 454 pyrosequencing.

5.4.3 Fungi

Information within the literature on primers previously used for fungal 454 pyrosequencing is limited. ITS1F (used by us for rumen fungal profiling) has been used with ITS2 (White et al., 1990) for pyrosequencing various fungal communities (Buee et al., 2009; Hui et al., 2011; Jumpponen and Jones, 2009; Jumpponen et al., 2010; Lentendu et al., 2011) (Table 5-1). However, we found that ITS1F/ITS2 produced two dominant amplicons, as well as, and other smaller non-specific amplicons *in-vitro*. The larger dominant amplicon (rumen fungal origin) was less intense than the smaller dominant amplicon (plant fungal origin). It was anticipated that pyrosequencing with this fungal primer pair would lead to biased sequencing of plant fungal sequences and was discounted from further consideration. An alternative fungal primer pair previously used for pyrosequencing was funSSUF and funSSUR (Handl et al., 2011). Although this primer pair generated an amplicon of suitable length (470 bp), it would amplify the conserved 18S rRNA region making species discrimination difficult. Furthermore, it was found not to be specific to fungi *in-silico* and was discounted from further consideration. Alternative primers for use with ITS1F, which would also amplify the variable ITS region(s), were investigated. Primers GM2 (Brookman et al., 2000) and Neo5.8SRev (Edwards et al., 2008) were both tested *in-vitro* with ITS1F. ITS1F/GM2 generated expected amplicons of 200-300 bp, as well as, other non-specific amplicons, hence was discounted as a candidate for 454 pyrosequencing. ITS1F/Neo5.8SRev was tested *in-vitro* and found to amplify expected amplicons of 300-350bp, with some faint non-specific larger

bands which were thought not to interfere with 454 pyrosequencing. However, when this primer pair was used for pyrosequencing, short sequences of 50-150 bp were generated instead of the expected 300-350 bp and the primer pair was abandoned. Primer ITS400RW (GRA-A5 report (http://www.livestockemissions.net/reports,listing,10,201011-intitial-priority-livestock-research-projects.html) was investigated with ITS1F and found to produce a single amplicon of ca 350 bp suitable for rumen fungal pyrosequencing (Table 5-1).

Table 5-1 PCR primers evaluated for pyrosequenicng of rumen microbial communities.

Forward primer	Reverse primer	Target	Outcome
27F (AGAGTTTGATCMTGG)	534R (TTACCGCGGCTGCT)	Bacteria	Primers used for bacterial pryosequencing
Ar109F (ACKGCTCAGTAACACGT)	Parch519 (TTACCGCGGCKGCT)	Archaea	Non specific to archaea *in--silico*
Ar109F (ACKGCTCAGTAACACGT)	691R (GGATTACARGATTTCAC)	Archaea	Non specific to archaea *in-silico*
Ar109F (ACKGCTCAGTAACACGT)	Arch518 (GGTDTTACCGCGGCKGCTG)	Archaea	Non specific amplicons *in-vitro*
Ar109F (ACKGCTCAGTAACACGT)	EK510R (CCTGCCCRGCCCTT)	Archaea	Non specific amplicons *in-vitro*
Ar109F (ACKGCTCAGTAACACGT)	A533b (TTACCGCGGCGGCTGGCA)	Archaea	Non specific amplicons *in-vitro*
Ar912r (CTCCCCCGCCAATTC CTTTA)	Ar344f (ACGGGGYGCAGCAGGCGCGA)	Archaea	Primers used for archaeal pyrosequencing
ITSF1 (CTTGGT CATTTAGAGGAAGTAA)	ITS2 (GCTGCGTTCTTC ATCGATGC)	Fungi	Non specific amplicons *in-vitro*
funSSUF (TGGAGG GCAAGTCTGGTG)	funSSUR (TCGGCATAGTTTATGGTTAAG)	Fungi	Non specific to fungi *in-silico* & targeted conserved 18S rRNA region
ITSF1 (CTTGGT CATTTAGAGGAAGTAA)	GM2 (CTGCGTTCTTCATCGAT)	Fungi	Non specific amplicons *in-vitro*
ITSF1 (CTTGGT CATTTAGAGGAAGTAA)	Neo 5.8S Rev (CGAGAACCAAGAGATCCA)	Fungi	Non specific amplicons *in-vitro*
ITSF1 (CTTGGT CATTTAGAGGAAGTAA)	ITS400RW (ATTGTCAAAAGTTGTTTTTAWA TTAT)	Fungi	Primers used for fungal pyrosequencing

5.4.4 PCR amplification of microbial communities

Bacterial, archaeal and fungal DNA were amplified from total nucleic acid using the FastStart HiFi PCR system (Roche Diagnostics). Each PCR reaction contained 100-200 ng of template DNA, 1 x FastStart HiFi PCR buffer, 0.1 uM each of forward and reverse primer (Table 5-1), 0.8mM dNTPs and 1U FastStart HiFi Taq polymerase. Each primer also included sequences to facilitate the sequencing of products in the Roche/454 system. All forward PCR primers consisted of a related set of primers with different “barcode” sequences which enabled the identification of individual samples. All PCRs were done in a MJ Research PTC-225 Peltier thermal cycler (GeneWorks, Adelaide, Australia) with the following amplification conditions: for bacteria an initial denaturation at 95°C for 5 min followed by 25 cycles of denaturation at 95°C for 45 s, annealing at 40°C for 30 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 10 min; for archaea an initial denaturation at 95°C for 4 min followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, with a final extension step at 72°C for 7 min; and for fungi an initial denaturation at 95°C for 4 min followed by 30 cycles of denaturation at 95°C for 60 s, annealing at 50°C for 30 s, and extension at 72°C for 45 s, with a final extension step at 72°C for 7 min.

PCR amplicons were analysed for specificity by electrophoresis on a 2% agarose gel. PCR amplicons were purified using Agencourt AMPure XP (Beckman Coulter) and quantified according to the "Amplicon Library Preparation Methods Manual GS Junior Titanium Series" protocol (Roche Diagnostics). Pooled samples (n=12) were sequenced using the Roche/454 GS Junior Genome Sequencer and Titanium chemistry according to manufacturer's instructions (Roche Diagnostics).

5.4.5 Data analysis

The rumen bacterial, archaeal and fungal communities from low (n=22) and high (n=25) RFI cows on the winter pasture, as well as, the rumen bacterial communities of the same cows on the summer pasture were analysed. Sequences were assigned to Operational Taxonomic Units (OTU) with 97% within group similarity, a level which is approximately equivalent to a species designation. OTU tables were produced in Qiime v 1.4.0 (Caporaso et al., 2010), using the "pick_otus_through_otu_table" and "summarize_taxa_through_plots" scripts with standard defaults and taxonomy assignment determined using BLAST (Altschul et al., 1990). Bacterial and archaeal OTU were identified using the greengenes database (4th Feb 2011), while fungal OTU were identified using the UNITE (Abarenkov et al., 2010) database (21st Nov 2011) (http://qiime.sourceforge.net/home_static/dataFiles.html). OTU tables were filtered to remove the possibility of sequencing errors and chimeras using the "filter_otu_table" script, eliminating OTU with less than five sequences and present in less than two samples.

5.5 Statistical analysis

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 microbial communities and identify OTUs accounting for differences observed in microbial communities associated with diet and/or feed efficiency. Bray-Curtis measures of similarity (Bray and Curtis, 1957) were calculated to examine similarities between rumen microbial communities from pyrosequencing 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 microbial communities were significantly different between treatments (dietary and/or feed efficiency). 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. Bacteria OTUs contributing significantly to the dissimilarity between treatments were calculated (Diss/SD>2). For the archaea and fungi communities significant OTU associated with feed efficiency were determined in Qiime using ANOVA with the "otu_category_significance" script.

Unconstrained ordinations were done to graphically illustrate the relationships between rumen microbial communities and diet and/or RFI genotype using principal coordinate analysis (PCO) (Gower, 1966).

6 Results

6.1 Rumen bacterial communities

Pyrosequencing of bacterial communities generated 407,453 reads with an average of 4,300 reads per sample. The majority of bacteria detected within the rumen belonged to the phyla Bacteroidetes (48.3%) and Firmicutes (33%). The majority of Bacteroidetes were classified as unclassified Bacteroidales (13.4%) and *Prevotella* (29.6%). The majority of Firmicutes identified belonged to unclassified Clostridiales (9.2%), *Lachnospiraceaea* (6.6%) and *Ruminococcaceae* (6.6%). Rumen bacterial communities were significantly different (global R=0.996, P=0.001) with respect to diet regardless of feed efficiency genotype. However, feed efficiency genotype also had a significant influence on rumen bacterial communities (global R=0.212, P=0.001), irrespective of dietary treatment. When cows were fed a poor quality summer pasture, bacteria belonging to the phyla Fibrobacteres, Cyanobacteria, Lentisphaerae, Proteobacteria, Spirochaetes and Verrucomicrobia were more abundant (Fig 6-1).

SIMPER analysis showed that rumen bacterial communities differed by 81.69% between cows fed the winter and summer pastures. Twenty-seven OTU contributing to the top 5% of differences between cows fed the two pastures, and showing a greater than three fold difference between treatments, were identified (Table 6-1). Twelve OTU were more abundant in the rumen of cows fed the low quality summer pasture. These OTU were related to Alphaproteobacteria, Bacteroidales, *Ruminococcaceae*, *Prevotellaceae*, Chlamydiae, *TP21* and *YS2*. Fifteen OTU were more abundant in the rumen of cows fed the high quality winter pasture. The majority of these bacteria belonged to the Firmicutes and could be classified further to *Lachnospiraceae*, *Ruminococcaceae*, *Clostridiales XIII Insertae Sedis*, *Butyrivibrio*, *Coprococcus*, *Ruminococcus*, *Lachnospira pectinoschiza* and *Eubacterium cellulosolvens*. Bacteroidetes related to *Prevotella* and Bacteroidales were also more abundant in the rumen of cows fed the winter diet. These differences are visually demonstrated in the PCO ordination in Fig 6-2.

When examined by pasture type only, SIMPER analysis showed that the rumen bacterial communities differed by 68.53% between high and low RFI cows fed the winter pasture. Twenty-eight OTU contributing to at least a three fold differences between high and low RFI cows fed the winter pasture were identified (Table 6-2). Eleven OTU were more abundant in the rumen of high RFI cows, while 17 were more abundant in the rumen of low RFI cows. Firmicutes and Bacteroidetes were primarily associated with the high RFI cows on the winter diet and included Bacteroidales, Clostridiales, *Ruminococcaceae*, *Butyrivibrio* and *Prevotella*. A broader range of phyla were associated with the low RFI cows on the winter diet including Firmicutes, Bacteroidetes, TM7, Fibrobacteres, Lentisphaerae and Armatimonadetes. *Ruminococcaceae* (OTU 37339) and two *Butyrivibrio* (OTU 43458 and 74135) were strongly associated with the high RFI cows, while Bacteroidales (OTU 25020) and *Fibrobacter succinogenes* (OTU 52361) were strongly associated with low RFI cows when fed the winter pasture.

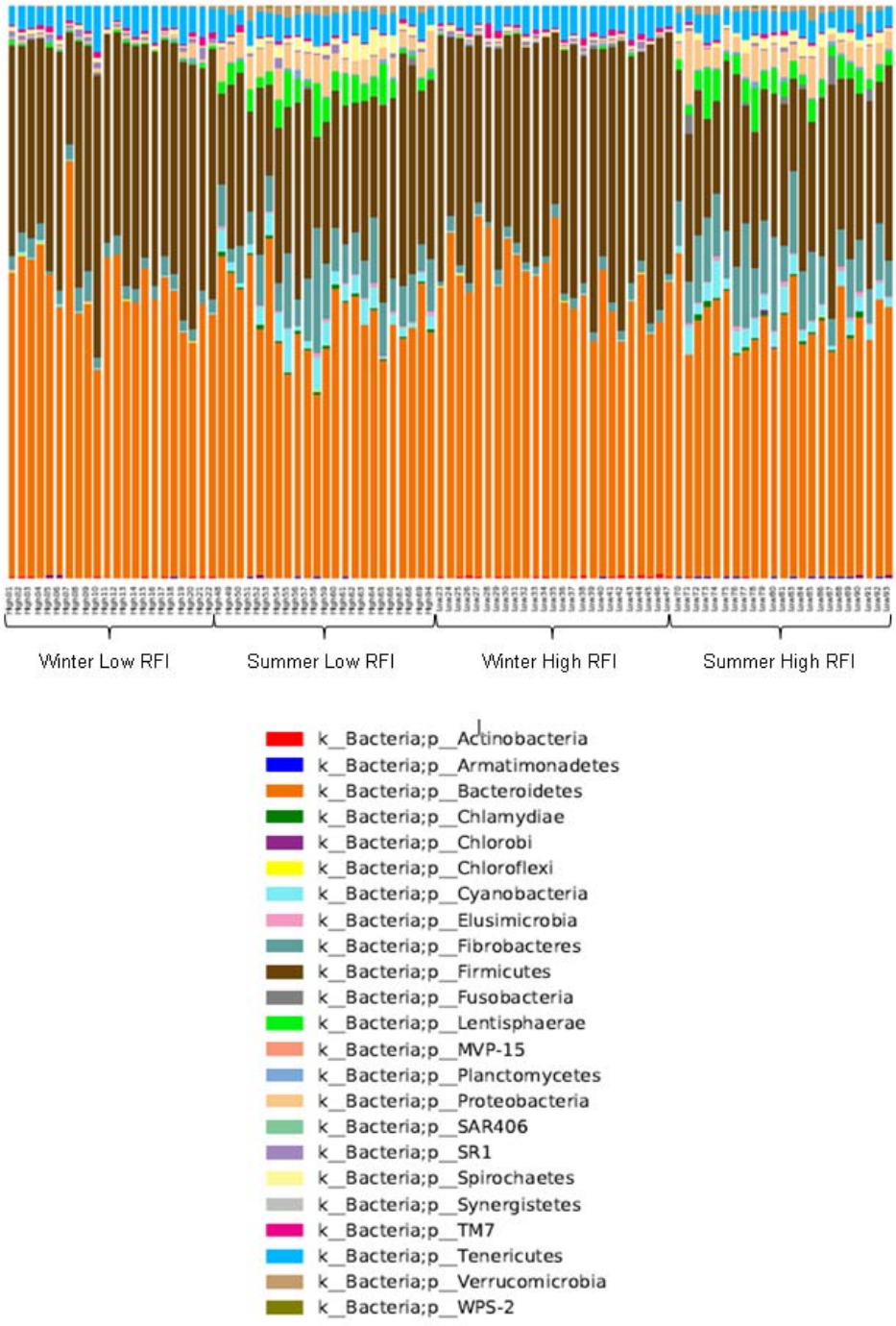

Figure 6-1 Abundance of rumen bacteria classified to level of phyla for individual cows divergent in feed efficiency which were fed a high quality winter or low quality summer pasture.

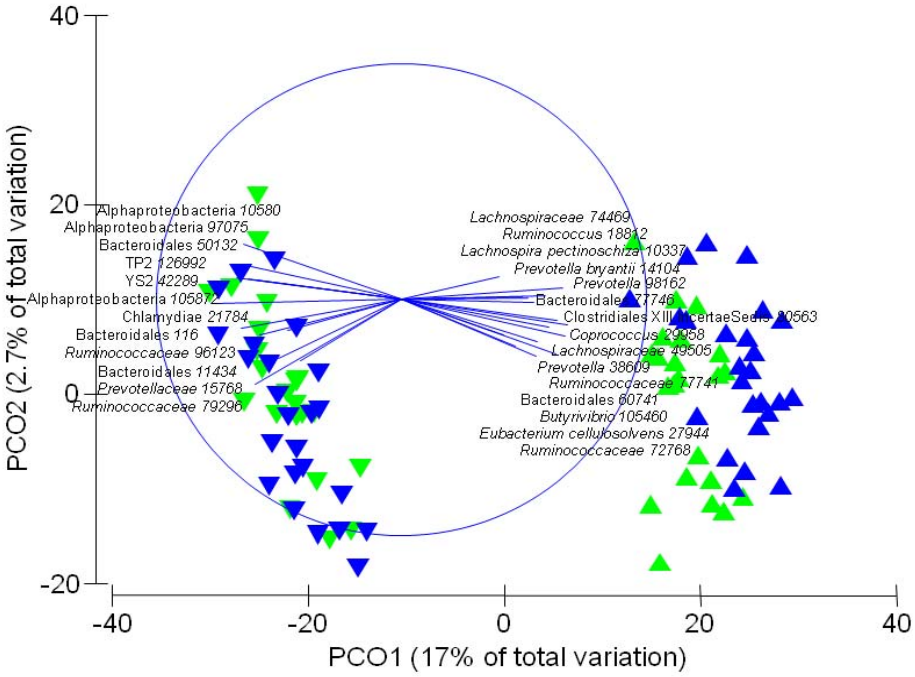

Figure 6-2 PCO ordination of rumen bacterial communities from cows fed a high quality winter (▲) and low quality summer (▼) pasture. Overlaid onto the PCO are vectors of significant OTU identified by SIMPER indicating the association of OTU with a particular diet. Rumen bacterial communities are also identified by feed efficiency: low RFI (green) and high RFI (blue)

For cows fed the summer pasture SIMPER analysis showed that the rumen bacterial communities differed by 72.44% between high and low RFI cows. Seven OTU contributing at least a three fold differences between high and low RFI cows fed the summer pasture were identified (Table 6-3). Two OTU were more abundant in the rumen of high RFI cows, while 5 were more abundant in the rumen of low RFI cows. *Ruminococcus albus* (OTU 12625), Bacteroidales (OTU 20852), *Ruminococcus* (OTU 100270) and *Paludibacter* (OTU 107222) were strongly associated with the low RFI cows.

Table 6-1 Bacterial OTU identified as being significantly associated with dietary treatment

OTU	Winter diet Av.Abund	Summer diet Av.Abund	Diss/SD	Fold W/S[a]	Classification: kingdom; phyla; class; order; family; genus; species
116	0.07	0.61	2.41	0.115	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;
10580	0.02	0.7	2.99	0.029	Bacteria;Proteobacteria;Alphaproteobacteria
11434	0	0.58	2.15	0.000	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;
15768	0.1	0.52	2.02	0.192	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae
21784	0.01	0.64	2.93	0.016	Bacteria;Chlamydiae;Chlamydiae
26992	0.13	0.7	2.01	0.186	Bacteria;Verrucomicrobia;TP21
42289	0.02	0.57	2.24	0.035	Bacteria;Cyanobacteria;4C0d-2;YS2;
50132	0.03	0.54	2.07	0.056	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;
79296	0.04	0.62	2.48	0.065	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae
96123	0	0.73	2.75	0.000	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae
97075	0.02	0.51	2.14	0.039	Bacteria;Proteobacteria;Alphaproteobacteria
105872	0.01	0.74	3.09	0.014	Bacteria;Proteobacteria;Alphaproteobacteria
10337	0.74	0.01	3.98	74.000	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Lachnospira;Lachnospira pectinoschiza
14104	0.58	0	2.02	undefined	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Prevotella;Prevotella bryantii
18812	0.68	0.01	2.25	68.000	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminococcus;
27944	0.65	0	3.57	undefined	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Eubacterium;Eubacterium cellulosolvens
29958	0.65	0.06	2.82	10.833	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Coprococcus;
38609	0.61	0.08	2.15	7.625	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Prevotella;
49505	0.85	0.05	3.31	17.000	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae
60741	0.67	0.08	2.38	8.375	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;
72768	0.78	0.1	2.59	7.800	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae
74469	0.82	0	2.85	undefined	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae
77741	0.72	0.1	2.69	7.200	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae
77746	0.58	0.03	2.15	19.333	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;
80563	0.56	0.09	2.19	6.222	Bacteria;Firmicutes;Clostridia;Clostridiales;ClostridialesFamilyXIII.Incertae Sedis
98162	0.59	0.1	2.01	5.900	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Prevotella;
105460	0.56	0.07	2	8.000	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Butyrivibrio;

[a] Fold difference represents the ratio of average OTU abundance for cows fed the winter pasture against cows fed the summer pasture. Fold values >1 indicate higher abundance in winter pasture fed cows while values <1 indicate higher abundance in the summer pasture fed cows.

Table 6-2 Bacterial OTU identified as being significantly associated with feed efficiency when cows were fed a winter pasture.

OTU	High feed efficiency Av.Abund	Low feed efficiency Av.Abund	Diss/SD	Fold H/L[a]	Classification: kingdom; phyla; class; order; family; genus; species
1354	0.03	0.26	1.02	0.115	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Butyrivibrio;
15573	0.08	0.29	1.2	0.276	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Prevotella;
15757	0.07	0.28	1.09	0.250	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae
37339	0	0.37	1.38	0.000	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae
43282	0.06	0.27	1.07	0.222	Bacteria;Firmicutes;Clostridia;Clostridiales;
43458	0.07	0.27	1.15	0.259	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Butyrivibrio;
49513	0.08	0.33	1.29	0.242	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Prevotella;
71420	0.06	0.26	1.01	0.231	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;
74135	0.08	0.27	1.15	0.296	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Butyrivibrio;
92336	0.16	0.62	1.36	0.258	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;
106826	0.02	0.24	1.02	0.083	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae
18926	0.25	0.03	1.07	8.333	Bacteria;Armatimonadetes;SJA-176
25020	0.33	0.07	1.22	4.714	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;
25063	0.24	0.08	1	3.000	Bacteria;TM7;TM7-3;CW040;F16
28061	0.25	0.07	1.06	3.571	Bacteria;Fibrobacteres;Fibrobacteres;Fibrobacterales;Fibrobacteraceae;Fibrobacter;Fibrobacter succinogenes
30045	0.36	0.11	1.27	3.273	Bacteria;Lentisphaerae;Lentisphaerae;Victivallales;Victivallaceae
40108	0.22	0.07	1.01	3.143	Bacteria;Tenericutes;Erysipelotrichi;Erysipelotrichales;vadinHA31;RFN20;
43421	0.25	0.08	1.03	3.125	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae
44036	0.27	0.08	1.08	3.375	Bacteria;Firmicutes;Clostridia;Clostridiales;
52361	0.27	0.08	1.13	3.375	Bacteria;Fibrobacteres;Fibrobacteres;Fibrobacterales;Fibrobacteraceae;Fibrobacter;Fibrobacter succinogenes
62862	0.28	0.08	1.14	3.500	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Prevotella;
81059	0.28	0.05	1.04	5.600	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Prevotella;
81830	0.28	0.08	1.15	3.500	Bacteria;Firmicutes;Clostridia;Clostridiales;
89707	0.25	0.06	1	4.167	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Prevotella;
90058	0.49	0.14	1.57	3.500	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae
102897	0.35	0.08	1.41	4.375	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;
107248	0.25	0.08	1	3.125	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae
107276	0.27	0.06	1.14	4.500	Bacteria;Fibrobacteres;Fibrobacteres;Fibrobacterales;Fibrobacteraceae;Fibrobacter;Fibrobacter succinogenes

Table 6-3 Bacteria OTU identified as being significantly associated with feed efficiency when cows were fed a summer pasture.

OTU	High feed efficiency Av.Abund	Low feed efficiency Av.Abund	Diss/SD	Fold H/L[a]	Classification: kingdom; phyla; class; order; family; genus; species
69684	0.08	0.3	1.12	0.267	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Prevotella;
102609	0.07	0.27	1	0.259	Bacteria;Fibrobacteres;Fibrobacteres;Fibrobacterales;Fibrobacteraceae;Fibrobacter;Fibrobacter succinogenes
7091	0.42	0.14	1.19	3.000	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae
12625	0.4	0.08	1.2	5.000	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminococcus;Ruminococcus albus
20852	0.68	0.2	1.25	3.400	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;
100270	0.52	0.1	1.72	5.200	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminococcus;
107222	0.26	0.06	1.07	4.333	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Paludibacter;

[a] Fold difference represent the ratio of average OTU abundance for high feed efficiency (low RFI) against low feed efficiency (high RFI) cows. Fold values >1 indicate higher abundance in high feed efficiency cows, while values <1 indicate higher abundance in low feed efficiency cows.

6.2 Rumen archaeal communities

Pyrosequencing of archaeal communities generated 251,600 reads with an average of 5,000 reads per sample. The majority of archaea detected within the rumen belonged to the class Methanobacteria (93%), all belonging to the family *Methanobacteriaceae*. Thermoplasmata (6%) and Methanomicrobia (<1%) were also detected (Fig 6-3). Rumen archaeal communities were significantly different between high and low RFI cows on the winter diet (global R=0.099, P=0.002). SIMPER analysis showed that the rumen archaeal communities differed by 23.74% between the high and low RFI cows fed the winter pastures. Ten OTU contributing significantly to differences between feed efficiency genotype were identified by ANOVA (Table 6-4). Methanobacteria related to *Methanosphaera stadtmanae* and *Methanobrevibacter* sp were more abundant in high RFI cows, while Thermoplasmata related to methanogenic archaeaon clones were more abundant in low RFI cows (Fig 6-4).

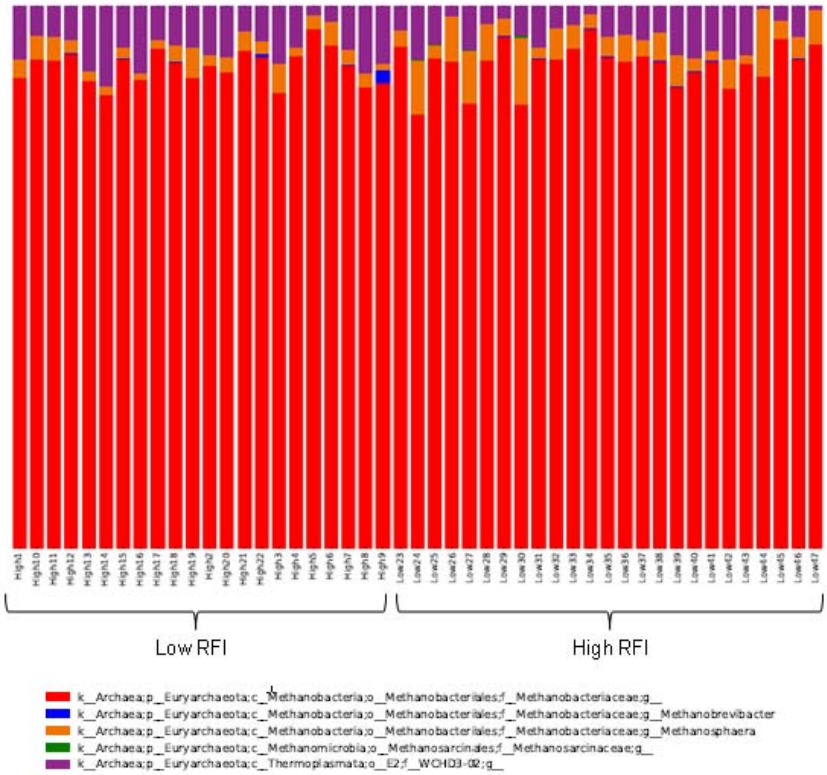

Figure 6-3 Abundance of rumen archaea classified to level of genus for individual cows divergent in feed efficiency which were fed a high quality winter pasture.

Table 6-4 Archaeal OTU identified as being significantly associated with feed efficiency when cows were fed a winter pasture

OTU	Prob	Fold H/L	Accession Number	BLAST Description	E value	% Identity
112	0.001	1.22	NR042785	Methanobrevibacter millerae ZA-10	0	100
135	0.001	0.65	GQ906571	Methanobrevibacter sp. YE288	0	99
97	0.014	0.42	AY196684	Methanosphaera stadtmanae	0	97
20	0.018	2.53	GQ339875	Methanogenic archaeon CRM1	0	94
129	0.023	0.29	AY196684	Methanosphaera stadtmanae	8E-161	94
1	0.023	1.91	DQ445723	Methanogenic archaeon CH1270	1E-97	91
124	0.027	2.93	GQ339877	Methanogenic archaeon WGK1	3E-104	92
120	0.028	1.58	DQ445723	Methanogenic archaeon CH1270	0	96
16	0.039	0.61	AY196684	Methanosphaera stadtmanae	0	98
72	0.046	0.49	GQ339877	Methanogenic archaeon WGK1	0	95

Representative sequences for significant OTUs (P<0.05) were identified using BLASTn. Fold difference represent the ratio of average sequence numbers for cows with high feed efficiency (low RFI) against cows with low feed efficiency (high RFI). Fold values >1 indicate higher abundance in high feed efficiency cows, while values <1 indicate higher abundance in low feed efficiency cows.

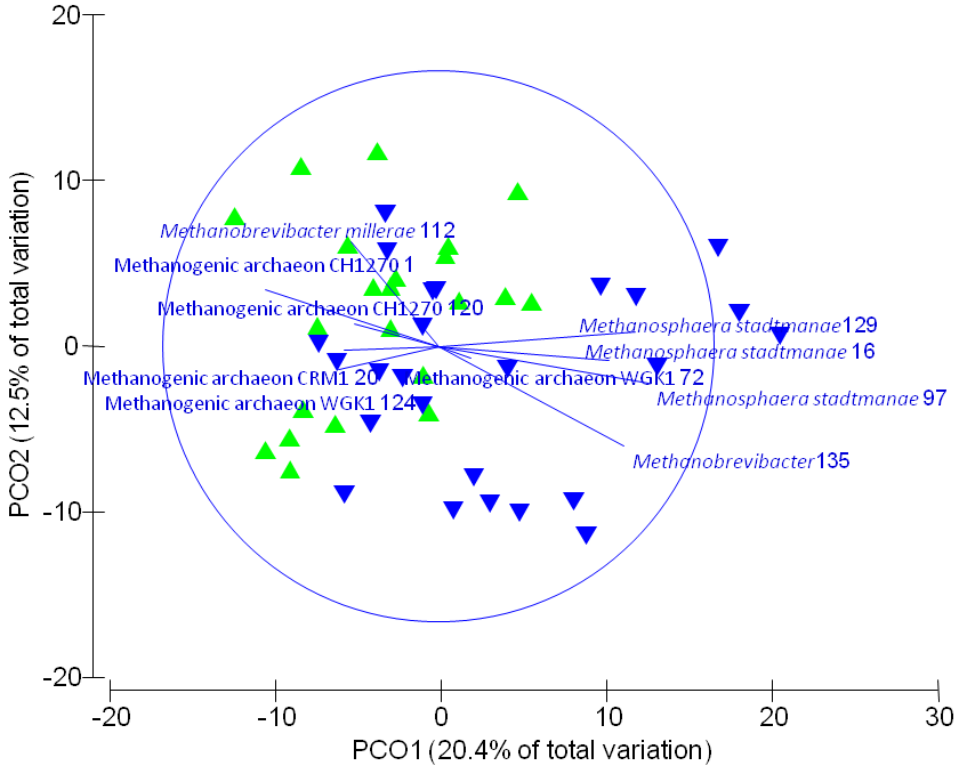

Figure 6-4 PCO ordination of rumen archaeal communities from divergent RFI cows fed a high quality winter pasture. Low RFI (▲) and high RFI (▼). Overlaid onto the PCO are vectors of significant OTU identified by ANOVA indicating the association of OTUs with feed efficiency genotype.

6.3 Rumen fungal communities

Pyrosequencing of fungal communities generated 318,667 reads with an average of 6,700 reads per sample. The rumen fungal communities were significantly different (global R=0.398, P=0.001) between high and low RFI cows on the winter pasture. Although, visual differences were observed in the rumen fungal communities between high and low RFI, these differences were only identifiable as unknown Neocallimastigales using the UNITE database (Fig 6-5). Furthermore, several OTU were identified as non rumen fungi (Glomeromycota, Ascomycota and Basidiomycota) using this database. However, when these OTU were put through

NCBI BLASTn they were all classified as Neocallimastigales, indicating an improved database needs to be developed for investigating rumen fungal pyrosequencing data.

SIMPER analysis showed that the rumen fungal communities differed by 71.05% between the high and low RFI cows fed the winter pastures. Eighteen OTU were identified as being significantly associated with RFI (Table 6-5). Uncultured rumen fungi (n=6) were more abundant in the high RFI cows, while *Anaeromyces* related species (n=12) were more abundant in the low RFI cows (Fig 6-6).

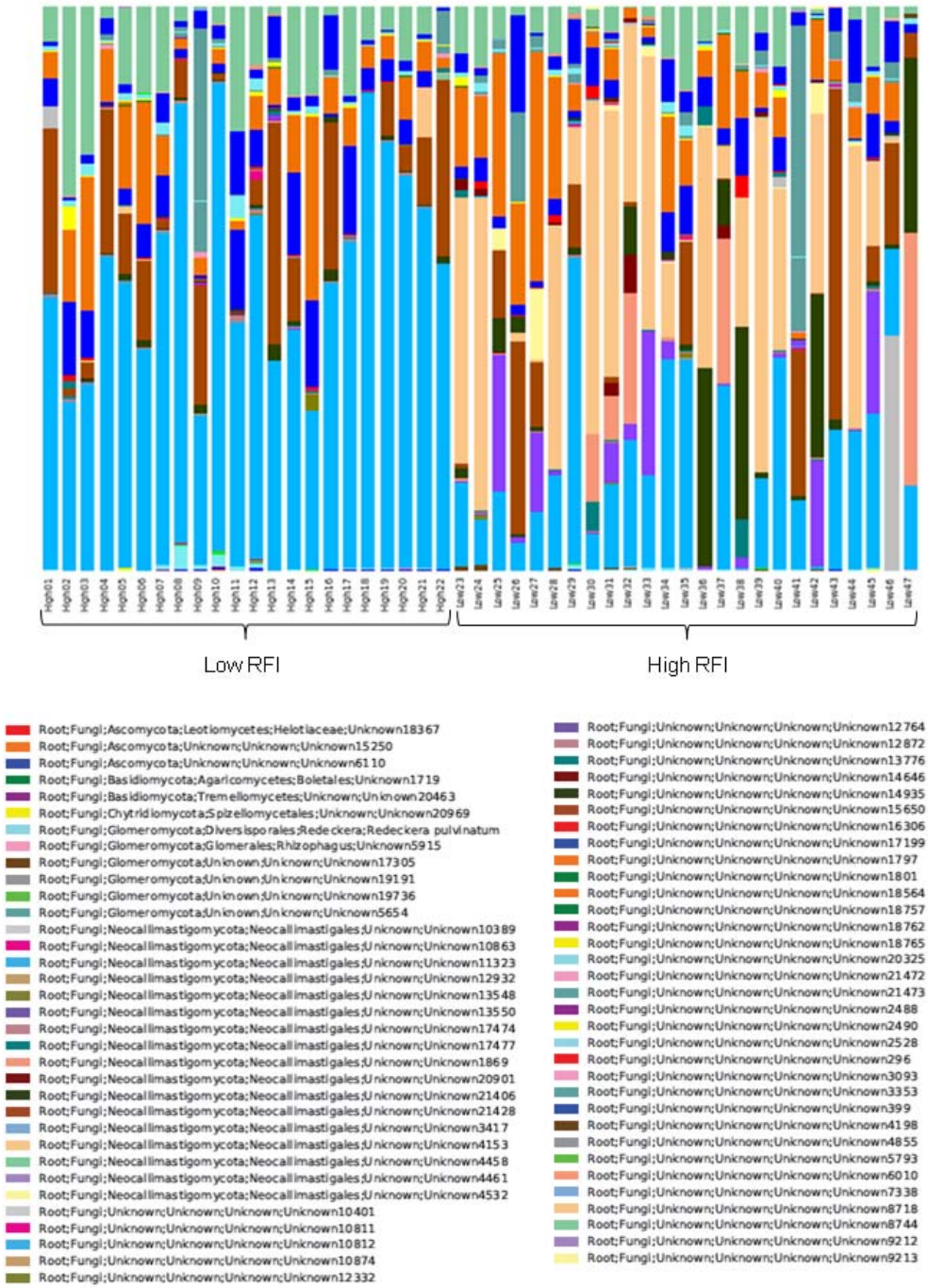

Figure 6-5 Abundance of rumen fungi classified to level of genus for individual cows divergent in feed efficiency which were fed a high quality winter pasture.

Table 6-5 Significant fungal OTU associated with RFI in cows feed a high quality diet.

OTU	Prob	Fold H/L	Consensus phyla (Unite DB)	Accession Number	BAST Description	E value	% Identity
51	0.028	0.01	Neocallimastigomycota	JF423672	Uncultured fungus	3E-139	97
136	0.011	0.03	Neocallimastigomycota	JF423509	Uncultured fungus	6E-137	97
301	9.5E-06	3.60	Neocallimastigomycota	JQ326217	Anaeromyces sp. JISSA-BRL-6	8E-61	82
478	0.026	3.92	Neocallimastigomycota	JQ326217	Anaeromyces sp. JISSA-BRL-6	2E-56	81
716	0.012	37.79	Unknown	AY091485	Anaeromyces sp. W-98	2E-67	83
766	0.048	2.63	Neocallimastigomycota	AY091485	Anaeromyces sp. W-98	1E-48	79
890	0.002	2.62	Neocallimastigomycota	AY091485	Anaeromyces sp. W-98	4E-59	81
1487	0.051	7.02	Neocallimastigomycota	JQ326217	Anaeromyces sp. JISSA-BRL-6	1E-49	79
1733	0.010	4.60	Neocallimastigomycota	AY091485	Anaeromyces sp. W-98	3E-55	81
1876	0.023	0.00	Neocallimastigomycota	JF423509	Uncultured fungus	4E-108	92
1901	0.038	0.00	Neocallimastigomycota	JF423509	Uncultured fungus	4E-128	95
2175	0.011	12.01	Neocallimastigomycota	GQ850325	Uncultured Anaeromyces 157	3E-50	80
2318	0.011	4.02	Neocallimastigomycota	AY091485	Anaeromyces sp. W-98	2E-52	80
2510	0.028	0.01	Neocallimastigomycota	JF423509	Uncultured fungus	2E-141	97
2674	0.024	0.01	Neocallimastigomycota	JF423658	Uncultured fungus	2E-132	95
3133	0.026	14.48	Glomeromycota	GQ850325	Uncultured Anaeromyces	2E-46	80
3140	0.011	8.05	Ascomycota	GQ850325	Uncultured Anaeromyces	8E-51	80
3621	0.012	11.11	Neocallimastigomycota	GQ850325	Uncultured Anaeromyces	2E-47	80

Representative sequences for significant OTUs (P<0.05) were identified using BLASTn. Fold difference represents the ratio of average sequence numbers for cows with high feed efficiency (low RFI) against cows with low feed efficiency (high RFI). Fold values >1 indicate higher abundance in high feed efficiency cows, while values <1 indicates higher abundance in low feed efficiency cows.

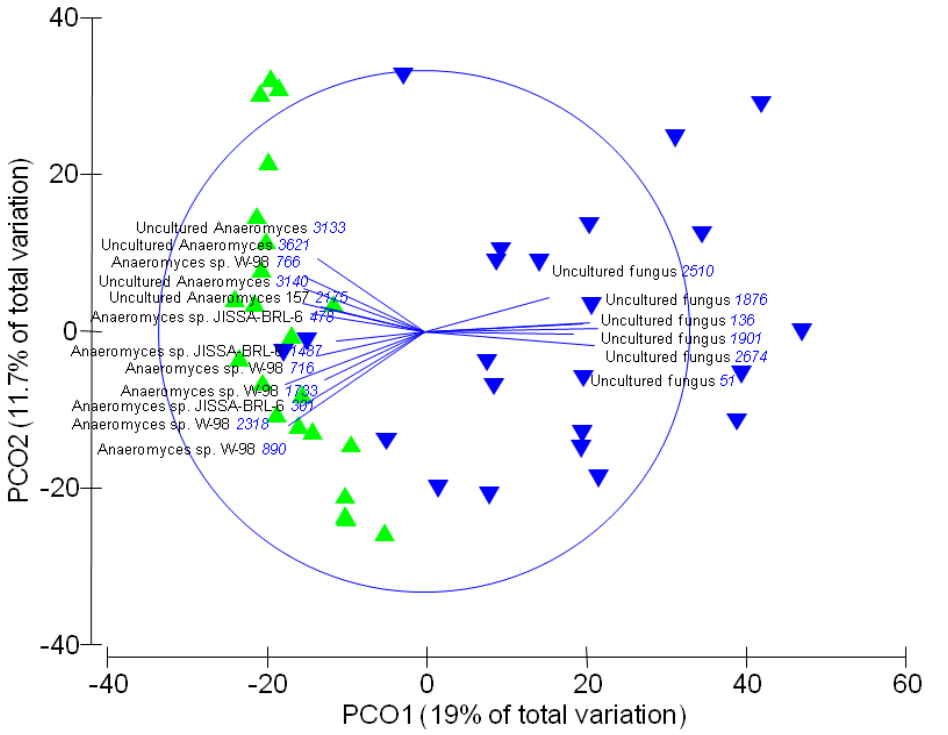

Figure 6-6 PCO ordination of rumen fungal communities from divergent RFI cow lines fed a high quality winter pasture. Rumen fungal communities are identified by low (▲) and high (▼) RFI lines. Overlaid onto the PCO are vectors of significant OTU identified by ANOVA indicating the association of OTUs with feed efficiency genotype.

6.4 Discussion

In this short term-term project we have developed and refined pyrosequencing methodology for the phylogenetic investigation of the rumen bacterial, archaeal and fungal communities. Furthermore, we have used these methodologies to investigating the influence of feed efficiency genotype and diet on rumen microbial communities which could be linked to changes observed in enteric methane production. The results of pyrosequencing showed that the rumen bacterial communities differed significantly with both diet and RFI genotype, and that diet had a greater influence. Rumen archaeal and fungal communities also differed significantly between high and low RFI cows on the high quality winter pasture. These data are consistent with our previous microbial profiling observations based on T-RFLP (B.CCH.1008). This demonstrates that high through-put microbial profiling technology, despite being of lower resolution, is a valid low cost screening tool for initial investigation of dietary and genetic methane mitigation methodologies, which can then be fully characterised using deep sequencing technology. Although pyrosequencing is becoming more accessible and affordable, the bioinformatics required to analyse the vast amounts of data generated is a constraint which does not make it applicable to large scale screening studies. However, a two staged approach of initial screening (microbial profiling) followed by full microbial community structure characterisation (deep sequencing) is justified and appropriate. Furthermore, the phylogenetic information generated by deep sequencing can allow subsequent quantitative screening tools to be developed to organisms of interest for future large scale studies.

Of the pyrosequncing methodology developed in this study the most challenging was for the rumen fungal communities. Fungal pyrosequncing studies published to date are not specific to rumen fungi, or target the highly conserved 18S rRNA region making species discrimination difficult (Buee et al., 2009; Handl et al., 2011; Hui et al., 2011; Jumpponen and Jones, 2009; Jumpponen et al., 2010; Lentendu et al., 2011). Alternately, targeting the variable genome regions spanning the ITS would result in the generation of multiple amplicons unsuitable for pyrosequencing. A further difficulty encountered with fungal pyrosequencing was the accurate classification of fungal data generated. This was due to the limited availability of good fungal classification databases suitable for rumen studies. A custom built database could be developed, but was beyond the scope of the current study. In the present study we used the UNITE database (Abarenkov et al., 2010), however, due to its limitation many of our OTU were misclassified as plant fungi. When those OTU sequences were further investigated using NCBI BLASTn we found them to be of rumen fungal origin (Neomallicastigomycota). Hence, all significant RFI related OTU identified in this study were further classified using BLASTn. Despite this effort many OTU could not be classified with 97% similarity to a species level. Classification of fungi will improve over the next few years as databases expand due to increased interest in fungal ecology particular in other ecosystems such as in soils.

Jones et al. (2011) showed that low and high RFI cows differed significantly ($P<0.05$) in methane production (0.34 $\pm$ 1.017 g CH_4/kg LW and 0.46 ± 0.023 g CH_4/kg LW respectively) when fed a high quality winter pasture but not when fed a low quality summer pasture (0.26 ± 0.013 g CH_4/kg LW and 0.26 ± 0.018 g CH_4/kg LW respectively). Changes observed particularly in the archaeal communities between the low and high RFI on the winter diet could relate to differences in methane emissions, as it is these communities that are directly involved in methane production. We found that *Methanosphaera stadtmanae* was less abundant in low RFI animals producing less methane. *M. stadtmanae* has been reported to be less

frequently associated with low RFI cows (Zhou et al., 2009), although methane production was not measured. We also found methanogenic archaeon related to CH1270 to be more abundant in the low RFI cows on the winter pasture. These cows produced significantly less methane than the high RFI cows on the same diet. Methanogenic archaeon related to CH1270 have been previously shown to be negatively correlated with methane yield (Zhou et al., 2011).

Significant changes in the bacterial and fungal communities between the low and high RFI on the winter diet were also observed. These changes could be indirectly linked to reduced methane production as these organisms provide substrate for the methanogens. We found that Firmcutes and Bacteroidetes were primarily associated with the high RFI cows on the winter diet and included Bacteroidales, Clostridiales, *Ruminococcaceae*, *Butyrivibrio* and *Prevotella*. A broader range of phyla were associated with the more feed efficient low RFI cows on the winter diet including Firmicutes, Bacteroidetes, TM7, Fibrobacteres, Lentisphaerae and Armatimonadetes. Rumen fungal sequences significantly associated with low RFI animals grouped closest to the genus *Anaeromyces* (ca 80% similarity), although these organisms are unlikely to be *Anaeromyces* but rather an unclassified group of rumen fungi. It was also observed that several sequences related to other uncultured rumen fungi (>92% similarity) were more abundant in high RFI cows. Research into the role rumen fungi may play in enteric methane production is less advanced than that for rumen bacteria and archaea. Hence there is a lack of data available for the accurate classification of rumen fungal sequences. Despite this difficulty we found that the rumen fungal communities were approximately 70% dissimilar between the divergent methane producing cows, which was on par with the level of dissimilarity observed in the rumen bacterial communities. By contrast the rumen archaeal communities, the usual focus of investigation in relation to methane production, were only 24% dissimilar. Our results indicate that the rumen fungi have a greater role to play in enteric methane production than previously thought, and should not be overlooked when developing methane mitigation methodologies.

Although no significant differences in methane production were observed between high and low RFI on the summer diet, in general cows on the summer diet produced less methane than cows on the winter diet (Jones et al., 2011). Some of the significant diet related changes observed in bacterial communities may be linked with this observation. OTU more abundant in the rumen of cows fed the low quality summer pasture were related to Alphaproteobacteria, Bacteroidales, *Ruminococcaceae*, *Prevotellaceae*, Chlamydiae, *TP21* and *YS2*. The majority of OTU more abundant in the rumen of cows fed the high quality winter pasture belonged to the Firmicutes and could be classified further to *Lachnospiraceae*, *Ruminococcaceae*, *Clostridiales XIII Insertae Sedis*, *Butyrivibrio*, *Coprococcus*, *Ruminococcus*, *Lachnospira pectinoschiza* and *Eubacterium cellulosolvens*. Bacteroidetes related to *Prevotella* and Bacteroidales were also more abundant in the rumen of cows fed the winter diet. Despite dietary difference there were significant differences observed in bacterial communities linked with RFI genotype, although these were not generally common between diets indicating that diet had a greater effect than genotype on rumen microbiota composiotion. Nevertheless, in our study we found the *Fibrobacter succinogenes* and *Ruminococcus albus* were more abundant in the low RFI regardless of dietary treatment. These cellulolytic organisms, which aid digestion, may be the reason these cows are partially more feed efficient.

7 Conclusion

Deep sequencing methodology has been developed to investigate the rumen bacterial, archaeal and fungal communities. Deep sequencing of rumen microbial communities from cows divergent in feed efficiency have identified organisms related to genotype and diet, but more importantly linked to significant reductions in enteric methane production. Together these data provide a detailed picture of the symbiotic relationships among rumen micro-organisms rather than a targeted and isolated look at one particular group of organisms. Although it is the methanogenic archaea which are generally considered most important in enteric methane production these micro-organisms should not be studied in isolation. In our study we found that the bacterial and fungal communities were vastly more variable between cows divergent in methane production than the rumen archaea.

The data generated in this project could be used to develop targeted quantitative assays for the investigation of organisms deemed of interest. As most micro-organisms have not been cultured to date the importance of unclassified micro-organism in methane mitigation should not be overlooked. The significant difference detected in the rumen microbiota by pyrosequencing, related to feed efficiency genotype and diet, are in support of the significant difference detected in the broader rumen microbial communities by microbial profiling. This indicates that the two technologies are complementary in evaluating and developing methane mitigation strategies in ruminant livestock.

8 Recommendations

- It is important to monitor the broader rumen microbiota (to include bacteria, archaea and fungi), as part of research on genetic and dietary methane mitigation strategies as this underpins our ability to understand changes in enteric methane production.
- Many studies only focus on the bacterial or archaeal communities when investigation methane mitigation strategies. We have shown that microbial community structure does not alter in isolation, but that there is a complex symbiotic relationship among micro-organisms. Furthermore, we have shown that rumen fungi can be vastly variable between cows divergent in their methane production, justifying further research in this area. Although, rumen fungal classification is currently limited by available databases this situation will improve rapidly with the expansion of fungal studies in various other ecosystems.
- Further work needs to be done to investigate rumen microbiota in animal trials demonstrating successful and significant methane mitigation strategies
- Specific and quantitative diagnostic assays should be developed to micro-oganisms linked with significant reductions in enteric methane production.
- Microbial profiling in conjunction with deep sequencing 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.

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