# The Rumen Methanogen Community and Diurnal Activity in Pasture Based Dairy Cows of the South Island

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A thesis
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by
Pavanpreet Kaur Benepal

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**Rumen Methanogen Effects of Different Diets in Pature Based Dairy Cows of South Island**

by

Pavanpreet Kaur Benepal

The South Island of New Zealand offers a novel medium for studying methanogen community because dairy cows are fed almost exclusively on pasture and yet have unusually high milk production due to high dry matter intakes from high quality pastures. This study was conducted to investigate the population structure and diurnal activity profiles of the resident methanogen community in pasture fed cows in this system, using the effect of various dietary supplementations. Since there are no current techniques available to satisfactorily measure actual methane production within the diurnal period in free grazing animals, molecular techniques were adapted and used for this research. Denaturing gradient gel electrophoresis (DGGE) was used for the intial screening of the populations. Similar to previous studies, *Methanobrevibacter* sp.was found to be the predominant methanogen. Though the supplements (grain, fat and monensin) used have previously been reported to alter rumen methanogen community, there were very few observed differences in the methanogen community structure detected with DGGE in the present experiment. It was concluded that this method is relatively insensitive in representing any smaller rumen methanogen shifts in response to dietary changes in these pasture based systems, possibly because these supplements did not act to eliminate methanogen groups, but to alter their activity, and this method was not suitable for assessing any changes within the diurnal period.

qPCR and qRT-PCR targeting the *mcr*A gene were then used for highly specific quantification of methanogen quantity and gene expression. However, the available methods for extracting RNA from rumen samples were not satisfactory to yield the necessary high quality RNA from rumen samples with high quality forage diets. A highly effective method was developed by adaptation from two existing methods of Whitford *et al*., (1998) and Gambino *et al*., (2008) which could simultaneously extract RNA and DNA from the rumen

samples. It was demonstrated to be highly effective for *mcr*A cDNA (mRNA) detection, a critical requirement for assessing activity of methanogens in this study.

The applicability of this qPCR and qRT-PCR technique to quantify changes in methanogen numbers and gene expression was then tested on pen fed, ruminally fistulated cattle where the diet was allocated either once or twice daily, and rumen samples were obtained every 4h for 24h periods. *mcr*A gene expression, an indicator of methanogen activity, was significantly reduced ($p \leq 0.05$, $t=4.90$) in twice daily fed cattle, but no significant ($p \leq 0.05$) change in methanogen numbers was detected. A clear diurnal pattern of methanogen activity in forage fed cattle was established, which is the first report of its kind. This technique was then used in a cross over design experiment with ruminally fistulated cows grazing high quality pastures, and serial diurnal rumen sampling, comparing a treatment group of either an administered methanogen inhibitor or an unsupplemented control. The method detected a decrease in methanogen numbers in the treatment group after 15d of fish oil supplementation ($p \leq 0.05$, $t=5.90$) but the greatest effect was observed on methanogenesis activity ($p \leq 0.05$, $t=7.90$). There was a clear diurnal pattern of methanogen activity related to the grazing behaviour of animals, with a significant increase ($p \leq 0.05$, $t=3.83$) in *mcr*A expression observed after 8h post prandially. This understanding of diurnal methanogen activity adds significantly to the knowledge of enteric methane production, and may guide the future methane mitigation strategies using targeted supplementation strategies within the diurnal cycle to mitigate the short term production of methane in ruminants.

**Keywords**: methane, DGGE, *mcr*A, qPCR, qRT-PCR, pasture, kale, fish oil, feeding frequency, diurnal pattern, grains, monensin, fat, grazing, rumen, gene expression, VFA, ruminants.

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Thank You Everybody!!!!

Pavan

# List of Abbreviations

| | |
|---|---|
| ATP | Adenosine triphosphate |
| cDNA | Complimentary deoxyribonucleic acid |
| $CH_4$ | Methane |
| $CO_2$ | Carbon dioxide |
| CP | Crude protein |
| CRC | Controlled release capsule |
| CT | Cycle threshold |
| DGGE | Denaturing gradient gel electrophoresis |
| DM | Dry matter |
| DMI | Dry matter intake |
| DNA | Deoxyribonucleic acid |
| GC clamp | Guanine-cytosine clamp |
| GE | Gross energy |
| GEI | Gross energy intake |
| GHG | Green house gases |
| $H_2$ | Hydrogen |
| IgG | Immunoglobulin G |
| LCFAs | Long chain fatty acids |
| MAUC | Mean area under curve |
| MCFA | Medium chain fatty acid |
| *mcr* | Methyl coenzyme M reductase |
| ME | Metabolisable energy |

| | |
|---|---|
| mRNA | Messenger ribonucleic acid |
| NAD | Nucleotide adenine diphosphate |
| NADH | Nucleotide adenine diphosphate hydrogenase |
| NDF | Neutral detergent fibre |
| NI | North Island |
| NZ | New Zealand |
| PCR | Polymerase chain reaction |
| PVC | Polyvinyl chloride |
| PUFA | Polyunsaturated fatty acid |
| qPCR | Quantitative polymerase chain reaction |
| qRT-PCR | Quantitative reverse transcriptase polymerase chain reaction |
| RF | Rumen fluid |
| RFI | Residual feed intake |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal RNA |
| RRT | Rumen retention time |
| $SF_6$ | Sulphur hexafluoride |
| SI | South Island |
| SSCP | Single strand conformation polymorphism |
| TMR | Total mixed ration |
| VFAs | Volatile fatty acids |
| WRC | Whole rumen contents |
| 16S rDNA | Genomic deoxyribonucleic acid |

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# Chapter 1
# Introduction

The increased demand for livestock products has led to a growth of the livestock sector. Production of milk has increased by 118% and of meat products by 298% over the last 25 years (Steinfeld and Wassenar 2007). But the increased production is coupled with an increase in greenhouse gas (GHG) emissions. Agriculture was responsible for 10–12% of total global non-carbon dioxide GHG emissions in 2005 with a 17% increase in methane ($CH_4$) and nitrous oxide ($N_2O$) from 1990 to 2005 and both gases contributing equally to the increase (Smith *et al.* 2007). Methane is considered 23-25 times more powerful than carbon dioxide ($CO_2$) in its global warming potential (Forster *et al.* 2007). The enteric methane fermentation from ruminants accounted for about 32% of total non-$CO_2$ emissions from agriculture in 2005 (Grainger and Beauchemin 2011; Smith *et al.* 2007). It has been proposed that if methane emissions grow in direct proportion to projected increase in livestock numbers, then global methane emissions from livestock production are expected to increase 60% by 2030 (FAO 2003; Grainger and Beauchemin 2011). The situation is even more sensitive for New Zealand (NZ) because it has an economy based upon agriculture which accounts for 48% of GHG emissions from NZ and methane emissions (mostly from ruminants) contributed 35% of GHG emissions from NZ in 2008 (Pinares-Patino *et al.* 2009).

Though the methanogens produce methane, a significant GHG, yet their presence in gastrointestinal tract of ruminants is not without reason. The ruminant effectively utilises complex carbohydrates, present in the form of cell wall constituents, in a manner not possible for monogastrics, and this extends the range of environments from which they can produce meat, fibre or milk. This unique digestive ability of ruminants is due to the presence of various anaerobic microorganisms comprising bacteria, protozoa, fungi and archaea in their digestive tract, the majority of which are symbiotically associated (Hobson 1997). These microorganisms ferment complex plant constituents under anaerobic conditions and provide the host with energy sources in the form of volatile fatty acids (VFAs) (Peters *et al.* 1990). Along with VFAs, hydrogen ($H_2$) and $CO_2$ are the major end products of fermentation by rumen bacteria, fungi and protozoa (Martin *et al.* 2010). The $H_2$ though, does not accumulate in the rumen because it is rapidly consumed by other microorganisms present in the rumen through the process called interspecies Hydrogen transfer (Moss *et al.* 2000). This benefits the growth and activity of $H_2$ producing bacteria by removing excess $H_2$ (substrate inhibition) and ensures continued degradation of fibrous plant material (Hegarty and Gerdes 1999;

Klieve 2009). The methanogens are the most efficient $H_2$ consumers in the rumen and reduce $CO_2$ to form methane. But this methane produced in rumen also causes a loss of 2-12% of the digestible energy of animals which could otherwise be utilized by animal (Johnson and Johnson 1995). This is significant in the commercial animal production and thus there has been a considerable interest to mitigate methane production in ruminants.

In the South Island (SI) of NZ, production system is different from other pasture based systems because it relies almost exclusively on a pasture based diet, but has a much higher production associated with this. In forage based systems, dry matter intake (DMI) is the critical factor for achieving high production because in other production systems such as the total mixed ration (TMR), any shortfalls in energy or protein can be managed by feeding the required amount of concentrates. In SI, the pasture is of an unusually high digestibility (mean monthly ME≈11-12 MJ/kg DM) with high crude protein (Gibbs and Laporte 2009), which supports high DMI by animals, and therefore high production per hectare.

The high pasture quality is produced by a sophisticated management system where the pre and post grazing herbage mass is maintained from 3200 to 1450kg DM/ha (Gibbs and Laporte 2009). This reduces the amount of leftover dead matter, ensuring availability of a higher quality pasture to animals and also increases pasture utilization (Lambert *et al.* 2004) by maintaining a higher stocking rate. North Canterbury in the South Island has the highest NZ average number of cows per hectare (3.31), followed by South Canterbury (3.20) and North Canterbury also has the largest average herd size (757). Along with this high stocking rate, the highest average production per dairy herd (285,412 kg of milk solids), per hectare (1,249kg) and per cow (377kg) were also recorded in North Canterbury (LIC 2011), South Island average herd sizes are increasing faster than North Island, likely due in part to increased profitability due to higher production.

This pasture management system is almost always associated with a specific daily grazing management that uses a single allocation of pasture, and this in turn alters the intake patterns of the cows to a short period of daily intake in which they eat the bulk of their ration (Gibbs and Laporte 2009). This does change the rumen patterns of energy and protein metabolism, and so is likely to also alter methane production. In addition, the success of this pasture management system in producing high yields of energy dense swards has encouraged the export of this approach to other temperate pasture based systems internationally, including South America and Ireland. As a consequence, an increasing number of dairy cattle are raised under this system.

It has been observed that pasture based diet might have higher methane emissions per kg DMI, but when measured over unit of product produced, the net carbon emissions might actually be lower than TMR based systems (Nagel *et al.* 2003). Further, the quality of pasture is a major governing factor for optimum DMI and the resultant methane emissions (Benchaar *et al.* 2001; Boadi *et al.* 2004). It has been shown that at high intakes, methane production per kg DM of diet can be reduced by 20 to 40% (Johnson and Johnson 1995), and by increasing milk production the methane per kg milk produced can be decreased by ≥16% (O'Mara 2004). Thus, the South Island production system offers a novel medium for analysing the methanogen community in high producing animals supported by a higher DMI from high quality pastures.

But no study of the rumen methanogen community in cattle, or the effects of the grazing system typically used on methanogen community structure or activity profiles within the diurnal cycle, has been conducted to date in this unique and fast growing system. A significant challenge is posed by the requirement to investigate the animals in actual grazing conditions because both the actual dynamics of rumen methanogens and any consequent mitigation strategies can only be satisfactorily studied under normal grazing conditions.

# Chapter 2
# Review of Literature

## 2.1 Techniques for studying methanogenesis

In order to investigate methanogenesis and how the exaggerated diurnal pattern of rumen activity may open possible mitigation strategies in South Island pasture based systems, it is important to study the rumen methanogen community and the methanogenesis under actual grazing conditions. The techniques available for measuring methane production are discussed.

### *2.1.1 Estimation techniques*

The techniques which have been developed for enteric methane emission measurement can be classified into direct and indirect measurements. The direct measurements include methane measurements through total or partial enclosure of animals while indirect methods include use of tracers or estimations based upon rumen fermentation characteristics (Pinares-Patino and Clark 2008).

#### 2.1.1.1 Respiration calorimetry

This is a technique in which direct measurement of gas emissions from animals can be done. The animals are housed in closed chambers and samples of gas can be collected as per required interval. This method has been used for measuring methane emissions in ruminants across a range of studies (Denman *et al.* 2007; Kinsman *et al.* 1995; McCrabb and Hunter 1999). Significant differences in methane emissions between control and Bromochloromethane supplemented animals (Denman *et al.* 2007) or ciliated and ciliate free cattle (Whitelaw *et al.* 1984) were detected using this technique. But a limitation of this technique is that the animals are kept in confined space and have to be fed manually which is similar to the stall fed animals but may not accurately imitate the pasture grazing systems. As it has been recognised (Pinares-Patino *et al.* 2007) that voluntary feed intake varies with the quality of pasture and manner of feeding (stall fed vs. grazing), which further effects other rumen parameters such as rumen fill, rate of fermentation and passage, the overall methane production from an animal in controlled conditions may be different as compared to the actual grazing conditions (Pinares-Patino *et al.* 2003a; c). Further, the extensive use of this technique is limited by the number of animals which could be analysed and the relative costs.

#### 2.1.1.2 Sulphur hexafluoride ($SF_6$) estimation technique

The $SF_6$ tracer technique was developed at Washington State University by Johnson and Johnson (1995). In this technique a known source of $SF_6$ , a calibrated brass permeation tube of known weight, dimensions and $SF_6$ release rate, is placed in the rumen prior to the experiment (Ulyatt *et al.* 1999). The experimental animals are fitted with a halter which supports an inlet tube placed close to the nose and is connected through a capillary tube and valve to a PVC collection canister. The methane emission rate ($Q_{CH4}$) is calculated as:

$$Q_{CH4} = Q_{SF6} \text{ X } ([CH_4 \text{ sample}] - [CH_4 \text{ ambient}]) / ([SF_6 \text{ sample}] - [SF_6 \text{ ambient}])$$

where, $Q_{SF6}$ is the calibrated rate of permeation from the $SF_6$ tube and $[CH_4]$ and $[SF_6]$ are concentrations of methane and sulphur hexafluoride, respectively, in the collection yoke and background concentrations.

Many studies in pastoral systems of New Zealand, Australia and other countries have used this technique for methane production estimation (Cavanagh *et al.* 2008; Munger and Kreuzer 2008) and there are some reports that this technique is broadly comparable to respiration calorimetry methods (Boadi and Wittenberg 2002; Grainger *et al.* 2007). It has been used for measuring methane emissions from ruminants for national inventory purposes using a large number of animals in actual grazing conditions (Cavanagh *et al.* 2008; Clark *et al.* 2008; Swainson *et al.* 2008). It has been able to show significant differences in methane emissions from animals fed fodder of varying digestibility (Chaves *et al.* 2006; DeRamus *et al.* 2003), or upon supplementations (Grainger *et al.* 2008; Jordan *et al.* 2006b). But some studies (Ulyatt *et al.* 1999; Wright *et al.* 2004) have found 2-3 fold variability in methane estimations using calorimetry and $SF_6$ technique. Therefore, the reliability of this technique has been questioned. Also, the methane production estimation using this technique has been found to be dependent upon the permeation rate of the $SF_6$ tube. It has been found to be higher for the tubes with higher permeation rates than those with lower permeation rates (Pinares-Patino and Clark 2008). Thus it is quite possible that animals with tubes having higher permeation rates would record higher methane production levels than those with lower permeation rate tubes. Researchers have even found differences between the pre-experimental and post-experimental permeation rates of the tubes (Pinares-Patino and Clark 2008).

An important point to consider is that this technique does not account for the methane released through flatus. Normally the methane production from hindgut is about 13%, but there has been evidence to show that under cold conditions, a higher feed intake or intake of highly digestible fodder, where there is an increase in ruminal passage rate, hindgut may

account for up to 32% of the total methane production (Kennedy and Milligan 1978). Therefore this technique needs to be corrected for any dietary modifications. This is significant in the South Island Production system where animals have high DMI of highly digestible fodder. Grainger *et al*., (2007), found that the methane production rate from animals fed different diets actually depended upon the technique used for methane estimation i.e. chamber vs. $SF_6$. Williams *et al*., (2011) have reported that even the atmospheric concentration of methane can alter the methane production estimates. These shortcomings raise some serious issues regarding the use of this technique in studies where subtle differences over a diurnal period have to be estimated accurately.

Importantly, the errors associated with this method necessitate large sample group numbers, and multiple day sampling. These requirements invalidate the use of the method for investigation of rumen methane production within the diurnal cycle.

#### 2.1.1.3 Inverse dispersion technique

This technique was developed by Gao *et al*., (2011) to measure the methane from a herd of feedlot cattle. This technique was reported to be suitable for analysing the methane production pattern from a herd over a diurnal period. The diurnal pattern was reported to be related to the grazing pattern of animals. But this technique has its limitations. It is not possible to analyse individual methane productions from animals given different treatments in a herd measurement and cannot accurately measure the actual rumen methane production within the diurnal windows due to the characteristic lag between rumen methane production and its eructation.

Another tunnel method (Lockyer 1997) has been used to measure methane emissions from sheep and calves. The animals are put in portable tunnels, and the emissions can then be measured from the enclosed atmospheric changes. The methane emissions can be measured from animals grazed on different types of pastures or under different treatments with a high degree of accuracy (Judd *et al.* 1999; Murray *et al.* 2001; Murray *et al.* 1999). This method however has its limitation in the ability to confine adult cattle under portable tunnels. While this method has been used for small ruminants, there are no extant cattle studies, and similar to the inverse dispersion technique, it also uses multiple animals and relies on eructated methane.

#### 2.1.1.4 *In vitro* technique

This technique has been used extensively in understanding the methanogen community and its behaviour under modified environment. It can be performed by two ways. One method is

when the rumen contents are taken from the rumen and incubated for a period of 1-2 hours. The gas produced within that incubated sample is then analysed by Gas Chromatography (GC) in order to estimate the potential of methane production from that sample. Other method is through using a Rumen Simulation Technique (RUSITEC) (Czerkawski and Breckenridge 1977; Durand *et al.* 1988). In this the rumen contents are collected and put in chambers where they can be incubated for a long period by simulating the rumen conditions. Simulation of rumen environment is achieved by adding buffers to maintain the pH. The chambers are equipped with gas collection apparatus and samples of gas can be collected and analysed by GC. The impact of external modifications such as addition of fats or other chemicals on different microbial communities inside the chambers can also be analysed (Dohme *et al.* 2000; 2001; Dong *et al.* 1997; Machmuller *et al.* 1998; Neumann *et al.* 1999). This technique has provided valuable information to understand the impact of various mitigation options on the rumen methanogenic community (Dong *et al.* 1999; Durand *et al.* 1988; Hess *et al.* 2003; Newbold *et al.* 2005; Sliwinski *et al.* 2003; Wallace *et al.* 1981).

Though this technique has provided good foundation information, it cannot possibly replicate actual rumen conditions. The samples drawn from rumen contents can only tell the potential of that sample for methane production but not the actual methane production at that time. Rumen has a very dynamic environment where there is a constant influx and outflow of various biological chemicals such as saliva, acids, enzymes, etc. along with rapid exchange of cations and anions (Wales *et al.* 2004). $H_2$ which is the major end product of rumen fermentation is not allowed to accumulate and is being rapidly utilized for the fermentation to proceed continuously. This may not be the case in case of *in vitro* technique. There is every possibility that $H_2$ may accumulate over time and may cause substrate inhibition thereby affecting the rate of fermentation. It may lower the pH and it has been reported that not all microorganisms function optimally at a lower pH (Russell and Wilson 1996). Further, as discussed in section 2.1.2, methanogens have proven difficult to culture in laboratory conditions because of their very specific requirements for substrates (Wolin *et al.* 1997). Thus it is possible that many organisms who have an active role to play in rumen methane production in an actual rumen environment may not be contributing significantly under *in vitro* conditions. This may have a significant impact on the conclusions which we can draw from such experiments. This technique therefore is limited towards its use for conducting studies in animals under actual grazing conditions.

Since the methods commonly used to measure methane production are not suitable for analysing diurnal methane production in pasture based systems, an alternative approach could

be the use of molecular techniques. But before discussing the molecular techniques for studying methanogen community, it is important to understand the specific methanogen structure and biochemistry which form the basis for any molecular study.

### *2.1.2 Structure and biochemistry of methanogens*

The methanogens are classified under a domain Archaea which has been defined as a separate domain from bacteria and eukaryotes based on the specific 16S ribosomal sequences (Schafer *et al.* 1999; Woese *et al.* 1978). Chemically, the archaea differ from bacteria by the presence of membrane lipids consisting of diether or tetraether linked isoprenoids (De Rosa and Gambacorta 1988) and lack of muramic acid containing peptidoglycans, though some species may have a pseudomuramic acid (Kandler and Hippe 1977). Another significant difference between the archaea and bacteria is in the structure of archaeal ribosomes. The subunit structure of archaeal ribosomes has a closer resemblance to the eukaryotes than the true bacteria and the transcription machinery i.e. the structure of DNA dependent RNA polymerase is also different (Schafer *et al.* 1999).

Within this domain, archaea are highly diverse organisms (Boone *et al.* 1993). In addition to methanogens there are two other physiologically distinct groups of archaea; the thermoacidophiles and the halophiles which differ widely in their energy transducing mechanisms. Some rely on aerobic as well as anaerobic respiration while some halobacteria use photosynthetic processes for producing some of their energy (Lewalter and Muller 2006)

The methanogens are an ubiquitous group of microbes and can exist in extreme conditions from highly thermophilic or mesophilic to as low as 2$^{\circ}$C temperatures, from non-saline to halophilic, strictly anaerobic to those having an ability to survive in aerobic and water conditions (Zinder 1993). Their distinct structural and functional characteristics e.g. the presence of specific membrane lipids which maintain a very low ion permeability of membranes and allow chemiosmotic charge separation under high temperature or at very low pH conditions help in the survival of methanogens in extreme conditions (Zinder 1993).

Eight species of methanogens have so far been reported to be cultured from the rumen, namely: *Methanobrevibacter ruminantium, Methanosarcina barkeri, Methanosarcina mazei, Methanobacterium formicicum, Methanobacterium bryantii, Methanobrevibacter olleyae, Methanobrevibacter millerae,* and *Methanomicrobium mobile* (McAllister *et al.* 1996; Zhou *et al.* 2011). Only *Methanobrevibacter ruminantium* and *Methanosarcina barkeri* have been found at populations greater than $10^6 ml^{-1}$in rumen fluid and are thus the only two assumed to play a major role in rumen methanogenesis (Boadi *et al.* 2004; McAllister *et al.* 1996; Moss *et*

*al.* 2000; Ouwerkerk *et al.* 2008). Many uncultured species/ strains of methanogens have been isolated with culture independent techniques like 16S rRNA gene clone libraries and DNA sequence analysis (Klieve 2009; Klieve *et al.* 2009; Ouwerkerk *et al.* 2008; Wright *et al.* 2008). These species owing to very specific requirements for particular substrates and physiological conditions are difficult to culture.

Methanogens produce methane through a process called methanogenesis and use a very narrow range of substrates which include $H_2$ and $CO_2$ along with other substrates like formate, acetate, methanol, methylamines, dimethylsulfide or some alcohols (Wolin *et al.* 1997) The formation of methane is a character unique to the methanogens. This ability is due to the presence of three coenzymes in methanogens which have not been found in other microorganisms namely, coenzyme $F_{430}$, coenzyme M and a factor B. All of these coenzymes are involved in various oxidation and reduction processes during methane formation (Baker 1999; Boadi *et al.* 2004). Methanogenesis can occur through 3 pathways (Boone *et al.* 1993):

a. $CO_2$- reducing
b. Methylotrophic- by performing methyl group transfer
c. Aceticlastic-by cleaving acetate and reducing the methyl group to methane while oxidizing carboxyl group to $CO_2$

The reactions during formation of methane from $CO_2$ and $H_2$, the major pathway for methanogenesis under anaerobic conditions in rumen (Hungate 1967; Hungate *et al.* 1970; Janssen 2010) and the aceticlastic pathway can broadly be depicted by the flow chart in Figure 2.1.

The reduction of $CO_2$ to methane proceeds via coenzyme bound intermediates methanofuran (MFR), tetrahydromethanopterin ($H_4$MPT) and coenzyme M (H-S-CoM) which are subsequently reduced and oxidized along with specific electron carriers coenzyme $F_{430}$ , the H-S-HTP (N-7-mercaptoheptanoyl-O-phospho-L-threonine) and the coenzyme $F_{420}$ (Eirich *et al.* 1978; Thauer *et al.* 1993).

The formation of methane from methanol is similar except that from methanol there is either direct formation of methane through coenzyme M, or by the methanol oxidation to $CO_2$ through a pathway which initially proceeds in the opposite direction to the $CO_2$ reduction pathway, before transfer of the methyl group to coenzyme-M, which is then reduced to methane (Ferry 1993). Thus, the last step in the formation of methane from all pathways proceeds through coenzyme M which is reduced by the methyl-CoM reductase (*mcr*) enzyme

and a cofactor $F_{430}$ exclusive to the archaea which acts as a prosthetic group to the enzyme *mcr* (Thauer 1998).

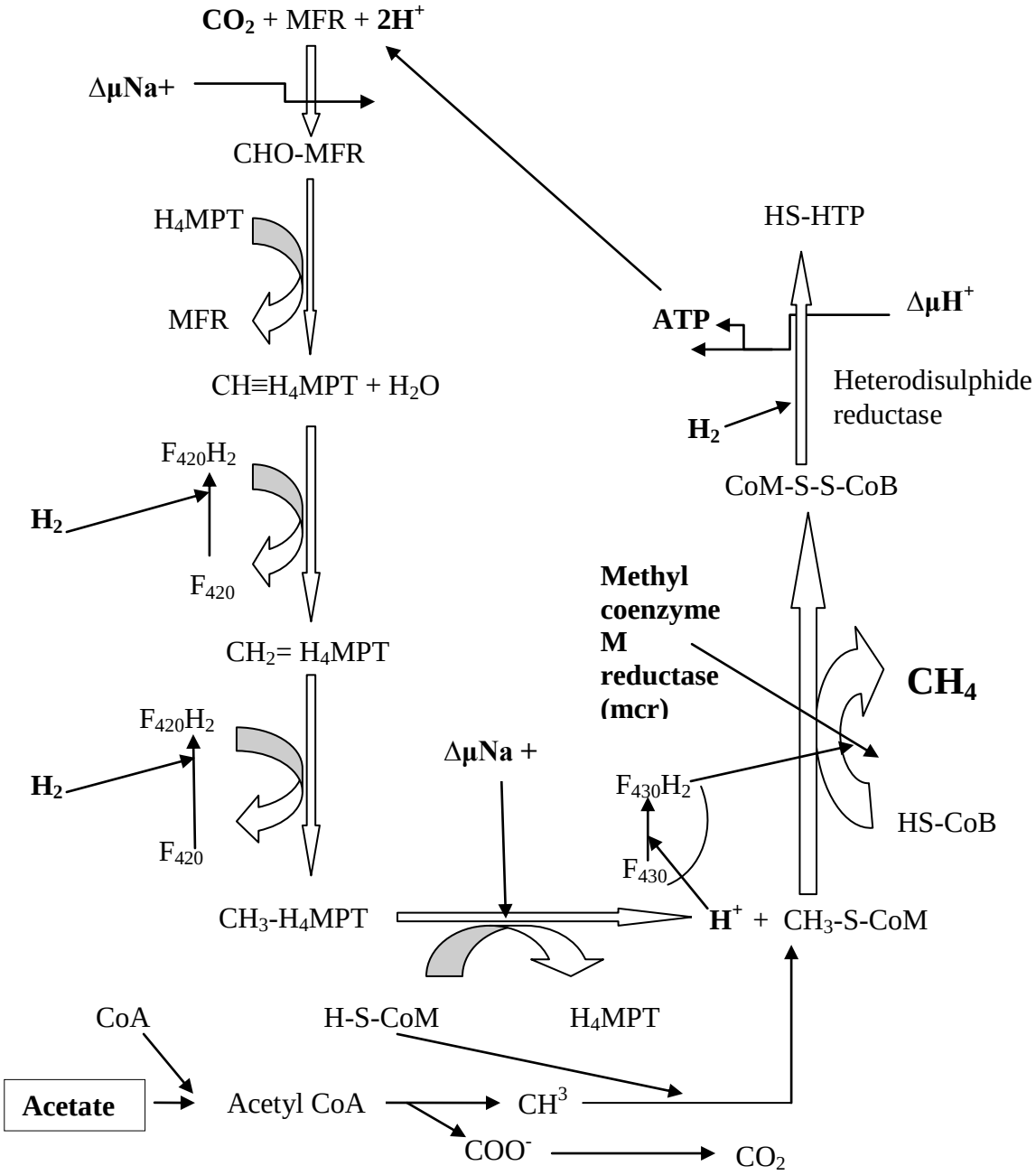



**Figure 2.1 Metabolic pathway of methanogenesis from $CO_2$, $H_2$ and acetate.**

**MFR- methanofuran, $H_4$MPT- tetrahydromethanopterin, HS-CoM-Coenzyme M, HS-HTP-N-7-mercaptoheptanoyl-O-phospho-L-threonine, $F_{420}H_2$- reduced coenzyme $F_{420}$, ΔμNa+-elecrochemical sodium ion gradient, $\Delta\mu H^+$-electrochemical hydrogen potential (Thauer *et al.* 1993).**

It can thus be concluded that methanogens have highly conserved 16S ribosomal sequences which make them a distinct group from other microorganisms. Also, they are able to produce methane because of the presence of exclusive coenzymes in them, especially a coenzyme M which is reduced by an enzyme *mcr* in the final step of methane production from all substrates

through all pathways. These unique sequences can thus be targeted to perform culture independent molecular analysis of the methanogens.

### *2.1.3 Molecular techniques*

The study of structure and function of microbial communities in their natural environment by using genomic techniques is often referred to as metagenomics. It involves molecular techniques targeting the conserved area of ribosomal RNA (rRNA) or rRNA gene which encodes this RNA and has made possible the study of earlier unidentified and uncultured microorganisms, thus improving upon our understanding of the microbial biodiversity and population dynamics (Firkins *et al.* 2007). This assumes greater significance in the study of methanogenesis because the methanogens are fastidiously anaerobic organisms and some are difficult to culture (Wright *et al.* 2008). Since the 16S ribosomal RNA (rRNA) and their encoding genes are conserved in archaea as well as other prokaryotes (Pace *et al.* 1986 ; Wheelis *et al.* 1992), their study has helped to classify and discover new species of microbes. A significant number of species of the rumen methanogenic community have been determined using the conserved 16S rRNA gene (Ouwerkerk *et al.* 2008; Skillman *et al.* 2006; Whitford *et al.* 2001; Wright *et al.* 2007; Wright *et al.* 2008; Yu *et al.* 2008c; Zhou *et al.* 2009; 2010; Zhou *et al.* 2011).

The different molecular technologies have made it possible to understand the molecular mechanisms of methanogenesis and its potential impacts on enteric methane emissions (Guo *et al.* 2008; Hart *et al.* 2009; Mohammed *et al.* 2011). They present a direct way of estimating the effect of any mitigation strategy on methanogen populations or their methane producing activity and thus an indirect way of estimating the actual methane emissions(Hart *et al.* 2009; Popova *et al.* 2011).

The methodology involves extraction of nucleic acids, amplification by polymerase chain reaction (PCR) using strain specific or group specific primers and then analysis of the PCR products using fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), restriction fragment length polymorphism (RFLP), single strand conformation polymorphism (SSCP) etc. Molecular techniques which have been used to study rumen molecular ecology are summarized (Deng *et al.* 2008) in figure 2.2 and are discussed below.

#### 2.1.3.1 DNA/RNA based techniques

The 16S rRNA/rDNA techniques are based upon extraction of nucleic acids, amplification, cloning and sequencing to classify the organisms based upon their phylogeny. The similarity

search can be performed online using many sequence databases available on the internet such as Basic Local Alignment Tool (BLAST) (http://www.ncbi.nlm.nih.gov/Blast) in GenBank (Benson *et al.* 2005; Madden *et al.* 1996). Based upon the knowledge of common sequences, group specific probes can be designed which are then used in hybridization techniques for amplifying/cloning and sequencing studies to study the activity and spatial distribution of an organism or a group of microorganisms (Amann *et al.* 1992; Amann *et al.* 1995; Deng *et al.* 2008).

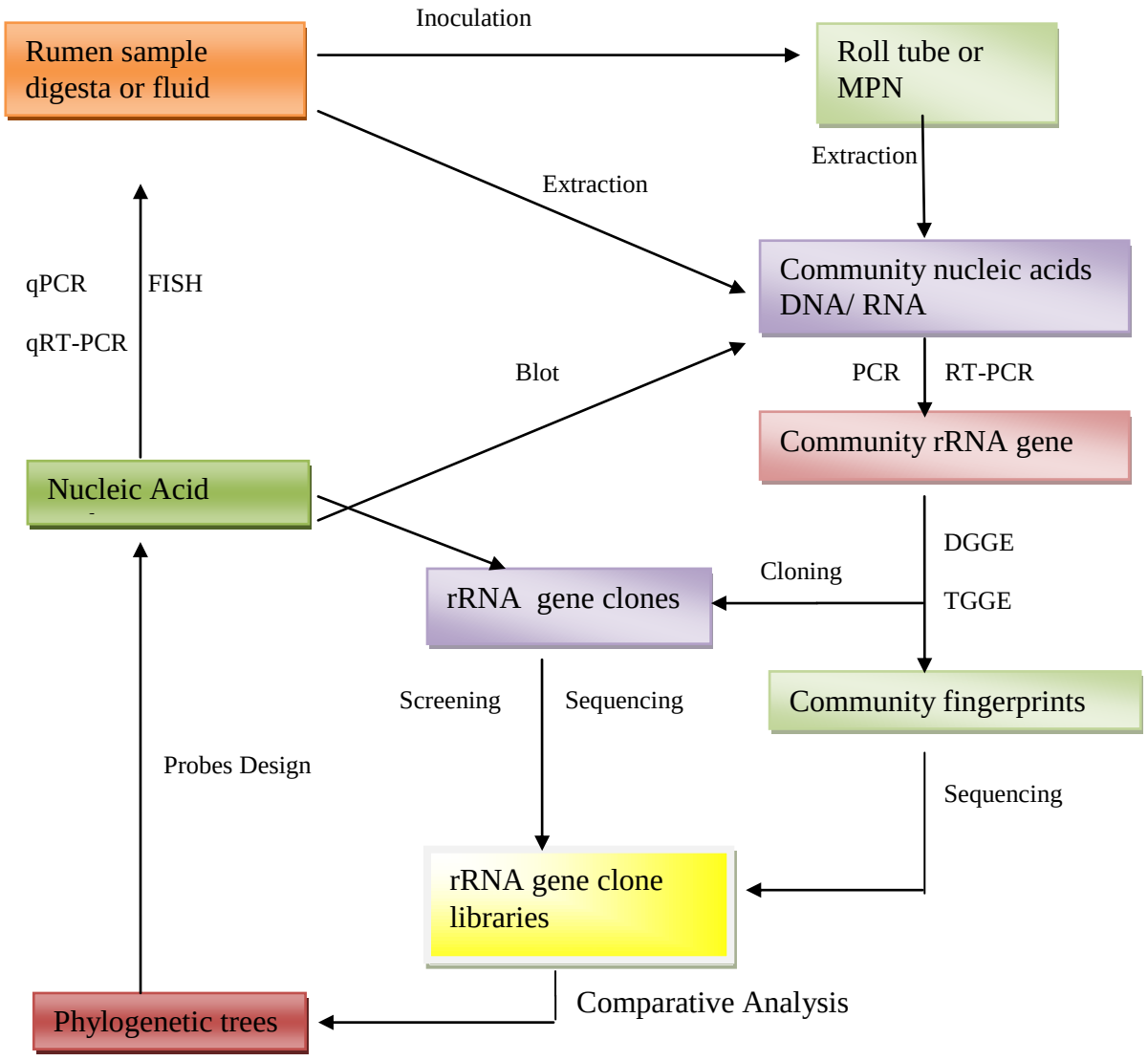



**Figure 2.2 Molecular schematics based on 16S rRNA/rRNA gene used to analyze rumen microbial ecosystem (Deng *et al.* 2008).**

#### 2.1.3.1.1 Polymerase chain reaction (PCR)

PCR is an *in vitro* method for enzymatically synthesizing defined sequences of DNA. The reaction involves use of two oligonucleotide primers that hybridize to complimentary strands and flank the target DNA sequence that is to be amplified. The elongation of the primers is

catalysed by a heat stable DNA polymerase such as the Taq DNA polymerase (Saiki *et al.* 1988). Repetitive series of cycles involving template denaturation, primer annealing and extension of annealed primers by DNA polymerase results in exponential accumulation of a specific DNA fragment. The use of this technique allows for even trace amounts of genetic material to be amplified for further evaluation.

This technique is being extensively used in ruminants as well as other ecological systems as the initial step for DNA amplification before creating clone libraries for phylogenetic analysis, studying the cultural diversity in a particular set of conditions through fingerprinting techniques such as DGGE, SSCP etc. (Hales *et al.* 1996; Hook *et al.* 2009; Kocherginskaya *et al.* 2001; Wanapat *et al.* 2009; Watanabe *et al.* 2004). However, the specificity of primer pairs is important. It has been reported that a same sample can give different predominant phylotypes with different primer pairs. As an example, in a study by Skillman *et al*., (2006), using same samples the primer pair 21f/958r amplified mostly *Methanosphaera stadtmanae* like sequences, while the primer pair Arch f364/Arch r1386 amplified mainly *Methanobrevibacter* sequences. It has also been found that while using universal archaeal primers, bacterial 16S rRNA gene sequences can also be amplified (Zhou *et al.* 2009; Zhou *et al.* 2011). However, after evaluations of different hypervariable regions of archaeal 16S rRNA genes for specificity in profiling of methanogens, it has been reported that the primers 344 F-GC and 522R which target the V2V3 region of methanogens, cover the widest range of methanogen species in the PCR based denaturing gradient gel electrophoresis (DGGE) analysis (Ouwerkerk *et al.* 2008; Yu *et al.* 2008a).

#### 2.1.3.1.2 Denaturing gradient gel electrophoresis (DGGE) and single strand polymorphism confirmation (SSCP)

DGGE is a genetic fingerprinting technique that is used to analyse complex microbial communities from a diverse range of environmental samples. It involves amplification of target DNA/cDNA through PCR using primers specific to the species of interest and then separation of amplified double stranded DNA fragments up to 500 base pairs (bp) in length using a denaturing gradient gel (Muyzer *et al.* 1993b). The denaturation is achieved in the gel through use of denaturing chemicals (urea, formamide) in the polyacrylamide gel, with the concentration of denaturants increasing from top to bottom of the gel. Since the PCR amplified fragments are similar in size, their separation is achieved by their discrete melting temperatures (Kocherginskaya *et al.* 2005). The melting temperature and hence the denaturing ability of a species specific sequence is governed by the ratio of guanine (G) and cytosine (C) composition to adenine (A) and thymine (T) component. Higher is the GC to AT ratio in a fragment, more able it is to remain intact in a denaturing gradient and can therefore travel

further down in a denaturing gradient. When a fragment reaches the point in the gel where it is denatured by the concentration of denaturants, it stops. This results in formation of banding patterns which vary with the community being studied. For a higher degree of resolution during analysis of banding patterns in a DGGE gel, a GC clamp is attached to 5' end of one of the primers during PCR amplification (Muyzer *et al.* 1996). The GC clamp prevents complete separation of two DNA strands in the gel thereby avoiding smeared bands.

The band patterns produced from amplification of conserved 16S rRNA gene (which encodes the rRNA) may reflect the community composition of the target organisms. By amplifying the 16S rRNA, the active members of that community can be studied. This technique has been used widely for studying community molecular diversity of a wide variety of ecosystems including rumen microbes such as protozoa, bacteria and archaea. With this technique it is possible to do a rapid and simple monitoring of microbial community structures (Cheng *et al.* 2009) over a range of studies which may involve dietary and environmental patterns (Cheng *et al.* 2009; Hook *et al.* 2009; Wanapat *et al.* 2009; Watanabe *et al.* 2004). Specific bands showing variability amongst different diets or treatments can be isolated and identified by cloning and sequencing. This can help in establishing species-substrate linkages. With the use of cDNA as a template, it is also possible to visualise the active populations and patterns of their gene expression (Popova *et al.* 2011).

This technique has been successfully used in earlier studies to detect changes in methanogen community after change in diets. There were altered PCR-DGGE profiles of methanogens upon fat supplementation reported by Yu *et al*., (2008), with an increased presence of *M. stadtmanae* while that of *Methanobrevibacter* sp AbM4 decreased. Similarly, Zhou *et al*., (2010) also observed a strong shift in the PCR-DGGE profiles of rumen methanogens from one that consisted primarily of *M. ruminantium* in cattle fed a growing diet (low cereal concentrate) to a mixture of different species in cattle fed a finishing diet (high cereal concentrate). In another study, DGGE was used to detect the effects of chloroform, a powerful inhibitor of methanogens (Knight *et al.* 2011).

However, it has been suggested that DGGE profiling does not capture the full microbial diversity because only abundant populations are detected (Kocherginskaya *et al.* 2001) and is also subject to PCR bias. Klieve *et al*., (2007) also noted that that apparently intense bands might be misleading because of multiple sequences from potentially diverse species recovered per band due to difference of only a few base pairs. Also, some species may contain several copies of the same gene differing slightly in sequence and therefore, one species may produce several bands (Muyzer and Smalla 1998).

An alternative fingerprinting technique available is the single strand polymorphism confirmation (SSCP) which separates PCR amplicons having the same length but different nucleotide sequences on the basis of the conformation of single-stranded DNA (Schwieger and Tebbe 1998). In this technique, one type of strain or species would produce two bands, one for each strand of DNA. Thus it is quite possible that more than one species might be represented in one band and also that one species may present itself in multiple bands which are the same limitations as that of DGGE. Further, given the fact that one species would be presented in two bands, this technique may have more applicability in systems where the methanogen community to be analyzed is not very diverse. But as has been reported in many earlier studies that the methanogen community in rumen is very diverse with almost 25 bands exhibited in a single DGGE gel (Mohammed *et al.* 2011; Zhou *et al.* 2010), it may not be possible to apply SSCP for the rumen methanogen community profiling. Also, the animals in this study are fed on pasture based diets which are reported to have even higher diversity than concentrate based diets or the TMR systems (Ouwerkerk *et al.* 2008).

Further, in a study to compare soil communities for a direct comparison between DGGE, SSCP and another technique terminal restriction length fragment polymorphism (TRLFP), it was found that there was no difference between these three techniques for obtaining the community profiling (Smalla *et al.* 2007). Thus, DGGE can offer a relatively simple and systematic approach for initial profiling and comparison and it is always possible to reduce the experimental errors with improvement in technology and protocol. To make it more sensitive, it has also been recommended that the bands should be verified by sequencing and any changes detected because of different treatments, should be verified by quantitative techniques such as real–time PCR (Firkins *et al.* 2008).

#### 2.1.3.1.3 Real –time PCR (qPCR)

In the conventional PCR methods, the PCR products are quantified at the end of the PCR reaction whereas in quantitative or real-time PCR (qPCR), the detection and hence quantification of each amplicon is done at the end of each cycle during the phase of exponential amplification of products where theoretically each cycle results in doubling of the product (Denman and McSweeney 2005). There are different approaches for performing a quantitative PCR. The first step in the design of any assay generally involves the use of a fluorescent DNA binding dye e.g. SYBR® Green which binds to the minor groove of double stranded DNA. The dye radiates higher fluorescence when bound to a double stranded DNA as compared to when it is in free solution. As the amplification reaction proceeds and more double stranded amplicons are produced, the amount of fluorescence detected also increases.

A more specific technique involves the use of specific oligonucleotide probe which is complementary to one strand of the amplicon e.g. the TaqMan® assay. A flouorophore is attached to the 5' end of the probe and a quencher to the 3' end. The probe binds to the amplicon during each annealing step of the PCR. When the Taq polymerase extends from the primer which is bound to amplicon, it cleaves the 5' end of the probe effectively separating the fluorophore from the quencher. This increases the amount of detected fluorescence.

For quantification, the cycle at which fluorescence is detectable above the background level during exponential phase of amplification is termed the cycle threshold (CT). Quantification is either done directly by comparing and plotting against a standard curve generated using known quantities of similar DNA, or by comparison between the target gene and a 'house-keeping' or 'reference' gene. Theoretically there is quantitative relation between the amount of starting material and the amount of amplicon at any given cycle within the exponential phase of amplification. As such a difference of a single CT value represents a twofold difference in starting material whereas a difference of 3.331 CT value represents a 10 fold difference in starting material. For direct quantification, the experimental value is plotted against the standard curve, and the starting concentration of that experimental sample can then be deduced.

While TaqMan assay has the advantage of being highly specific in product amplification, needing minimum optimization and thus providing very robust assays, it is cost prohibitive for a smaller sample size. SYBR® Green on the other hand is cost effective for lesser number of samples but it can give non-specific amplification. Because, it will bind indiscriminately to any double stranded product, it is very important to amplify only specific piece of DNA and exclude any non-specific product or substantial formation of primer dimers. To check specificity of amplified product, a melting or dissociation curve analysis should be performed at the end of reaction by slowly increasing the temperature from 60°C to 95°C which causes denaturation of products and loss of fluorescence signal. A single type of product will have same melting temperature but a different melting point of product depicts non-specific amplification. Enhanced sensitivity in a SYBR® Green assay can further be achieved by care in designing of primers to eliminate any chances of secondary structure formation and ensuring the specificity of primers by initial amplification and examination on agarose gel, sequencing and optimisation of the reaction conditions (Denman and McSweeney 2005).

The real-time PCR technique has been widely applied in the rumen microbial system for monitoring shifts in bacterial populations over dietary changes (Tajima *et al.* 2001a), after microbial manipulations (Klieve *et al.* 2003) and to monitor ciliate and fungal populations

(Denman and McSweeney 2006; Ouwerkerk *et al.* 2002; Sylvester *et al.* 2004). This technique has also been used to study shifts in archaeal community in soil ecosystems, paddy fields (Luton *et al.* 2002; Watanabe *et al.* 2007) and rumen (Denman *et al.* 2007; Guo *et al.* 2008).

A limitation to use of this technique could be that the copy number of the target gene can differ among targeted methanogens (Zhou *et al.* 2011). As an example, the genome of *Methanobrevibacter smithii* contains two copies of 16S rRNA gene, while that of *Methanosphaera stadtmanae* possesses four (Klappenbach *et al.* 2001). Therefore it was suggested that in order to estimate the absolute number of methanogens present in a sample, an adjustment factor that considers the copy number in the cells must be considered (Zhou *et al.* 2011).

Many recent studies have quantified total methanogens and different methanogen phylogenetic groups from varied samples by targeting the *mcr*A gene (Denman *et al.* 2007; Guo *et al.* 2008; Popova *et al.* 2011; Steinberg and Regan 2009) which is present only in methanogens and unlikely to have multiple copies (Springer *et al.* 1995; Steinberg and Regan 2009). It has been suggested that using primers targeting methanogen specific genes such as *mcr*A instead of 16S rRNA will increase the specificity of amplified target (Luton *et al.* 2002; Zhou *et al.* 2011).

#### 2.1.3.1.4 Reverse transcriptase polymerase chain reaction (RT-PCR) and reverse transcriptase real-time PCR (RT-qPCR)

The detection and analysis of RNA is an important aspect of most of molecular biology studies. Generally, RNA is first converted to a complimentary DNA (cDNA) molecule by using reverse transcriptase enzyme. This cDNA is then used as a template for performing PCR. This combination of both the techniques is collectively referred to as RT-PCR. The cDNA from different samples over different treatments or time periods can also be subjected to quantification through quantitative (qPCR) when it is called reverse transcriptase quantitative PCR (qRT-PCR).

In methanogen studies, RT-PCR has been used to create 16S rRNA libraries, quantification of gene activity in rumen, soil, paddy field and other ecological systems (Denman *et al.* 2007; Tajima *et al.* 2001b; Watanabe *et al.* 2007; Watanabe *et al.* 2009). It has also been used to perform an analysis of relative gene expression of methanogens through DGGE analysis of amplified *mcr*A mRNA (Popova *et al.* 2011).

Previous studies have reported that total methanogens numbers are not necessarily correlated with total rRNA copies or methane production (Guo *et al.* 2008; Machmuller *et al.* 2003a).

Therefore, a 16S quantitative PCR is not a very sensitive method for detecting the methanogen dynamics relative to a mitigation strategy. But the sequence of the gene responsible for synthesis of mRNA complimentary to the *mcr* enzyme is known (Attwood and McSweeney 2008; Attwood *et al.* 2008). This specific mRNA can be thus be targeted for qRT-PCR to study the amount of *mcr* mRNA transcribed in the cell under given set of ecological conditions.

The expression of *mcr*A gene has been used as an indicator of the methanogenic activity of methanogens population (Guo *et al.* 2008) and also as a phylogenetic marker to enumerate the methanogen population (Denman *et al.* 2007). Also analysis of the *mcr*A gene whose function is consistent in all methanogens, ensures the accurate investigation of any possible mitigation strategy because this technique allows the analysis of all methanogens which may fill the niche previously occupied by methanogens sensitive to an inhibitor (Attwood and McSweeney 2008). This technique has in fact been applied by Guo *et al* (2008) in *in vitro* mixed rumen samples where a significant decrease in methane production and methanogenic activity upon the addition of tea saponins was found. The methane production was estimated by analyzing gas samples using gas chromatography and methanogen activity was estimated by performing qRT-PCR.

But, this technique has not been extensively used in rumen methanogen studies and even less in *in vivo* conditions. Most of the studies have been done in *in vitro* only. A major limitation to the wider application of this technique is the requirement for high quality and quantity RNA (Fleige and Pfaffl 2006) which is even more challenging in rumen samples (Yu and Morrison 2004). It is further encumbered by the high cost of commercial kits available for RNA extraction. Therefore it is important to develop a reliable and economical method for extracting high quality RNA which would pave the way for wider application of this technique.

#### 2.1.3.1.5 Pyrosequencing

This is a more recent technique which has the capacity to sequence the whole metagenome present in a sample using culture independent approaches (Brulc *et al.* 2010). It has been discussed that molecular fingerprinting techniques like DGGE can detect only abundant population and less predominant species may not be differentiated due to PCR bias (Kocherginskaya *et al.* 2001). The cloning techniques commonly used for sequencing may also not show the full diversity because of inability of cloning vector to bind to a certain kind of DNA fragments (Brulc *et al.* 2010). Pyrosequencing has been reported to eliminate all such limitations. Because several thousand species sequences can be obtained per sample,

microorganisms present below 0.1% of population can also be detected (Callaway *et al.* 2010). It can be used to amplify the DNA of a particular community using specific primers or amplify the whole metagenome by nonspecific amplification (untargeted massive parallel sequencing) which can reduce the PCR bias (Ross *et al.* 2012). This technique has been used to study microbial diversity in a wide range of communities such as methanogen community in coal mines (Guo *et al.* 2012), bacteria and fungi in poultry aerosols (Nonnenmann *et al.* 2010), rumen bacterial diversity in cattle as well as pre-ruminant calves (Dowd *et al.* 2008; Li *et al.* 2012). It has also been used to identify changes in microbial diversity with change in diet composition (Callaway *et al.* 2010; de Menezes *et al.* 2011). It is also possible to analyse the relative proportion of a particular species under specific diet composition. Thus combining this technique with a quantitative analysis may play important role in understanding the inter-species relationships in rumen which is a very complex and dynamic environment (Wright and Klieve 2011).

However, at present the significant costs involved in this technique have limited wider application, but with development of the methodology it may become cost effective for use in large sample numbers.

## 2.2 Factors affecting methanogenesis

In order to measure the amount of methane produced from an animal in a production system, many factors need to be considered which may be animal, environment or diet related and they may exert their influence individually or upon interaction with other factors.

### *2.2.1 Dietary factors*

#### 2.2.1.1 Dry matter intake (DMI)

Methane production is directly influenced by DMI. Molano and Clark (2008) reported a high correlation ($r^2 = 0.83$) between DMI and methane production. But according to Johnson and Johnson (1995), when the DMI increases, the percentage of gross energy lost as methane decreases. It had earlier been reported by Blaxter (1967) that when feed intake is increased from maintenance to above maintenance level, the total production of methane increased but the amount of energy lost as methane per unit of feed consumed decreased by 12-30%. Similarly, the increase in intake of forages from 54.6 to 77.21g DM/kg $^{-0.75}$, decreased the methane production from 7% to 6.5% of the gross energy of diet (McAllister *et al.* 1996). They explained that an increased DMI causes an increase in the amount of organic matter degradation in the rumen along with an increase in fractional passage rate of solids and liquids. But if expressed as a percentage of dietary intake, the ruminal digestion decreases as

DMI increases (McAllister *et al.* 1996). The effect of diet composition is more pronounced at a higher level of feed intake and the effect of increased level of intake is greater with diets containing concentrates as compared to forages. This fact was substantiated by Benchaar *et al*., (2001) who reported that increasing DMI from 9 to 17 kg/d decreased methane energy losses as % of GE intake by 9% for the 100% alf-alfa hay diet as compared to a reduction of 23% on supplementation of hay with concentrates.

#### 2.2.1.2 Feed quality

Ruminants having access to diets rich in cereal concentrate emit less methane compared to those on forage based diets (Chandramoni *et al.* 1999; Durand *et al.* 1988). This is because concentrates are a readily fermentable source of carbohydrates which cause an increased rate of fermentation and thereby shift the pattern of fermentation towards increased propionate production over methane as well as lower acetate: propionate ratio. Acetate and butyrate production results in a net release of $H_2$ and favors methane production, while propionate formation is a competitive pathway to methanogenesis for $H_2$ use in the rumen (Martin *et al.* 2010). The feeding of highly soluble carbohydrates also decreases the rumen pH and this inhibits the activity of methanogens as well as protozoa (McAllister *et al.* 1996; Station 1963; Tellier *et al.* 2004a; b; Yang *et al.* 2001b). Lowering pH can also kill protozoa, destroying the habitat of around 20% of methanogens (Stumm *et al.* 1982) or effect some species more than the other and alter the rumen microbial population (Cottle *et al.* 2011). In an *in vitro* experiment, Malik *et al*., (2010) reported an increase in total VFAs and decrease in protozoal numbers, acetate proportion and methane production upon including first cut lucerne fodder (high quality leguminous fodder) at 30% and 45% levels. The effects of a lower pH on altering the rumen microbial population will however need to be tested over a longer period of time in actual grazing conditions because of the ability of microorganisms to adapt themselves to their environment.

Amongst the completely forage diets as is common in pasture fed animals, the quality and processing of forages effects the methane emissions (Benchaar *et al.* 2001). Dry and mature forages have a higher neutral detergent fibre (NDF) content and a lower concentration of soluble carbohydrates, which decrease the ruminal passage rate causing higher methane production per kg of DMI. Coarsely chopped fodders having a bigger particle size and a lower density also increase the rumen retention time and will invariably lead to higher methane production per kg of DMI. Feed particles having smaller particle size and higher density have a higher rumen clearance rate which leads to a shift in fermentation site to the large intestine where comparatively lower amount of methane is produced per the amount of feed fermented

(Gibb *et al.* 1999; Heinrichs *et al.* 1999; Imamidoost and Cant 2005; Kaske *et al.* 1992; Krause and Combs 2003; Krause *et al.* 2003; Nishida *et al.* 2007; Soita *et al.* 2003) due to prevalence of microorganisms called reductive acetogens which may outcompete the methanogens to use $H_2$ for reductive acetogenesis (Demeyer *et al.* 1989; Demeyer and Graeve 1991; Fievez *et al.* 2001). Further, as discussed above in section 2.2.1.1, when the rumen retention is low, the DMI is also decreased and may cause decreased methane emission per unit of DMI but a lower DMI affects the production of animal. At higher rumen clearance, though DMI is also increased and methane emissions from animals increase but net methane emissions per unit of product produced are decreased. Replacement of a mature fibrous forage with an immature, highly digestible forage decreased methane production by 15% and the processing of forages caused 21% reduction (Benchaar *et al.* 2001). It has been stated that differences in rumen retention time (RRT) change the number, composition, maintenance energy requirements and diversity of rumen microorganisms and their VFA production pattern (Cottle *et al.* 2011). Thus modifying RRT has been suggested as a way to affect the methane production and VFA production in rumen consequently affecting the energetic efficiency of animal (Cottle *et al.* 2011).

The energy lost through methane is also higher from grass forages as compared to the leguminous forages per unit of product produced (McAllister *et al.* 1996). McCaughey *et al*., (1999) reported that on feeding an alfa-alfa and grass diet or a grass only diet over two different seasons, the DMI increased with the alfa-alfa and grass diet because of a higher digestibility and rate of passage with leguminous diets. When expressed as percentage of gross energy intake, the methane production decreased by nearly 10% in the alfa-alfa diet per unit of product (weight gain). McCaughey *et al*., (1999) had also found a significant difference in pasture quality and resulting DMI over different grazing periods and varying methane emissions accordingly. The methane emissions in August (spring) having better pasture quality were lower as compared to the July (winter) emissions numerically but they did not vary significantly. Similarly the methane emissions were 33% lower for silage based diets over the hay based diet (Benchaar *et al.* 2001). Therefore, we can conclude that diet composition as well as its processing plays a very important part in the amount of methane emission from an animal.

## *2.2.2 Animal factors*

### 2.2.2.1 Species

Differences in methane production between the ruminant species have been reported with cattle being the highest methane producers, followed by deer and then sheep. The New

Zealand National Inventory estimates for methane ($CH_4$) yield in cattle are 21.6g $CH_4$/kg DMI, 21.25g $CH_4$/kg DMI in deer and 20.9g $CH_4$/ kg DMI in sheep. The reasons for these differences could be attributed to the difference between digestive physiologies of these species as in their rate of digesta passage, microbial population, fermentation patterns, rumen environment etc., albeit all are ruminants. On the other hand, kangaroos produce negligible or very low amount of methane per unit of digestible DMI from same type of feed as fed to cows or sheep (Kempton *et al.* 1976; Ouwerkerk *et al.* 2005; Von Engelhardt *et al.* 1978).

#### 2.2.2.2 Age

Methane production tends to increase with the age in ruminants. Significant differences have been reported between methane emissions between animals less than 1 year in age and the mature animals in cattle, sheep as well as deer. Knight *et al*., (2008) reported 8% less emissions from lambs than mature ewes (21.9 *v.* 23.8±0.95 g $CH_4$/kg DMI). The emissions from lambs increased with age along with an increase in live weight and DMI. Similar results have been reported by Lassey *et al*., (1997) and Ulyatt *et al*., (2005) in sheep, by Cavanagh *et al*., (2004) in beef cattle and by Swainson *et al*., (2008) in deer.

Since the microbial population establishes in the rumen of young lambs by first 3 weeks (Fonty *et al.* 1987; Joyce and Rattray 1970) and in calves the rumeno-reticulum is fully mature by first 11 weeks of life (Godfrey 1961a; b), it is suggested that the reasons for this variance in methane production cannot be assigned to the presence or absence of microbial population. These variations could be due to difference between digesta kinetics of the young and mature animals. The young animals have a high digesta passage rate (Okine *et al.* 1989; Pinares-Patino *et al.* 2003c) which causes decreased cellulose fermentation in rumen for a given DMI, a decreased supply of $H_2$ and thus lower methane production in the rumen. The feed digestion shifts to large intestine where there is lower production of methane per unit of the feed fermented due to relative prevalence of reductive acetogens which may outcompete the methanogens in utilizing $H_2$ for reductive acetogenesis (Demeyer *et al.* 1989; Demeyer and Graeve 1991; Fievez *et al.* 2001; Graeve and Demeyer 1988).

#### 2.2.2.3 Animal to animal variation

Variations in methane emissions between animals feeding on same kind of diets have been reported by several workers (Ulyatt *et al.* 1999). Grainger *et al*., (2007) reported a 17.8% coefficient of variation (CV) between different animals in their methane emissions substantiating the previous studies where varied but significant CV in methane emissions between animals were found; Blaxter and Clapperton (1965) - 7-8%, Boadi and Wittenburg (2002) - 15.5% , Lassey *et al*., (1997) - 11.5% and McNaughton *et al*., (2005) - 25%.

But, other studies (Cavanagh *et al.* 2008; Pinares-Patino *et al.* 2003c; Vlaming *et al.* 2007) have reported that though there were differences between animals in methane emissions, the repeatability of the results was not very high. In fact the same animals changed ranking within the same experiment from low to high producing or vice-versa. Therefore, it was difficult to classify an animal as a low or high emitter in comparison to other animals over a limited period of time. A possible reason could also be the use of $SF_6$ technique for measuring methane emissions which is prone to error (discussed earlier in section 2.1.1.2).

The available literature suggests that these between animal variations could be accounted by an animal's feed conversion efficiency or residual feed intake (RFI) i.e. differences in feed intake, digestion, metabolism, activity and thermoregulation can influence the DMI for a particular production level which is correlated with the amount of methane produced (Herd *et al.* 2004; Munger and Kreuzer 2008; Pinares-Patino *et al.* 2003b). Hegarty *et al*., (2007) found a significant relation between the RFI and the daily rate of methane production (MPR) and calculated a daily reduction in methane production of 13.38g with a reduction of 1 kg/day RFI at *ad libitum* feed intake. Similar results of 24-28% lower methane production in animals having lower RFI as compared to a medium or high RFI were reported by Nkrumah *et al*., (2006). Other factors e.g. a difference in feed intake, digestion, activity on day to day basis could also be responsible for within animal variations.

#### 2.2.2.4 Production status

The energy requirement of an animal is influenced by its production status and is met by an increase or decrease in DMI. This may influence the net methane emissions from the animal e.g. it has been reported that dairy cows at the peak lactation emit ≈ 430g $CH_4$/day and it can decrease to ≈ 250g $CH_4$/ day as the milk yield declines (Cottle *et al.* 2011; Eckard *et al.* 2009). A major contributor to this decline would be the decrease in DMI of animal in proportion to decreased production.

### *2.2.3 Environmental factors*

#### 2.2.3.1 Temperature

Moss *et al*., (2001) found that methane production was higher at the lower temperature of 5°C as compared to 24°C of ambient temperature. They related this to an increased DMI leading to increase in substrate availability in rumen, an increase in microbial activity and a higher level of methane produced per kg of DM degraded (32.89 vs. 31.00 l/kg DMI). McGinn *et al*., (2008) also attributed the lower $CH_4$ emissions in Australian feedlot animals as compared to Canadian animals, to a difference in the environmental temperature causing a heat stress and

thus a lower DMI. Similarly, Von Keyserlingk and Mathison (1993) reported a 25% greater methane production in sheep managed at 4.7°C over those managed at 21°C. They attributed this increase in methane production to an 8% increase in DMI, and when expressed as percentage of digestible energy there still was 14% more production of methane in the cold conditions.

In contrast, a 20% and 30% decrease in methane production in adult sheep was found by Graham *et al*., (1959) when temperature was decreased from 33°C to 8°C and by Kennedy and Milligan (1978), in cold adapted sheep, respectively. An increase in ruminal passage rate of fluid and particulate matter by 54% and 68% respectively, was also observed along with a higher level of feed intake under cold conditions thereby decreasing the apparent organic matter digestibility, which was assumed to be the reason behind a lower methane production. Okine *et al*., (1989) also reported a 29% decrease in methane emission when fractional passage rate was increased by 63%. Similarly, it has been stated that mature beef cows have an average methane production of ≈350g/ day in the tropics and ≈240g/ day in the temperate zones (Cottle *et al.* 2011; Eckard *et al.* 2009).

Further, West (2003) reported a 0.85kg decrease in DMI with every one degree (°C) increase in mean air temperature beyond the critical threshold and Fox and Tylutk (1998) concluded that lactating animals were more susceptible to heat stress than the cold temperatures in terms of effect on DMI. From the above reports it is hard to conclude the exact effect of temperature but it can safely be assumed that the effect of temperature manifests itself through an effect on the DMI. The animals may have a higher DMI at a comfortable temperature but as the temperature increases beyond the comfort level it may cause heat stress and result in decreased DMI. Therefore, while deciding on the effect of temperature on methane production, many more factors and their interaction with each other need to be considered.

#### 2.2.3.2 Season

The effect of season on methane production is supposed to manifest itself through the change in pasture quality along with a change in ambient temperature. The production of methane during different seasons: spring- September, early summer-November, autumn or late summer-March and winter-June/July in both ewes and dairy cows was estimated by Ulyatt *et al*., (2002)**.** The methane emissions varied significantly on DMI basis and were highest in September (22.4g/kg DMI) and June (20.2g/kg DMI) as compared to November (13.7g/kg DMI) and March (12.3g/kg DMI). As regards the pasture composition, the soluble sugars content was highest in September >November >March >June, the crude protein level was highest in September and March, the fat content differed less except being lowest in June,

whereas the NDF content as well as DM digestibility were highest in June >March >November >September. Thus, neither of these variations in pasture composition over different time periods could individually explain the variation in methane emissions.

### *2.2.4 Physiological factors*

The optimum conditions for methanogenesis in rumen are a temperature of 39°C, a redox potential below -300mV and a pH of 6-8 (Moss *et al.* 2000; Thauer *et al.* 1993; Zinder 1993) and a variation in these conditions may have a bearing on methane emissions.

#### 2.2.4.1 Ruminal pH

The ruminal pH is normally maintained at 5.5-6.5. The pH is maintained through a combination of buffering agents introduced through saliva, ruminal epithelium, feed and water (e.g. bicarbonates, phosphates, etc.), along with an interplay of factors such as fluid dilution rate, the absorption and metabolism of VFAs and the rumen turnover rate (Wales *et al.* 2004). In general, the animals fed diets high in concentrate tend to have a lower ruminal pH as compared to the animals fed high fibre diets. This also leads to the prevalence of starch fermenting microbes over the fibre digesters because the cellulose digesting bacteria cannot function optimally below a pH of 5.8 (Russell 1998; Russell and Wilson 1996). There is also a decrease in acetate: propionate ratios because the starch fermenters tend to produce more propionic acid and the cellulose fermenters shift the fermentation towards more acetic acid and butyric acid production. The production of acetic acid tends to favour the production of methane because of production of more $H_2$ during acetic acid formation. Methane production was highly correlated ($r^2$ = 0.80) with acetate: propionate ratio at a pH range of 6.5-5.8 (Russell 1998).

Similarly, a high correlation between the acetate: propionate ratio and ruminal pH ($r^2$=0.82) *in vivo* and also between the *in vivo* acetate: propionate ratio and *in vitro* production of methane ($r^2$=0.78) was reported by Lana *et al*., (1998) when the steers were fed increasing amounts of concentrate and decreasing forage. Also, when the rumen fluid from cattle fed either forage or concentrate was incubated at decreasing pH values from 6.5 to 5.7, the methane production decreased from 48 to 7nmol mg protein$^{-1}$ min$^{-1}$ and from 14 to 2mol.mg protein$^{-1}$ min$^{-1}$, respectively.

Kessel and Russell (1997a) based on *in vitro* experiments had also reported that methane production ceased at a pH below 6 and as the pH rose above 6, the methane production returned. Therefore, they concluded that methanogens are not killed by low pH, but resume their activity after suitable conditions are regained. With the decrease in pH, there is a

simultaneous decrease in methane production due to the inability of methanogens to actively produce methane below a pH of 5.5 (Muller *et al.* 1999; Schuldiner and Padan 1992). In order to regulate their intracellular pH according to the extracellular pH, the methanogens extrude Na+ ions out of the cell and cellular processes are driven by a low sodium gradient across the cellular membrane. The cellular stock of ATP is depleted and consequently methanogenesis is decreased (Muller *et al.* 1999; Schuldiner and Padan 1992). Recently Hook *et al*., (2011) found no significant change in methanogen numbers when the diets of dairy cows were switched from a high concentrate to roughage based diet. However, they reported a significant positive correlation between average pH and methanogen numbers ($r^2$-0.97, P-0.018). Significantly, the reported pH range of *Methanobrevibacter ruminantium* is 5.5 to 7.0 and *Methanosphaera stadtmanae* was found to have a pH range and growth optima higher than other methanogens (Miller and Wolin 1985; Rea *et al.* 2007). It has been thus suggested that while *Methanobrevibacter ruminantium* is unlikely to be affected by pH fluctuations, the *Methanosphaera stadtmanae* methanogens may cease their activity at a low pH (Hook *et al.* 2011).

#### 2.2.4.2 Redox potential

The rapid fermentation of carbohydrates in the rumen creates a state of low redox due to the resulting reduced products. The $H_2$ ions produced as a result of fermentation, VFA production and further absorption and metabolism in the rumen epithelium have to be disposed of rapidly for the fermentation to proceed uninterrupted. Marounek *et al*., (1991) found that an increase in redox potential in anaerobic batch cultures using rumen inoculate from cow as well as sheep decreased both the methane as well as VFA production. Methanogenesis and propionate production are the major means of reducing equivalent disposal in rumen and are competitive in nature (Hino and Russell 1985). For the strictly anaerobic methanogens to use $H_2$ for reducing $CO_2$, the redox potential should be $\leq$ -300 mV whereas the reductive acetogens can use $H_2$ only at a higher redox potential (Fievez *et al.* 2001; Greening and Leedle 1989; Joblin 1999). A higher redox favours the reductive acetogens and leads to more acid production which is beneficial to the animal in terms of energy production.

Hungate (1966) had suggested that forages contribute natural reducing substances to the rumen environment and this conclusion was later supported by Males (1973) who found that the redox potential of rumen contents from sheep fed forage was much lower than from those fed a concentrate ration. The production of $H_2$, $CO_2$ and hence methane are also coupled with the deamination of reduced amino acids. An inhibition of methanogenesis led to the inhibition of bacterial hydrogenases due to an increased NADH/NAD ratio leading to the inhibition of

deamination and thus a decreased ammonia production *in vitro* (Hino and Russell 1985). Thus the reducing equivalent disposal and the ratio of NADH/NAD are important factors for fermentation and methanogenesis in the rumen.

### 2.2.5 Biological factors

#### Protozoal-methanogen interaction

The protozoa associated methanogens have been reported to contribute nearly 37% of the rumen methane emissions (Finlay *et al.* 1994). Also, rumen fluid with a higher number of protozoa, tends to have a higher rate of methanogenesis (Klieve and Hegarty 1999). Morgavi *et al*., (2008) reported that the defaunation decreased methane emissions in sheep by ≈ 20%. Schonhusen *et al*., (2003) also found that methane production decreased by 30% in the absence of protozoa, though this was also accompanied by a decreased digestibility in calves at the time of weaning. These effects are due to the association of methanogens with protozoa especially the Holotrichs in the rumen. This is a symbiotic relation because protozoa rapidly ferment readily soluble sugars thereby acting as a ready source of $H_2$ for the methanogens whereas the methanogens rapidly consume $H_2$ and prevent its accumulation which could be inhibitory for the protozoan metabolism.

The presence of protozoa *in vitro* also increased the amount of acetic and butyric acid relative to propionic acid and the main protozoon associated with this effect along with an increase in methane production was found to be *Entodinium caudatum* (Ranilla *et al*. 2007). Therefore the methanogens–protozoal interaction does play a part in ruminal methanogenesis and any effort to decrease methanogenesis has to be considered in conjunction with its effect on ruminal digestibility which has a bearing on its production performance.

### 2.2.6 Conclusion

To conclude, the rate of methane production at a given time depends on the interplay of a large number of external and internal factors working either in an inhibiting or in a stimulating fashion rendering it difficult to accurately correlate the methane production of an animal at a single unit of time to a single factor. However, after review of all factors affecting methane production, it can be concluded that their effect is manifested through an increase or decrease in DMI. The DMI is positively correlated with methane production. Since, for high production an optimum DMI is essential, choosing a methane mitigation strategy for a particular production system has to allow for either a high DMI or an increased feed/ fodder digestibility so that the production is not affected.

## 2.3 Mitigation opportunities for methane emissions

It is evident from the previous discussion that methane is an inevitable product of rumen fermentation. It would be advantageous if the metabolic $H_2$ which is converted into methane could be used in the synthesis of VFAs because methane has no nutritional value for the animal. The production of methane is actually a loss of energy to the animal accounting to about 2-12% of the dietary gross energy (Johnson and Ward 1996) which in turn effects the feed conversion efficiency. The exploration of ways to decrease methane emission from ruminants, because of the economic and environmental loss associated with it, has been going on for quite a while (Baker 1999; Bauchop 1967; Beauchemin *et al.* 2008; Beauchemin *et al.* 2007; Bryant and Murray 1974; Grainger and Beauchemin 2011; Klieve and Hegarty 1999; Petrie *et al.* 2009).

A recent review on enteric methane mitigation by Cottle *et al.*, (2011) (figure 2.3), summarized the mitigation options being tested by researchers. Ruminant methane mitigation options may broadly be divided into: (i) modifications of the rumen microbial population or the gastrointestinal tract environment; (ii) selecting the animal; (iii) managing the livestock environment.

As discussed earlier, methanogenesis in rumen is a means for using the free $H_2$ in rumen and if $H_2$ levels are not kept low, there may be inhibition of fermentation because of lack of reducing agents such as $NAD^+$ which are important for carrying out the carbohydrate degradation and energy generation for microbes. This will in turn hinder forage digestion and VFA production. Therefore, any attempt to reduce methanogenesis must ensure that the $H_2$ produced inside rumen does not accumulate. It has thus been proposed that the mitigation techniques for reducing methanogenesis may involve (Benchaar *et al.* 2001; Johnson and Johnson 1995; McGinn *et al.* 2004):

- Diverting $H_2$ away from methane production by creating alternative $H_2$ sinks e.g. biohydrogenation upon supplementation with lipids, shift towards reductive acetogenesis, etc.
- Inhibiting $H_2$ production inside rumen by decreasing the fermentation of organic matter in the rumen which may involve shifting the site of digestion from rumen to the intestine e.g. processing of forage, altering the composition or quality of feed offered.

- Directly inhibiting the methanogens to reduce the amount of methanogenesis e.g. through defaunation, antibiotics, ionophores, synthetic chemicals, natural compounds such as tannins, saponins, or through vaccination, etc.

For 'on-farm' adoption of any mitigation strategy it is desirable that it should minimise any loss of digestible energy to animal and may have the added benefit of producing end products useful to the animal or farmer. Some of the more common or practically feasible strategies are discussed here.

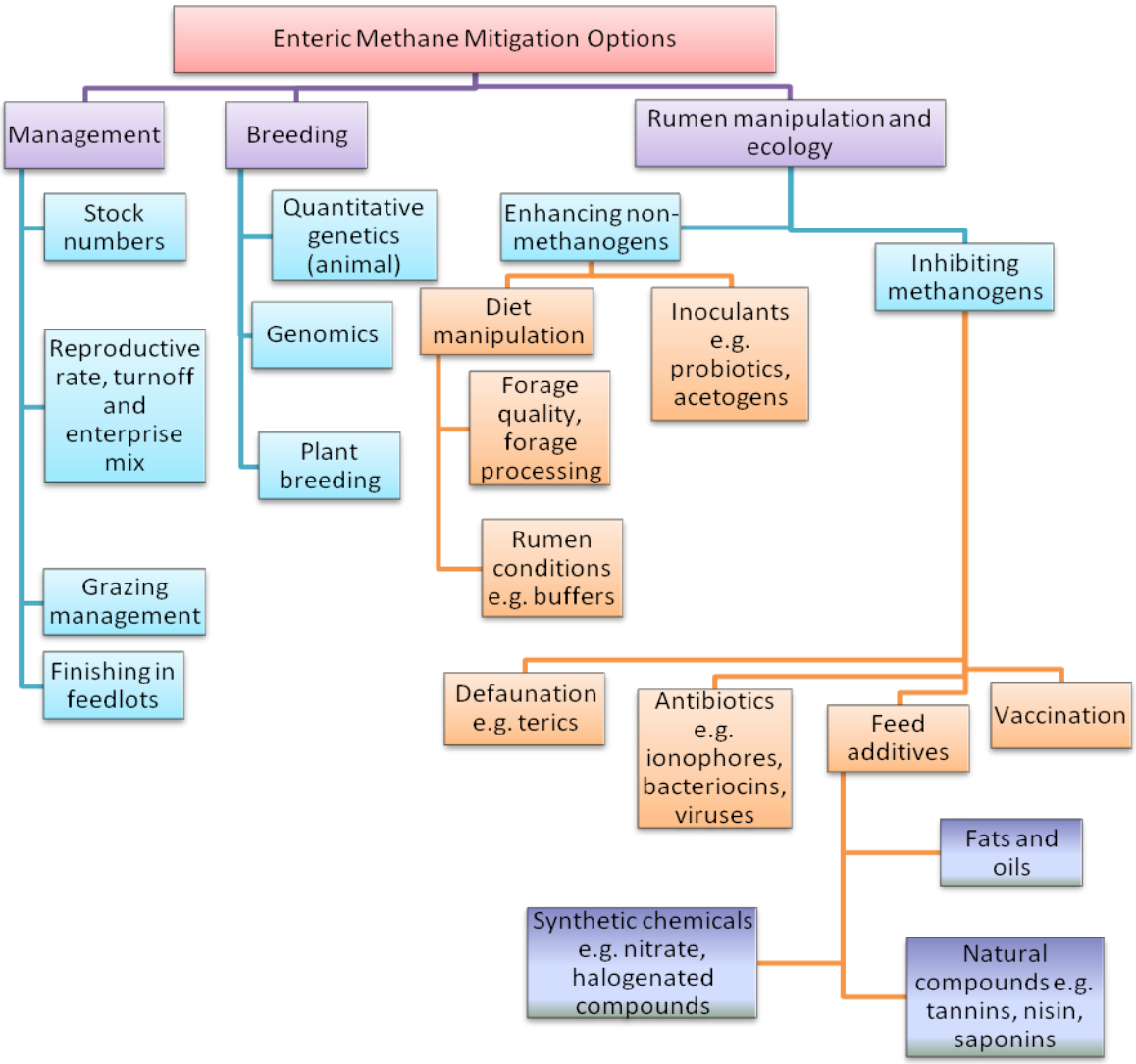



**Figure 2.3 Potential options for reducing enteric methane emissions (adapted from Cottle *et al.*, 2011).**

## *2.3.1 Inhibiting methanogens*

### 2.3.1.1 Ionophore antibiotics

Ionophore antibiotics are highly lipophillic substances and are commonly used in commercial beef and dairy cattle production. Monensin is the most commonly researched ionophore though others such as lasolacid, salinomycin, narasin, laidomycin are also used commercially and have been researched to some extent (Boadi *et al.* 2004; Guan *et al.* 2006; Lyle *et al.* 1981; McGinn *et al.* 2004) . The mode of action of ionophores is multi fold with influence on both the rumen fermentation as well as animal physiology.

A brief summary of the metabolic effects of ionophores on rumen fermentation as summarized by Bergen and Bates (1984) follows:

- Shift in acetate-propionate ratio towards more propionate.
- Some increase of lactate to propionate production via the acrylate pathway.
- Decreased ruminal protein breakdown and deamination; lower ruminal ammonia-N.
- Primary $H^+$ or formate producing, gram positive (Gram +ve) bacteria are inhibited.
- Decrease in methane production primarily due to lower availability of $H_2$ and formate, and a depressed interspecies $H_2$ transfer.
- Depression of lactic acid production under acidosis inducing conditions.
- Many gram negative (Gram –ve) bacteria survive especially succinate producers (source of propionate).
- Some evidence for depressed rumen content turnover.
- A mild inhibition of protozoa.
- Decrease in rumen fluid viscosity in bloated animals.

The ionophores exert these effects by disrupting the cellular metabolism of rumen bacteria mainly the Gram +ve bacteria which are mainly responsible for $H_2$ producing reactions in the rumen. They increase the membrane permeability of cells leading to disruption of the $Na^+$-$H^+$ antiporter system aside from the primary transport channels thereby affecting the ATP generating reactions and leading to depletion of intracellular ATP stores. The disruption of primary transport systems also interferes with the uptake of necessary cellular substrates further interfering in the cellular metabolism. The Gram -ve bacteria which are the main succinate and propionate producers are better adapted for survival under these conditions because they have capacity to generate ATP through electron transport chains which couple proton translocation with energy generation. This capacity for energy generation gives Gram

-ve bacteria a selective advantage over Gram +ve bacteria which can account for the observed shifts in acetate: propionate ratio.

Beauchemin *et al*., (2008) summarized the data on monensin treatments and have suggested that the efficacy of monensin in reducing methane emissions may be dose dependent and that the addition of monensin at a dose of <15-20 ppm had no effect on methane production per kg DMI in dairy cows but higher doses of 24-35 ppm reduced methane production by 3-8% in g/kg DMI in beef as well as dairy cows.

The main problem associated with monensin administration is that it reduces methane production for a short time. Guan *et al*., (2006) reported that both monensin or lasolacid (another ionophore) administration decreased methane production by 30%, but over a short time period of 2 weeks and by 27% up to 4 weeks. On the basis of their experiments, they suggested that the effect of ionophores on decreasing methane emissions is related to their inhibitory effect on the rumen protozoa population particularly the *Entodinium* spp. which are symbiotically associated with the methanogens. Since the protozoa were able to adapt to monensin enriched diets after 4 weeks, this was responsible for the receding effects on methane production observed after prolonged use of ionophores. Since the ionophores don't have a direct effect on methanogens but mediate through their suppressing effects on Gram +ve bacteria and protozoa, which also are effected by high concentrate diet, a significant influence of diet composition could be expected. Consequently, the adaptive responses were found to occur earlier in low concentrate diets as compared to high concentrate diets.

The influence of diet composition was also supported by the experiments of Mbanzamihigo *et al*., (1996) in sheep fed a high grain diet where methane production rates were lower than the controls for at least 35 days and by Green *et al*., (1999) in dairy cows fed total mixed rations where monensin containing controlled release capsules (CRC) changed some rumen fermentation parameters e.g. a decreased acetate: propionate ratio, decreased butyrate and increased pH. In contrast, Waghorn *et al*., (2008) found that when CRC of monensin were administered in pasture fed dairy cows, there was no effect on methane production over a period of 75 days. No effects were found on rumen metabolites, DMI and milk production. An actual average release rate of 170 mg/day in comparison to the anticipated 320 mg/day could also have compounded this lack of efficacy. Mutsvangwa *et al*., (2002) also reported no effect of monensin CRC on ruminal pH characters except reduced acetate: propionate ratio even in animals fed high grain diets. In a similar study (Hook *et al.* 2009), when animals on a total mixed ration were fed monensin at the rate of 24mg/kg DMI over a long period of 6 months, no change in amount or type of ruminal methanogens was detected.

These contrasting reports on monensin efficacy suggest that though monensin administration may be beneficial in grain based diets and for a short term period in pasture based diet, its long term efficacy is questionable and needs further thorough research.

#### 2.3.1.2 Defaunation

Protozoa play an active role in ruminal fermentation and primarily produce acetate, butyrate and $H_2$ as fermentation end products (Waghorn and Woodward 2004). Methanogens have been observed to be present either on the exterior surface of ruminal ciliate protozoa (Ohene-Adjei *et al.* 2007; Vogels *et al.* 1980) or as endosymbionts within ciliates (Finlay *et al.* 1994). Protozoa provide a habitat for up to 20% of rumen methanogens (Stumm *et al.* 1982) and therefore McAllister and Newbold (2008) found the concentration of methanogens in whole rumen contents was nearly 1.5 folds less in defaunated than faunated animals. The protozoa associated methanogens were reported to be responsible for 9-25% (Newbold *et al.* 1995) or 37% (Williams and Coleman 1992) of ruminal methane emissions and as a consequence, rumen fluid with high number of protozoa were reported to have higher rate of methanogenesis (Krumholz *et al.* 1983; Ushida *et al.* 1997). Since it has been reported that a high concentrate diet supports greater number of protozoa than a high forage diet, thus the effect of defaunation are also diet dependent (Moss *et al.* 2000; Ushida *et al.* 1987). Elimination of protozoa thus offers an opportunity to decrease methane production by indirect means i.e. by limiting amount of available $H_2$ as well as disrupting the symbiotic relation between protozoa and associated methanogens.

The toxicity of chemicals used for defaunation poses a significant animal health problem (Williams and Coleman 1992). Saponin containing plants offer a natural means of defaunation. The saponins are glycosides which selectively interact with cholesterol present in membrane of protozoa but have no effect on prokaryotic bacteria (Cheeke 1998) and their effect on decreasing methanogenic activity *in vitro* has been reported (Guo *et al.* 2008). Unfortunately, the effect of defaunation on methanogenesis cannot be considered in isolation. As suggested by Coleman (1986), the protozoa might be responsible for up to 50% of the fibrolytic activity in rumen and defaunation has been shown to adversely affect fibre digestion in rumen (Jouany and Ushida 1998). On the other hand, the elimination of *Entodinium caudatum* have been shown to reduce methane emission from rumen with no adverse effect on feed digestion (Kumar *et al.* 2009; Ranilla *et al.* 2007). Scientists have also suggested that protozoa have a negative effect on animal productivity in that the engulfment and digestion of bacteria by protozoa significantly decreases the flow of microbial protein from rumen to small intestine (Jouany and Ushida 1998; Moss *et al.* 2000; Wallace and McPherson 1987).

To conclude, the use of defaunation to decrease methane production has to be balanced against the effects on fibre and protein metabolism which is further dependent on the ratio of concentrate to forage in diet.

#### 2.3.1.3 Chemical analogues

Many synthetic chemicals analogues of coenzyme-M such as 2-bromoethanesulfonic acid (BES), bromochloromethane (BCM) have been found to have a direct inhibitory effect on the methanogens (McAllister and Newbold 2008). BES and BCM are reported to inhibit the coenzyme-M which is the enzyme involved in terminal step of methanogenesis. Since this enzyme is present only in methanogens, the usage of these methane analogues should not affect other ruminal bacteria and hence ruminal digestion or VFA production. Dong *et al*., (1999) observed that BES depressed methane production by 51% without significantly affecting organic matter digestibility and VFA concentrations in the artificial rumen through rumen simulation technique (RUSITEC) and recently in an *in vitro* study Lee *et al*., (2009) reported that BES at 5 mM inhibited methane production by more than 95% compared to the control, decreased the acetate to propionate ratio and did not affect the population of bacteria but reduced the population of total methanogens, especially the order Methanobacteriales (predominant population in rumen) and the order Methanomicrobiales, in a dose-dependent manner. However, the effect of BES addition has been reported to be transitory with methane emissions returning to pretreatment levels within a matter of days. A study in sheep showed that even though BES effectively depressed methane production, its effectiveness persisted for only 4 days even after regular infusion of the chemical (Immig *et al.* 1996), suggesting that adaptation of the methanogenic population occurred.

The addition of BCM has been shown to have long term efficacy. It reduced percentage of gross energy (GE) lost as methane from 3.9 to 0.6% in feedlot steers (Tomkins and Hunter 2004). Similarly, Denman *et al*., (2007) reported a 30% reduction in total methane emissions, an increase in propionate and other BCFAs upon feeding BCM to the animals for a period of 28 days and a decrease in the incidence of *Methanobrevibacter* sp. in the clone library generated from BCM treatment. Recently a 50% decrease in methane emissions was reported after adding BCM for a period of 90 days to cattle fed grain based diets (Tomkins *et al.* 2009).

Another halogenated compound, chloroform, has also been shown to decrease methanogenesis by acting upon *mcr* enzyme involved in the terminal step of methane production. But in a recent study rumen methanogens acquired resistance to even this compound after 1-2 weeks of daily administration and methane production recovered almost 62% to the pre-treatment levels (Knight *et al.* 2011). Though, this compound was able to

change the community profile of methanogens seen through DGGE, but, even other bacteria such as the reductive acetogens were affected by this compound. Also, this compound has been shown to have hepatotoxic and carcinogenic effects (Knight *et al.* 2011).

Therefore, the usage of these chemicals in livestock industries has been banned on account of their potential residues in animal products and concerns for health hazards.

#### 2.3.1.4 Addition of lipids

The supplementation of diets with lipids, which are not protected in the rumen, has been shown to reduce methane emissions from animals (Grainger and Beauchemin 2011; Grainger *et al.* 2008). Lipids are generally added to finishing diets on many farms around the world to increase the energy density of diets. Apart from their ability to reduce methane emissions by nearly 50% (Machmuller 2006), they have an added advantage of being perceived as a natural source of mitigation. Also, lipid addition reduces the dustiness of feed increasing its palatability, increases the absorption of fat soluble nutrients and can alter the composition of milk and meat products (Beauchemin *et al.* 2007). It has been recommended that the amount of fat added to diet should not exceed 6-7% otherwise it will affect the dietary digestibility and DMI, negating the advantages of increased energy density of diet. Based on 17 studies, Beauchemin *et al*., (2007) calculated that with every 1% addition of fat, the methane production (g/kg DMI) decreased by 5.6%. The level of added fat was able to explain 67% of the reduction in methane emissions relative to the control treatments. These results clearly set the lipid addition as an important mitigation option. The best type of lipids to be added to the diets, however, is a matter of question.

Dietary lipids have been proposed to exert their effect on methane inhibition directly or indirectly by (i) decreasing ruminal organic fermentation, (ii) diverting $H_2$ for biohydrogenation of the lipids containing unsaturated fatty acids (Czerkawski *et al.* 1966; Johnson and Johnson 1995), (iii) decreasing the ruminal bacteria and protozoa or a direct toxic effect on the methanogens, or a combination of these modes (Soliva *et al.* 2003). The effectiveness of long chain fatty acids (LCFA) in suppressing methane production has been thought to be proportional to their degree of unsaturation (Giger-Reverdin *et al.* 2003) which results in a greater partitioning of $H_2$ between biohydrogenation and reduction of $CO_2$. In contrast, as early as 1966 (Czerkawski *et al.* 1966) and in subsequent experiments thereon, a direct relation between the degree of unsaturation (number of double/ triple bonds) and the resulting decrease in methane production could not be demonstrated.

A direct toxic action of LCFA as well as medium chain fatty acids (MCFA) on methanogens, protozoa and bacteria has been ascribed as another reason for inhibition of methanogenesis (Hegarty 1999; Maczulak *et al.* 1981; Soliva *et al.* 2003). Addition of C14:0 and C12:0 directly affected the methanogen counts and simultaneously changed their population profile (Soliva *et al.* 2003). The mode of action for this direct toxic action has been suggested to be the adsorption of lipid particles onto the cellular surface, thereby increasing cellular permeability and interfering with cellular metabolism (Kabara 1978; Machmuller *et al.* 2003b). Machmuller *et al*., (1998), reported a decrease in methane production by ruminants by nearly 58% with the addition of myristic acid (C14:0) to their diet. Also, Matsumoto *et al*., (1991) reported a suppression of rumen ciliates particularly by C12:0 and C10:0 and Galbraith *et al*., (1971) showed that some species of Gram +ve bacteria were inhibited by MCFA addition. The Gram –ve bacteria were found to be less sensitive, again suggesting a role of cell wall structure in lipid tolerance. Due to absence of peptidoglycan polymer in the methanogen cell walls, the adsorption of fatty acids onto the cell surface may be enhanced which may interfere with their cellular metabolism (Dohme *et al.* 2000). The relative differences within methanogens in their cell wall structure may also explain the change in population profile of methanogens upon lipid addition (Boone *et al.* 1993; Machmuller *et al.* 2003b). Grainger and Beauchemin (2011) have recently summarized that type of fatty acid or nature of feeding e.g. as oilseed or liquid form does not have any effect on methane production though some seeds need to be processed before feeding for increasing digestibility.

But, Galbraith *et al*., (1971) had reported that for efficient adsorption, the fatty acids must be in solution and remain sufficiently lipophilic. To support this observation, the fats which have a lower melting temperature and are able to melt more efficiently at the rumen temperature e.g. coconut oil and palm kernel oil, which are particularly rich in lauric acid (C12:0), have been found to be more effective in methane suppression (Dohme *et al.* 2000).

The lipid particles may also directly compete with the rumen microbes for adsorption onto the feed particles decreasing the overall digestibility of feed. This effect on digestibility was observed to be more pronounced in diets having low concentrate content as compared to diets with high concentrate amount. It has been suggested that feeding non-esterified fatty acids may be more beneficial in high pasture based diets than concentrate diets (Machmuller 2006). Also, the presence of high calcium in diets may decrease the lipid solubility by causing saponification and consequently decrease their inhibitory effects (Machmuller *et al.* 2003b).

Numerous strategies involving addition of lipids having either LCFA or MCFA or varied concentrations of both have been tried by different researchers. Added fats having high

concentrations of MCFA (particularly C12:0 and C14:0) have been found to be more effective in reducing methane emissions. Based upon their sequential studies, Machmuller and Kreuzer (1999) reported that addition of coconut oil in animal diets at the rate of 3.5% and 7%, suppressed methane production by 28% and 73%, respectively, simultaneously reducing the ciliate protozoa counts by 88 and 97%, respectively. Further Dohme *et al*., (2000), upon investigations in RUSITEC with different fat sources having varied concentrations of MCFA reported that at a level of 5% addition, palm kernel oil and a genetically modified canola oil (having high concentrations of C12:0) were able to suppress methane production by 34% and 20%, respectively, and also achieved significant reductions in methanogens and ciliate numbers. Significantly, it was found that C12:0 and C14:0 are the predominant fatty acids associated with methane reduction and a ratio of 2:1 for them was recommended to be the best. This ratio is nearly approachable in coconut oil which was found to be very effective in methane reduction (21%) and had lower effect on fibre digestibility. Reportedly, the C12:0 has a larger impact on rumen fibre breakdown, but a combination with C14:0 decreases the level of C12:0 additions, thereby reducing the negative impact on fibre digestion. On the other hand, C14:0 has no effect on methane production independently but acts synergistically with C12:0 (Machmuller 2006). In spite of their obvious benefits, the MCFA are not routinely used for animal diets because of cost involved.

Lipid sources containing long chain fatty acids (LCFA) such as animal fats (tallow), sunflower oil, canola oil, soybean oil and oilseeds (sunflower seeds, cottonseed cakes) have also been found to reduce methane emissions while being cheaper. Reportedly, sunflower oil and canola oil reduced methane emissions from cattle fed high forage diets by up to 22% of GE intake when added at 45 g/kg DM (Beauchemin and McGinn 2006; McGinn *et al.* 2004). During further research by Beauchemin *et al*., (2007), sunflower oil was found to decrease methane emissions by 14% while having a minimal effect on fibre digestibility as compared to tallow and sunflower seeds, which though reduced methane emissions by 14% and 33%, but unfortunately reduced fibre digestibility by 15% and 20%, respectively and digestible energy intake by 3% and 12%, respectively. In another recent study (Grainger *et al.* 2008), supplementation of whole cottonseeds in dairy cows reduced methane emissions by 12% while increasing milk fat and milk solids. The reduction in methane emissions with LCFA is suggested to be partly due to decreased fibre digestion (Beauchemin *et al.* 2007; McGinn *et al.* 2004) and decreased DMI (Jordan *et al.* 2006a), and a major reason has been reported to be the direct toxic effect of LCFA on methanogens and fibrolytic bacteria (Beauchemin *et al.* 2007; Maczulak *et al.* 1981).

The addition of fat sources containing poly unsaturated fatty acids (PUFA) such as linoleic acid (C18:2n-6, LA), linolenic acid (C18:3n-3, LNA), eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA), which have potential health benefits, may also alter the composition and percentage of fat in milk which is desirable in the commercial sector these days (Belenguer *et al.* 2010; Kim *et al.* 2008; Shingfield *et al.* 2010). This desirable change in the composition of fatty acids in animal products actually prompted the research into the effect of using fish oil as a dietary supplement. Fish oil contains substantial quantity of PUFA such as LA, EPA and DHA (Fievez *et al.* 2003). Addition of fish oil has been shown to prevent biohydrogenation of LA and LNA to stearic acid (C18:0) by certain Gram +ve rumen bacteria such as *Butyrivibrio* sp., and *Clostridium* sp., through a direct toxic effect on them (Kim *et al.* 2008). These species classified as group B bacteria (Kemp and Lander 1984) are responsible for most of the biohydrogenation in rumen and hydrogenate LA (C18:2n-6) and LNA (C18:3n-3) to stearic acid (C18:0) whereas the group A bacteria hydrogenate these same PUFA into *trans*-11 18:1 (Huws *et al.* 2010). A DGGE analysis and qPCR of rumen fluid from steers fed fish oil at 0%, 1%, 2% or 3% of DMI showed a change in bacterial diversity as well as total DNA concentration upon inclusion of fish oil at 2% or 3% (Huws *et al.* 2010). However, the rate of decrease in biohydrogenation was found to be poorly correlated with actual DNA numbers of bacteria which may be due to its effect on unidentified species (Belenguer *et al.* 2010).

Fievez *et al*., (2003) observed up to 80% reduction in methane production of batch cultures *in vitro* 48h after addition of fish oil with no decrease in VFA production while a parallel culture with soybean oil inclusion showed 17% lower VFA production. When fish oil at two concentrations of EPA (18.1% vs. 5.4%) and DHA (11.9% vs. 7.5%) was added to the diets of steers fed on hay/concentrate diets (65/35, w/w), the fish oil with higher PUFA concentrations reduced methanogenesis without decreasing neutral detergent fibre (NDF) digestibility and also increased the concentration of propionate.

From the discussion above it can be deduced that PUFA present in fish oil have a toxic effect on methanogens as well as on protozoa and the gram +ve bacteria which provide $H_2$ to methanogens. This results in a decrease in methanogenesis.

To conclude, the efficacy of lipid addition in diets depends on the effective fatty acid concentration which is further dependent on the amount of lipid addition, composition of fatty acids, extent of esterification, mineral composition of diets, composition of diet available to the animal and initial rumen microbial community which further depends on the species and breed of host animal and feeding history (Machmuller 2006). In general a low amount (≈3%)

of added fat will have relatively low effects on digestibility depression while maintaining its beneficial effects. Secondly, the *in vivo* digestibility effects might be lower than the *in vitro* values because of a possible shift in fermentation to intestines and also the availability of excess $H_2$ in rumen while incorporating some $H_2$ into propionate may also shift the rumen fermentation towards reductive acetogenesis (Beauchemin *et al.* 2008; Dohme *et al.* 2000). Further, suppression of methanogens might aid in proliferation of reductive acetogens and thus a combination of two strategies might work for practical purposes. A judicious use of fats of the right quality and quantity combined with cost management may actually be a practical way of reducing methane emissions.

Fish oil being liquid at room temperature and containing high concentrations of beneficial PUFA is a promising supplement for reducing methanogenesis and needs to be tested in animals reared on pasture based system.

#### 2.3.1.5 Vaccination

Vaccination against methanogens has been researched as another mitigation approach. Almost 8% reduction in methane production in sheep vaccinated against three methanogens was reported by Wright *et al*., (2004). Williams *et al*., (2009) evaluated a broader spectrum vaccine targeting 5 methanogen species in 32 sheep which covered nearly 52% of the species/ strains of methanogens found in Queensland, Australia. Specific IgG titres were detected in plasma, saliva and rumen fluid but there was no effect on methane production or the number of methanogens. The plausible reason for this could be that a large proportion of methanogens cannot be cultured in the laboratory and culturing is required before a vaccine can be made (Wright *et al.* 2006). Also other strains/ species of methanogens can replace the ecological niche left by the species targeted by the antibodies (Williams *et al.* 2009). Further, the highly diverse methanogenic community present in animals reared under different conditions (Wright *et al.* 2007) increases the challenge for developing a broad spectrum vaccine suitable under varied production and geographical conditions (McAllister and Newbold 2008). In practical situations a successful vaccination against methanogens has not yet been achieved (Martin *et al.* 2010). Recently, scientists have sequenced the entire genome of *Methanobrevibacter ruminantium* which is projected to be an important step towards identification of suitable immunological targets (Leahy *et al.* 2010).

## *2.3.2 Enhancing non-methanogens*

### 2.3.2.1 Reductive Acetogens

The key to reducing methane emissions from the ruminants is to manage the $H_2$ produced in the rumen as an end product of fermentation. One of the strategies for this is to provide alternative sinks for $H_2$. As discussed above (section 2.3.1.4), lipids act as a sink by using $H_2$ for biohydrogenation. Another biological option is the promotion of reductive acetogens in the rumen. They use $H_2$ to produce acetate through the process called reductive acetogenesis. This is a highly desirable alternative to methanogenesis because the acetate produced can act as an energy source for the animal. Reductive acetogenesis is undertaken by a broad range of genetically diverse bacteria and is an active pathway for the removal of $H_2$ in a number of gut ecosystems such as in wood termites, kangaroos, rodents, humans and lambs just after birth (Joblin 1999; Klieve *et al.* 2009). However, in the rumen, reductive acetogens are few and cannot compete effectively with methanogens for $H_2$ ions, because they have a lower affinity for $H_2$ or a higher redox potential than methanogens. Rumen methanogens have 10-100 times lower threshold values for $H_2$ than reductive acetogens and thus keep the concentration of $H_2$ in rumen below the values necessary for reductive acetogens to survive (Fievez *et al.* 2001; Greening and Leedle 1989; Joblin 1999). Also, the negative change in Gibbs free energy ($\Delta G^o$) is more favorable for methanogenesis than reductive acetogenesis (Cottle *et al.* 2011).

Reductive Acetogenesis:

$$4H + 2CO_2 \longrightarrow CH_3COOH + 2H_2O + H^+ \quad \Delta G^o = -8.8 \text{ kJ/mol}$$
(Acetic acid)

Methanogenesis:

$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O \quad \Delta G^o = -67 \text{ kJ/mol}$$

$\Delta G^o$ is the change in free energy of the reactions (Cottle *et al.* 2011).

Studies have reported that the concentration of rumen reductive acetogens can vary from undetectable to $10^9$/g of rumen contents and the prevalence of these reductive acetogens depend on diet, animal age and time of sampling (Henderson *et al.* 2010). Microorganisms capable of producing acetate from $H_2$ have been found to originate from many groups having very different phylogenetic lineages. At least 10 species of rumen bacteria capable of reductive acetogenesis have been identified including *Acetitomaculum ruminis, Eubacterium limosum, Blautia schinkii, Blautia producta, Peptosteptococcus productus,* (Henderson *et al.* 2010; Joblin 1999).

Many of these reductive acetogens, however do not use $H_2$ and $CO_2$ as their sole energy source and can generate their energy from other substrates which means that they cannot out compete the methanogens (Cottle *et al.* 2011). Studies by Morvan *et al*., (1994) in the rumen of new born lambs revealed that rumen acetogenesis occurs in the first 24 h after birth, but is subsequently displaced by methanogenesis as the methanogens easily out compete the reductive acetogens at the low concentration of $H_2$ normally encountered in the rumen. Further, some reductive acetogens may require a minimum population in their environment, and/or a minimum $H_2$ threshold in order to compete successfully with methanogens (Joblin 1999).

As early as 1976, it was reported that kangaroos produce low amount of methane as compared to sheep per unit digestible DMI (Kempton *et al.* 1976; Von Engelhardt *et al.* 1978). Significantly, Ouwerkerk *et al*., (2005) found that the forestomach of kangaroos had high numbers of reductive acetogens but few methanogens. It can be deduced that reductive acetogens are able to compete very effectively with methanogens in the kangaroo forestomach and a possible but yet unsuccessful direction would be to promote reductive acetogenesis in rumen by simulating the conditions found in macropods inside the ruminants.

A study by Fonty *et al*., (2007) has showed that in lambs which were kept methanogen free, reductive acetogenesis played a significant part in $H_2$ removal, but once they were inoculated with methanogens, this contribution reverted back to the same level as other lambs having normal rumen populations. Thus, inhibition of methanogens remains to be shown under regular field conditions as to whether the existing rumen reductive acetogens will multiply to desirable population on their own or will have to be regularly inoculated. This may not be practical. In order to adopt reductive acetogens as alternative $H_2$ sink in the mature ruminants, methanogens have to be inhibited first by vaccination, or by regular dietary supplementation with lipids or chemical analogues, to increase the partial pressure of $H_2$ till the threshold desirable for reductive acetogens was reached. Previous attempts at inducing acetic acid production by inoculation with reductive acetogens have not been successful (Immig *et al.* 1996; Nollet *et al.* 1998) and further research is required.

#### 2.3.2.2 Concentrate supplementation

Increased supplementation of concentrates in ruminant diets has been shown to decrease methane production. This has been attributed to a decrease in acetic acid and a corresponding increase in propionic acid production (Demeyer and VanNevel 1975). Higher acetic acid levels have been shown to be associated with a higher methane production (Benchaar *et al.*

2001; Johnson and Johnson 1995). Decrease in methane production with decrease in acetate production is also explained by the fact that starch fermenting bacteria produce less $H_2$ (Stewart and Bryant 1988). Also, feeding soluble carbohydrates causes a decrease in rumen pH affecting the Gram +ve bacteria which are mainly responsible for $H_2$ production thereby limiting the $H_2$ supply to the methanogens. It can also have a direct effect on the pH sensitive methanogens. Further, feeding of highly soluble carbohydrates increases passage rate of feed particles out of rumen and reduces their ruminal digestion (Boadi *et al.* 2002; Leng 1993; Yang *et al.* 2001a). The enzymatic breakdown of starch to glucose followed by absorption of glucose in intestine is more beneficial for the animal than the process of fermentation and absorption of volatile fatty acids (Black 1971). In rumen, carbohydrate fermentation is associated with digestible energy losses comprising heat of fermentation loss (6%) and methane production loss (2-12%). Generally 0.35 mole of methane is produced per mole of fermented starch in the rumen whereas it is assumed that there is no loss of heat or methane production in small intestine (Channon and Rowe 2004; Hungate 1966). Black (1971) estimated that for concentrate diets, the net energy available to a lamb having no fermentation in rumen was 39% higher for maintenance and 22% higher for production than a lamb having normal rumen fermentation. Okine *et al*., (1989) observed a 30% decline in methane production when ruminal passage rate was increased by 54 to 68%. Similarly, Yan *et al*., (2000), found that increase in proportion of concentrate in diet decreased methane output when total digestible energy intake, dry matter intake and feeding level were kept constant.

In another study (Chandramoni *et al.* 1999), a 30:70 ratio of roughage to concentrate was recommended for more protein and energy retention and less methane emissions. Yanez-Ruiz (2008) studied the effect of feeding two different diets; grass hay *ad lib* and hay: concentrate:: 40:60, to weaning lambs and reported that the establishment of methanogens in the rumen and methane production were significantly lower in lambs fed hay and concentrate diet in the initial period of study but the differences diminished after they were grouped together and fed same kind of hay and roughage diet for 4 months.

However, an analysis by Blaxter and Clapperton (1965) showed no relationship between feed quality (DE) content and energy loss to methane for concentrate-roughage mixtures fed at maintenance. This could in part be explained by a modelling analysis by Benchaar *et al*., (2001) where total VFA production increased as the proportion of concentrate in diet increased. The production of acetic acid increased initially with a decreasing forage to concentrate ratio of 50:50 and after that it declined for a ratio of 30:70 whereas the production of propionic acid increased linearly with increase in concentrate level. The methane

production expressed in Mcal $d^{-1}$ also increased initially when the proportion of concentrate in diet was increased from 0 to 20% and it then declined with higher level of concentrate in diet. Further, it has been reported by Johnson and Johnson (1995) that when highly available carbohydrates are fed at limited intakes, high methane losses occur whereas at high intakes of highly digestible diets, low methane losses occur. The reported level of methane loss was 6-7% of energy intake when forages were fed at maintenance whereas it was 2-3% when high grain concentrate were fed *ad lib*.

In grazing steers, Boadi *et al*., (2002) studied the effect of grain supplementation at a level of 2, 4 and 4 kg $d^{-1}$ of steam rolled barley grain in early, mid and late grazing season respectively. Steers fed on early pastures having high pasture quality had 44% and 29% lower energy loss as methane than when fed on mid or late grazing pastures. They concluded that in grazing animals, pasture quality has a significantly higher effect on methane production than grain supplementation. In low quality feeds, proper nutrient utilization has to be achieved by other nitrogen and mineral supplements for decreasing methane production (Leng 1993).

To conclude, concentrate supplementation does offer a solution for methane mitigation in areas where low quality feed is used or when they are added at very high levels (>50%) but the feasibility of supplementation in terms of cost and net farm GHG emissions has to be established on an individual basis.

### *2.3.3 Management*

Since the present study targets cattle reared in South Island of New Zealand where pasture grazing is the main means feeding cattle during the milking season, this brief review on farm management targets strategies proposed for pasture management to achieve better production and net lower methane emissions.

#### Pasture management

Grazing beef or dairy animals have an added benefit in using land which may not be suitable for crops and producing commercial products (Buddle *et al.* 2011). As discussed by Boadi *et al*., (2004) there has been evidence that pasture based dairy farming systems can be as profitable as confinement systems (White *et al.* 2002). In grazing animals, achieving maximum DMI to achieve higher production is the most challenging aspect of nutritional management. It has been argued that net returns under managed intensive grazing are greater because of lower feeding costs associated with pasture forages (Boadi *et al.* 2004; Dartt *et al.* 1999; Hanson *et al.* 1998).

A study by Harper *et al*., (1999) showed that animals lost 1.9- 2.2% of their feed energy to methane when fed on a highly digestible, high grain diet, as compared to 7.7 -8.4% loss of feed energy to methane when they received low quality, high fibre diets. Generally, the methane emission increases with increase in DMI (Molano and Clark 2008). But at a higher level of DMI, the methane production per unit of product decreases with an increase in DMI (Johnson and Johnson 1995). The methane emissions per unit of DMI or per unit of commercial product can further be decreased by improving the quality of pasture. The effect of this improved pasture quality and a higher DMI towards decreasing methane emissions can be explained by an increased rumen passage rate and hence decreased ruminal digestion of high quality pasture (Buddle *et al.* 2011). As an example, a high passage and fermentation rate of lucerne silage (highly fermentable) as compared to grass silage (less fermentable) was observed by Dewhurst *et al*. (2003). Similarly, in an earlier study (McCaughey *et al.* 1999), DMI was high for cows grazing lucerne pastures than grass only pastures (11.4 vs. 9.7 kg DM/day; $P < 0.018$) and thus methane production was also high for lucerne pastures than grass only pastures (373.8 vs. 411.0 L $CH_4$ /day; $P < 0.008$). But, the net amount of feed energy lost through methane was lower for cows grazing lucerne than grass only pastures (7.1 vs. 9.5% of gross energy intake; $P < 0.001$).

Pasture quality was confirmed as a critical factor in managing on farm methane emissions in an experiment by Robertson and Waghorn (2002) when cows grazing low quality pastures had significantly higher methane emissions (7% of GEI) compared to those receiving a TMR (6.3% of GEI), while there was no difference between the two feeding regimens when cows grazed high quality pastures. Amongst the grass based pastures, a highly digestible grass was found to produce less methane than highly fibrous grass by DeRamus *et al*., (2003). Their experiment actually showed that adopting best management practices for grazed farm animals could lead to a 22% annual reduction in methane as compared to general operating practices.

In fact, adoption of better farm management practices for the pasture grazed animals is being considered a more realistic approach to methane mitigation (Beukes *et al.* 2010; Grainger and Beauchemin 2011; Waghorn and Hegarty 2011). These include selection of animals with high reproductive and production capabilities and culling of non-productive animals, good longevity to minimize replacement rate, attendance to animal health, use of improved forages and better grazing management to maximize pasture utilization and management of stocking rate to achieve high production/ha to which will decrease overall GHG emissions from farm. Using a typical pasture based New Zealand farm over different climate years to measure

whole farm GHG emissions, Beukes *et al*., (2010) used a mathematical model to illustrate that adopting better farm management strategies have the potential to decrease total farm GHG emissions by 27-32%.

As has been stressed by Grainger and Beauchemin (2011), it is particularly important in case of pasture grazed animals to consider any mitigation strategy on a whole farm and life cycle assessment (LCA) basis because adopting farm practices such as higher pasture digestibility and high producing animals will increase DMI and methane emissions in g/day, but when considered from commercial point of view i.e. methane emissions/unit of product formed, these practices will actually lead to lower overall methane emissions.

## 2.4 Summary

Recent developments in molecular analysis have paved the way for studying microbial diversity across different geographical areas, species and production systems and many new species or strains of methanogens have been sequenced based upon their 16S rRNA sequences. It has been generally reported that *Methanobrevibacter* spp. are the predominant methanogens in rumen but within a change in diet, the diversity of methanogens within that community might change. Diet quality and the DMI are highly correlated with methane production but it has also been reported that with increase in diet quality and DMI, the net $CH_4$ emissions decrease per unit of product. Thus the typical pasture system of South Island thus offers a novel opportunity to study methanogens in animals having high production from high DMI which is supported by very good quality pastures. Further, the general practice of giving fresh pasture breaks once daily encourages an exaggerated rumen diurnal pattern of pH and other parameters (e.g. VFA and $NH_3$), which would make likely a similar diurnal pattern of methanogenesis. Studying the methanogen ecology, quantity and expression of a typical South Island production system over a diurnal period is valuable to better understand methanogen community structure and activity. Techniques like respiration calorimetry, $SF_6$ technique and inverse dispersion technique are unsuitable to study methanogenesis over a diurnal period from individual animals on a large scale.

Molecular fingerprinting techniques such as DGGE offer means of initial screening of microbial community. qPCR and qRT-PCR are useful and feasible techniques to analyse methanogen community numbers and activity across different supplements. The development of *mcr*A primer which is highly specific for methanogens has increased the validity of these techniques. They can also be used to study the diurnal changes in methanogens and their expression under the influence of any external modifications to the rumen environment. But

the success of these techniques depends upon extraction of high quality and quantity RNA which might be a problem in the rumen contents from animals fed grass diets.

While choosing a mitigation strategy, it is necessary to ensure that any $H_2$ produced in rumen does not accumulate and is used up continuously for the fermentation to proceed continuously ensuring optimum feed utilization. Dietary strategies are the most researched at this point with addition of concentrates or fats being the most practical. Concentrates have the capability to reduce methane emissions but they are generally associated with overall higher carbon emissions, stressing on the need for developing alternate energy rich feed supplements. Fats have the capability of being successful mitigation agents provided they don't interfere with digestion of feed. A low amount (≈3%) of added fat will have relatively low effects on digestibility depression while maintaining its beneficial effects. Suppression of methanogens might aid in proliferation of reductive acetogens (due to increased partial pressure of $H_2$) and thus a combination of two strategies might work.

Fish oil being liquid at room temperature and containing high concentrations of beneficial PUFA is a promising supplement for reducing methanogenesis. The ability of fish oil to be fed to animals in liquid form along with water makes it an easy option to consider for supplementation in a pasture system. It has been shown to decrease number of methanogens as well as methane production in earlier *in vitro* studies. Though some *in vivo* studies have also been done but they have been generally based upon concentrate diets. Therefore, concentrate addition and fat supplementation seem to be the most promising supplements for the pasture system under study.

On this basis, this research was planned to achieve the following objectives:

1. **To describe the rumen methanogen community of South Island cattle grazing high quality forages.**
2. **To develop a suitable method for determining changes in the rumen methanogen community and activity within the diurnal cycle.**
3. **To study the effect of changed diets and management on the population diversity and activity of rumen methanogen in cattle within the diurnal period.**

# Chapter 3

# The Effect of Dietary Supplements on Rumen Methanogen Communities in Cattle Grazing High Quality Pastures

The dynamics of methanogen population in animals are strongly dependent on numerous factors such as diet, species, physiology or production state of animal, and others. Due to the critical importance of methanogenesis in energy budgeting of ruminants as well as any perceived environmental consequences, many studies have been conducted to gain understanding about rumen methanogens under different geographical, climate or dietary conditions.

Zhou *et al*., (2011), discussed that methanogens may be diverse in their phylogenetic relationships but they utilize similar energy generation pathways. These are the $CO_2$ reduction pathway; the $C_1$compound (e.g., methanol and methylamine) conversion pathway; or the acetate fermentation pathway. Also, each methanogen species has a substrate preference and most methanogens can use only one or two substrates (Zhou *et al.* 2011). Therefore, a particular diet might provide substrates which functionally determine the composition of methanogen population in that production system. As discussed in Chapter 1, the South Island (SI), New Zealand pasture production system is different than other TMR based or pasture based systems of the world in that it is based on very high quality pastures which support a high DMI and production.

But no study has so far reported the rumen methanogen community structure or activity in this system. Studies of the diversity of the rumen methanogen community in different systems around the world have generally reported that *Methanobrevibacter* spp. are the predominant methanogens in both cattle and sheep (Mohammed *et al.* 2011; Ouwerkerk *et al.* 2008; Wright *et al.* 2008; Zhou *et al.* 2010). Other species like *Methanosarcina barkeri*, *Methanosphaera stadtmanae* and *Methanobacterium* species have also been detected (Boadi *et al.* 2004; McAllister *et al.* 1996; Moss *et al.* 2000; Ouwerkerk *et al.* 2008; Zhou *et al.* 2011). Similar studies in Australia have reported many novel methanogens (Klieve 2009; Klieve *et al.* 2009; Ouwerkerk *et al.* 2008; Wright *et al.* 2008). A better understanding of the methanogen community structure in a typical SI production system would be of significant benefit in future mitigation strategy development.

Most of the culture independent studies into rumen methanogen communities have been carried out using molecular fingerprinting technique DGGE or by cloning and sequencing analysis or a combination of these (Ouwerkerk *et al.* 2008; Wright *et al.* 2007; Wright *et al.* 2008; Wright *et al.* 2006; Zhou *et al.* 2010). It is possible with DGGE to identify specific bands using cloning and sequencing analysis, and studies have been able to detect differences between DGGE community profiles upon different dietary supplementations (Mohammed *et al.* 2011; Zhou *et al.* 2009; 2010). In this study, the primers 344F-GC and 522R were used for PCR-DGGE analysis of methanogens targeting their V2V3 region which have been reported (Ouwerkerk *et al.* 2008; Yu *et al.* 2008) to cover the widest range of methanogen species as was desired for community analysis.

Cereal concentrates, fat and ionophore supplementation are all extensively reported (Guan *et al.* 2006; Okine *et al.* 1989; Soliva *et al.* 2003) to alter methane production in ruminants in different production systems internationally. Therefore, these three different supplements were used to induce changes in the rumen methanogen community in cows grazing under a typical SI production system, in order to investigate the structure of this community.

## 3.1 Materials and Methods

### *3.1.1 Animals, diets and experimental design*

Three experiments were performed using 10 lactating, ruminally fistulated dairy cows divided randomly into 2 groups of 5 each. All three supplementation experiments were carried out with interval of at least 3 months between them to negate the effect of any previous supplementation. The cows were Holstein-Friesian Jersey cross with a December live weight of 470kg with average seasonal milk solids production of approximately 425kg, broadly approximating a daily pasture intake of 16-18kg DM.

#### 3.1.1.1 Feeding and treatment

During each supplementation experiment, all the animals were grazed on ryegrass (*Lolium perennes*) and clover (*Trifolium repens*) pasture as control diet with a dry matter allowance of 16-18kg DM/day along with free access to drinking water. The experiments were performed in a 5 x 5 crossover design. One group of 5 animals was considered as treatment group and supplemented with the respective treatment along with standard pasture allocation while the second group of 5 cows was managed as control group and fed their usual dry matter allowance. This feeding pattern was followed for 14 days in order to provide an adaptation period for the animals on the experimental diet before samples were collected from them over a period of 4 days. After the first collection period, all the animals were given their routine

pasture allocation of 21 days in order for them to arrive at their pre-trial status. After the stabilization period, the groups were switched and previous procedures repeated.

***Grain supplementation***

The treatment group was given 1.5kg of cracked barley grain twice daily (16% of DMI) along with their pasture allowance while the second group of cows was managed as control group and fed only the pasture allowance.

***Fat supplementation***

The treatment group were fed 300g (1.8% of DMI) of granulated palm oil fat (HyFat, Agri-Feeds Pty Ltd., Mt Maunganui, NZ ) source along with their pasture allowance while the second group of cows was managed as control group and fed only the pasture allowance.

***Monensin supplementation***

Two pre-weighed tubes of Rumensin$^{TM}$ (Elanco Pty Ltd., Auckland, NZ) were placed in the rumen of treatment group cows through rumen fistulas so as to release an approximate 600mg (37mg/kg DM) of monensin in the rumen of animal each day while the second group of cows was managed as control group and fed their usual pasture allowance.

#### 3.1.1.2 Sample collection

The samples were collected from experimental and control animals once in the morning and once in the evening in order to observe any pre or post-prandial changes with an interval of 4 days i.e. for the first sample collection, the samples were collected on the first day (Monday) in the afternoon at 3pm (6 hours after fresh pasture break) and on fifth day (Friday) in the morning at 9am (just before fresh pasture break) and for the second collection period similar pattern was repeated after an interval of 21 days.

For sample collection, whole rumen contents were collected from the ventral sac of an animal's rumen through the rumen fistula and were immediately aliquoted into 4 microtubes (1.7ml capacity) per one animal. They were immediately centrifuged at 13,200rpm for 5min. The supernatant was drained off and samples were stored on ice before being transported and stored in a -20$^{\circ}$C freezer for further processing.

#### 3.1.1.3 Processing of samples

##### 3.1.1.3.1 Extraction of genomic deoxyribonucleic acid (DNA)

The genomic DNA was extracted from the samples by the standard bead beating method and phenol: chloroform precipitation method of Whitford *et al*., (2001) which is described in detail in appendix A.1. Briefly, 0.5g of whole rumen contents (WRC) stored at -20$^{\circ}$C were thawed and added to a bead beating tube containing 0.5g of 0.1mm zirconia beads (Daintree

Scientific, St. Helens, Tasmania) along with 700µl of TE buffer (pH 7.2, 10mM Tris-HCl, 1Mm EDTA), 700µl of phenol (pH of phenol phase 6.7±0.2, Sigma-Aldrich, Auckland, New Zealand) and 20µl of 10% sodium dodecyl sulfate (SDS) solution. The samples in tubes were then subjected to three bead beatings of 2min each in a Mini-Beadbeater-8™ (Biospec Products, BioLab, Auckland, New Zealand) with an intervening 2min interval on ice. This was followed by a chloroform extraction (700µl) at 10,000rpm for 10min at 4°C. The supernatant was pipetted out into a fresh microtube and incubated with 20µl of 10mg/ml RNase enzyme (REF 10109134001, Roche Diagnostics GmbH, Mannheim, Germany) at 39°C for 60min. After incubation 700µl of phenol was added and samples were centrifuged at 13,200rpm for 10min at 4°C. This was followed by a phenol: chloroform (350µl:350µl) extraction and a chloroform (700µl) extraction at 13,200rpm for 10min at 4°C. To the clear supernatant from this extraction, 0.25 volume of 3M sodium acetate and 2.5 volume of absolute ethanol, were added to precipitate DNA and incubated at -20°C for 1 hour. This was followed by centrifugation at 13,200rpm for 10min to pellet the DNA. The pelleted DNA was then washed with 1ml of 70% alcohol and then dried in a vacuum centrifuge at 60°C. The pellets were then reconstituted in sterile distilled water and stored at -20°C till further analysis.

The extracted genomic DNA samples were quantified with spectrophotometry for estimating DNA concentrations (NanoDrop Technologies, Thermo Fisher Scientific, Auckland, New Zealand). Absorbance was recorded at 260/280 nm and 260/230 nm. They were analysed for integrity by gel electrophoresis procedure in 1% agarose gel.

#### 3.1.1.3.2 Gel electrophoresis

The detailed protocol (Sambrook and Russell 2006) is described in appendix A.2. Briefly, gel electrophoresis was performed in 1% agarose gel containing ethidium bromide to check the integrity of extracted DNA as well as to check the size of amplified PCR products. 5µl of the standard, 1Kb ladder (Axygen, Raylab, Auckland, New Zealand) for genomic DNA or 100bp ladder (Axygen, Raylab, Auckland, New Zealand) for PCR products was loaded into the gel along with 5µl of sample mixed in loading buffer. The gel was run at 95V for 30min with 1X TBE as the running buffer. The gel was examined under UV light in Gel Doc™ XR+ System (catalogue no.-170-8195, Bio-Rad, Auckland, New Zealand).

#### 3.1.1.3.3 Amplification of methanogenic archaeal rDNA fragments

rDNA fragments of methanogenic archaea were PCR amplified from total DNA samples using two sets of methanogen specific primers in a nested PCR amplification in a Bio-Rad Thermocycler™ (Auckland, New Zealand) according to the protocol described in Ouwerkerk *et al* (2008). The DNA samples were quantified and the amount of genetic material to be

added into the PCR mix was adjusted by suitable dilutions in sterile distilled water to achieve similar concentration in µg/ml.

#### *3.1.1.3.3.1 Methanogen 16S rDNA specific PCR*

In the first step, amplification was done with Taq polymerase (Qiagen, Biostrategy Ltd., Auckland, New Zealand) using 16S rDNA primers 46F: 5' YTA AGC CAT GCR AGT (Ovreas *et al.* 1997) and 1017R: 5' GGC CAT GCA CCW CCT CTC (Barns *et al.* 1994). The protocol included initial denaturation at 95°C for 3min followed by 30 cycles of denaturation at 95°C for 45sec, annealing at 47.3°C for 45sec and extension at 72°C for 45sec with a final extension at 72°C for 5min.

***Reaction setup***

The master mix was prepared for all the samples along with negative and positive controls according to the composition described in appendix A.3. A 50µl reaction was set up having 1U/µl of Taq polymerase, 0.2mM of each dNTP, 2.5mM final concentration of $MgCl_2$ and primer concentration of 0.2µM each. 49µl of master mix was allocated into 0.2ml PCR tubes along with 1µl (≈400ng/ µl) of respective DNA template. The products were checked for amplification on a 1% Agarose gel electrophoresis and compared with a standard 100 bp ladder (Axygen, Raylab, Auckland, New Zealand).

#### *3.1.1.3.3.2 Methanogen V2V3 PCR*

The amplified products from the 16S PCR reaction were then subjected to a second PCR reaction for amplifying the variable V2V3 regions by using specific methanogen primers 344F-GC: 5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC GGG GHG CAG CAG GCG CGA (Raskin *et al.* 1994) and 522R: 5' GWA TTA CCG CGG CKG CTG (Amann *et al.* 1995). The reaction setup was the same as for 16S PCR except for different primers and is described in appendix A.3. The PCR reaction was performed using Taq Polymerase (Qiagen, Biostrategy Ltd., Auckland, New Zealand). The protocol included 20 cycles of denaturation at 94°C for 30sec, annealing at 65°C for 30sec with a 0.5°C decrease in annealing temperature after every cycle and extension at 72°C for 30sec. This was followed by 15 cycles of denaturation at 94°C for 30sec, annealing at 55°C for 30sec and extension at 72°C for 30sec. In the end there was a final extension at 72°C for 3min. The products were run on a 2% agarose-TBE gel to check for specific amplification and compared against a standard 100bp ladder (Axygen, Raylab, Auckland, New Zealand). The products from this PCR were then analysed on DGGE gels.

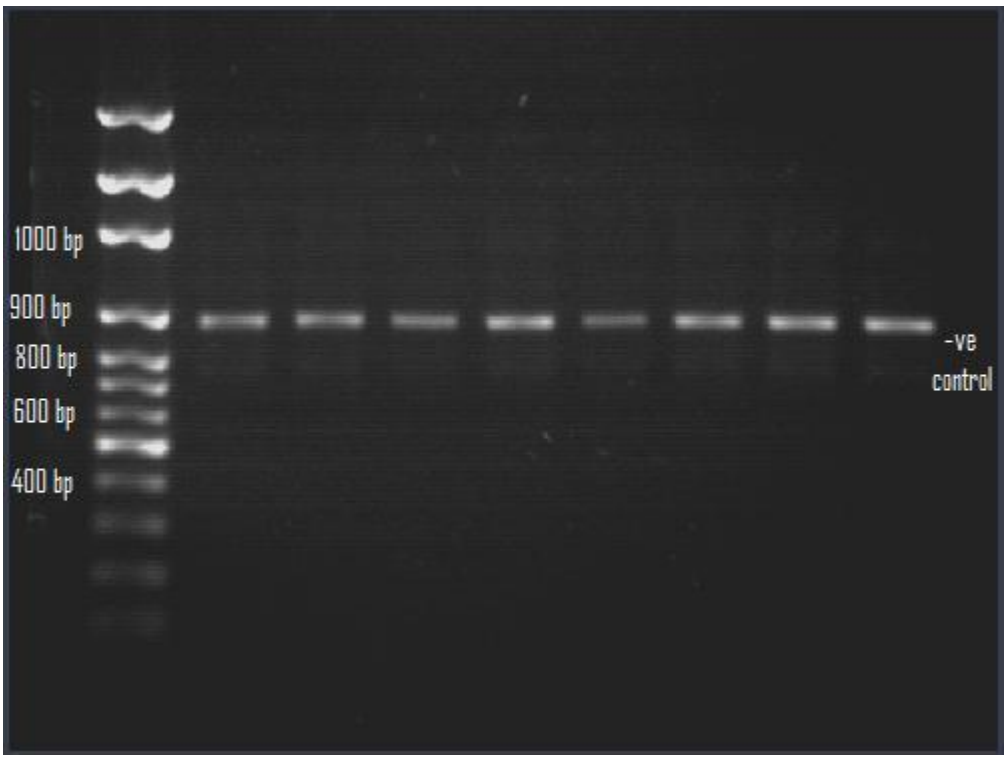



**Figure 3.1 Gel electrophoresis of methanogen 16S rDNA from different rumen samples amplified with 16S archael primers 46F and 1017R.**

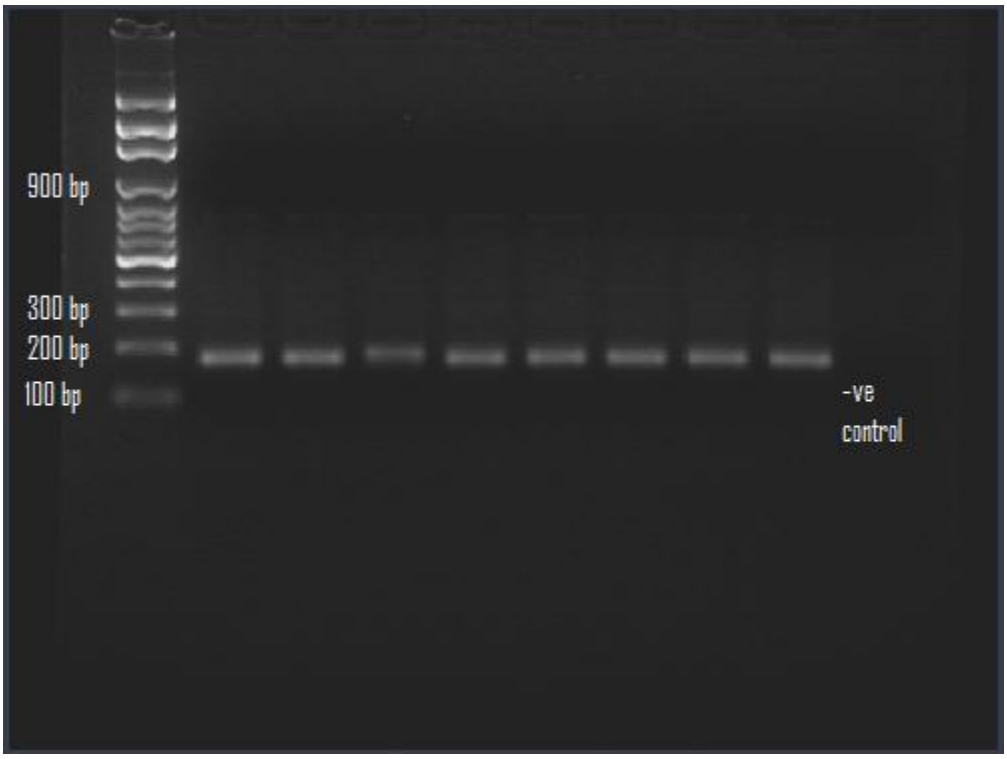



**Figure 3.2 Gel electrophoresis of methanogen 16S rDNA from different rumen samples amplified with archael V2V3 primers 344F-GC and 522R.**

#### 3.1.1.3.4 Verification of PCR products

The products from 16S archaeal PCR amplified with primers 46F and 1017R were analysed on 1% agarose gels and the bands obtained were compared against standard 100bp ladder where the sample bands ran close to the band in standard representing 900bp of the ladder as shown in figure 3.1. Similarly, the products from second archaeal V2V3 PCR with primers 344F-GC and 522R were analysed on 2% agarose gels and the bands obtained were compared against standard 100 bp ladder where the bands ran close to the band of standard representing 200 bp (expected product size 180bp) as shown in figure 3.2.

Further, to confirm the specificity of primers, the products obtained from Archaeal V2V3 PCR were cloned and sequenced using TOPO TA™ Cloning kit (Invitrogen, Catalogue no. K4500-01, Life Technologies, Auckland, New Zealand) through chemical transformation using pCR 2.1-TOPO vectors according to manufacturer's instructions. The protocol is described in appendix A.6.2. The sequences were subjected to BLAST™ search of NCBI database (Altschul *et al.* 1990) and they showed 100% matches with the sequences of *Methanobrevibacter* sp., *Methanosphaera stadtmanae* and a few uncultured methanogens. No non-specific product was detected confirming the specificity of primers.

#### 3.1.1.3.5 Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed with a Dcode™ Universal Mutation Detection System (Bio-Rad Laboratories, USA). Samples were amplified by PCR reaction (as in section 3.1.1.3.3) and analysed on polyacrylamide gels having 8% acrylamide with urea and formaldehyde as denaturants creating a gradient of denaturation from 30% to 60%. After assembling the parallel gradient gel sandwich, the glass plates were sealed with 8% acrylamide seal into which 30µl each of 10% ammonium persulphate and tetramethyl ethylene diamine (TEMED) were added. After the seal had set, 20ml each of the denaturing solutions were mixed with 70µl each of 10% ammonium persulphate and TEMED and pored through a gradient maker into the sealed glass plates. The gradient was created such that the denaturation capacity in gel increased from top to bottom. Gel was allowed to set for at least one hour and samples were loaded into the wells of gel. The samples were run along with standards in 0.5X TAE Buffer at 100V for 18 hours and stained by silver staining (protocol described in appendix A.4) adapted from Kocherginskaya *et al*., (2005). Images of the gels were obtained by scanning on a flatbed scanner (Hewlett and Packard, New Zealand).

***Validation of PCR/DGGE***

Extracted DNA samples of two animals from two different experiments (grain and fat supplementation) i.e. 4 in total, were taken and PCR was performed on them in triplicate

(technical). These 12 PCR products were then subjected to DGGE and the band profile was compared. Appearance of identical band profiles amongst technical triplicates indicated absence of any PCR or DGGE induced anomalies.

#### 3.1.1.3.6 Identification of bands

Based on the banding patterns observed in DGGE analysis, 12 bands were aseptically excised from the gels and DNA isolated from them using the method of Schweiger and Tebbe (1998) for polyacrylamide gel extraction. The extracted DNA was reamplified using primer pair 344F-GC and 522R without a GC-clamp. The PCR product was then run on 2% agarose gel to check the size of product and was purified using AXYPREP$^{TM}$ PCR cleanup kit (AXYGEN, Raylab, Auckland, New Zealand). The purified PCR products were cloned using a TOPO TA cloning kit (Invitrogen, Auckland, New Zealand) following the chemical transformation with pCR 2.1-TOPO vector and the transformants were screened on X-gal/LB medium containing ampicillin (100mg/ml). 10 colonies with inserts (white colonies) were randomly picked per band and grown overnight in LB broth containing ampicillin (100mg/ml). The plasmid DNA of the transformants containing insert was extracted using *Escherichia coli* (E.coli) plasmid mini prep procedure adapted from Birnboim and Doly (1979). Resriction enzyme digest was done with Eco R1 (20,000U/ml, R0101S, BioLabs, Auckland, New Zealand) to separate the inserts from plasmid and screened on a 2% agarose gel. The samples (plasmid mini-preps) with inserts were purified with AXYPREP$^{TM}$ PCR cleanup kit (Axygen, Raylab, Auckland, New Zealand) and 3 plasmids per band were submitted for sequencing. Sequencing was done using ABI Big Dye Terminator v3.1 cycler (Applied Biosystems, Auckland, New Zealand). The sequence reaction was performed with 10µl of solution containing 0.5µl of BigDye, 3.2pmol of M13 Forward (CGCCAGGGTTTTCCCAGTCACGAC), 2.0µl of 5X sequencing buffer, and 20ng of plasmid DNA as the template. DGGE was also performed on clones to compare them against the band requiring identification. The gene sequences obtained (180bp) were compared to the nucleotide databases for 100% match and identified through GenBank's basic local alignment search tool (BLAST) (Altschul *et al.* 1990). Sequences were assembled using sequence analysis software SEQMAN and aligned by neighbor-joining clustal W method in MEGALIGN (Lasergene, DNASTAR) (Burland 2000). A phylogenetic tree was generated based upon similarity index through MEGALIGN and bootstrap analysis performed with resampling 1000 times. Out of 12 bands sequenced 6 generated single sequences and 6 generated multiple sequences and they were labeled accordingly.

## 3.2 Results

### *3.2.1 Community identification from DGGE*

An average of 22-25 bands was obtained upon DGGE of all samples (figure 3.4). Out of these 12 bands could be sequenced. Because the primers used to generate products from DGGE band stabbings were the same as had been used for performing PCR, the sequences obtained were of short size (180bp) and strong phylogenetic relations could not be obtained through bootstrap analysis at 1000 times resampling.

Phylogenetic analysis of the sequences obtained from these 12 major DGGE bands (figure 3.3) indicated that most of the bands (8) represented sequences showing 100% identity match with *Methanobrevibacter* sp. along with 2 bands matching (100%) *Methanosphaera stadtmanae* and 1 band each were representing a species matching with order Methanobacteriales and family Methanobacteriacae. However, some bands showed presence of multiple sequences i.e. presence of more than 1 species in a single band. Some uncultured archaeon and uncultured methanogenic clones were also detected. The sequences were also compared with the bands obtained in standards for further confirmation of species identity. The band identification made through the limited phylogenetic analysis and comparison against standards was similar to the profile obtained by Mohammed *et al*., (2011) and Zhou *et al*., (2010) who had used similar primer pairs and a mix of pure cultures of different methanogen species as standards.

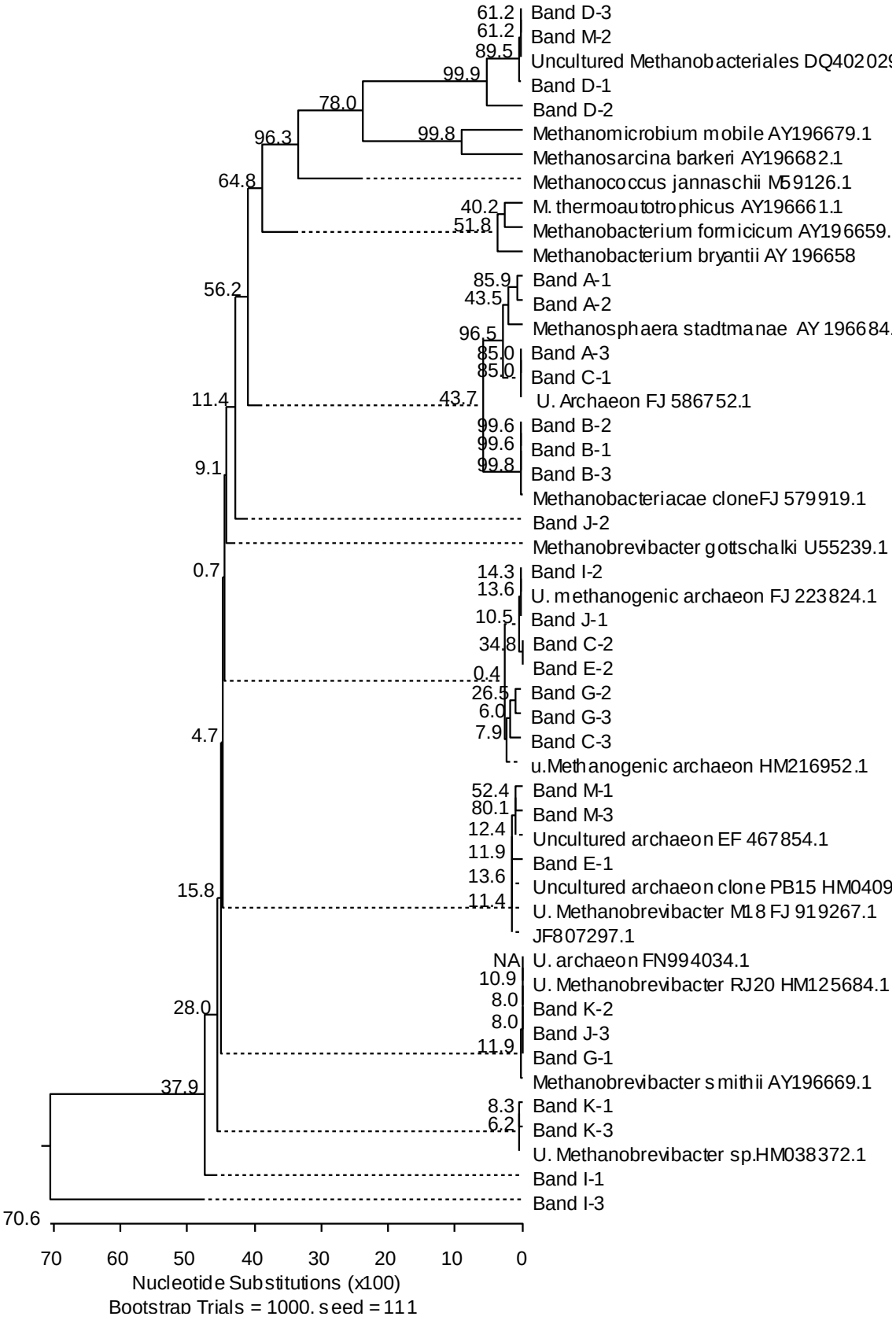
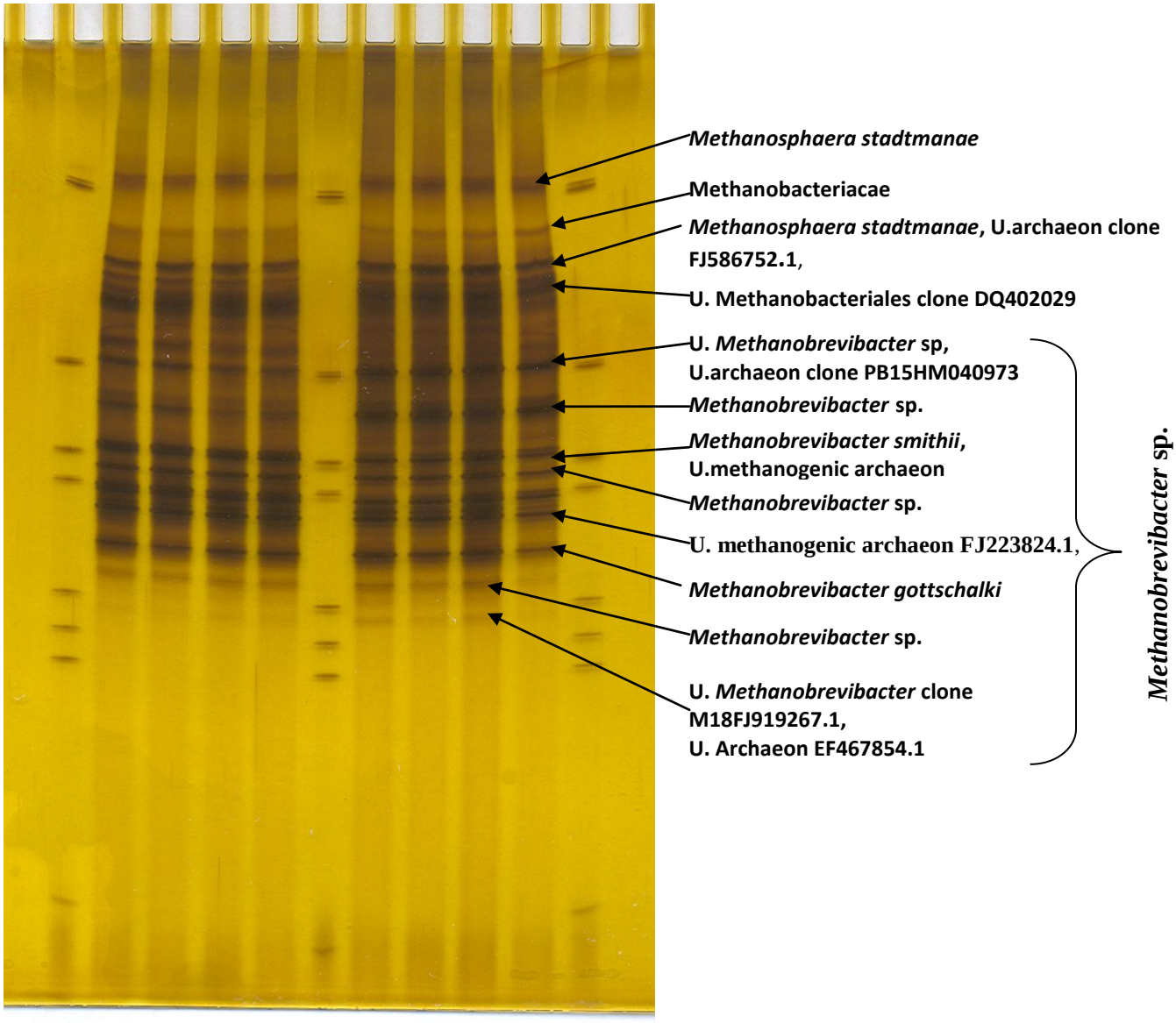



**Figure 3.3 Phylogenetic analysis of sequences obtained from DGGE band stabbing.**




U.- Uncultured strain/ species

**Figure 3.4 Identification of DGGE Bands.**

### *3.2.1 DGGE profiling upon dietary modifications*

DGGE profiling was done to detect any changes in the methanogen community. Each animal was represented in 2 control samples i.e. control morning (pre-prandial) and control evening (post-prandial) and 2 treatment samples i.e. treatment morning (pre-prandial) and treatment evening (post-prandial). Figures 3.5 to 3.8 show the DGGE gels for grain supplementation experiment, figures 3.9 to 3.12 for fat supplementation experiment and figures 3.13 to 3.16 for monensin supplementation experiment.

#### 3.2.1.1 Grain supplementation

In figure 3.5, the gel analysed represents both the morning and afternoon sample collections for control and grain supplementation period of animals 624 and 399, respectively. Approximately, 25 bands were detected clearly in the gel. For both the control as well as grain supplementation samples, no distinguishable difference was detected between the treatments. There was only a slight difference in the band intensity upon grain supplementation samples for animal 624 as highlighted in box 'a' of figure 3.5 and another difference in band intensity was detected in the morning sample of animal 399 upon grain supplementation as highlighted in the box 'b' of figure 3.5. The bands showing these changes represent *Methanobrevibacter* sp. indicating an effect of grain supplementation on this species in the two animals while no effect was observed on any other species.

In figure 3.6, the gel analysed represents both the morning and afternoon sample collections for control and grain supplementation period of animals 589, 641 and 704, respectively. Approximately, 25 bands were visible in this gel. In this gel the band no.3 representing *Methanosphaera stadtmanae* showed some changes as highlighted by boxes 'a', 'b' and 'c'. In box 'a' for the animal 589, the band was present in the control samples but absent in the treatment samples for both morning and evening sample collections whereas it was absent throughout in animal 641 as highlighted in box 'b' and was present in both control and treatment samples for both sample collection times in animal 704 as highlighted in box 'c'. Similarly, the band no.8 representing *Methanobrevibacter sp*. also showed some changes in band intensity as being lighter in the control samples of both times than the treatment samples, for animal 589 as highlighted by box 'd'; uniform in intensity for all samples of animal 641 (box 'e') and lighter in intensity for all samples of animal 704 (box 'f').

In figure 3.7, the band representing *Methanosphaera stadtmanae* was detected in all samples of 3 animals except the control samples of animal 616 (box 'd'). The band representing family Methanobacteriacae showed variable response in being totally absent in 616 (box 'c') and present in 715 (box 'b') except in control evening sample, whereas in 711 it was lighter in control and treatment evening sample but present in the treatment morning sample (box 'a'). Further, the band no. 8 representing *Methanobrevibacter sp*. was very light or absent in animals 711 & 715 (box 'g') but was present in control samples of 616 and absent in the treatment samples (box 'f'). Interestingly, in animal 616 the band for a species of order Methanobacteriales was present in control samples but it disappeared in the treatment samples (box 'e'). No significant change in DGGE profile was detected in any sample of animal 619 and 175 (figure 3.8).

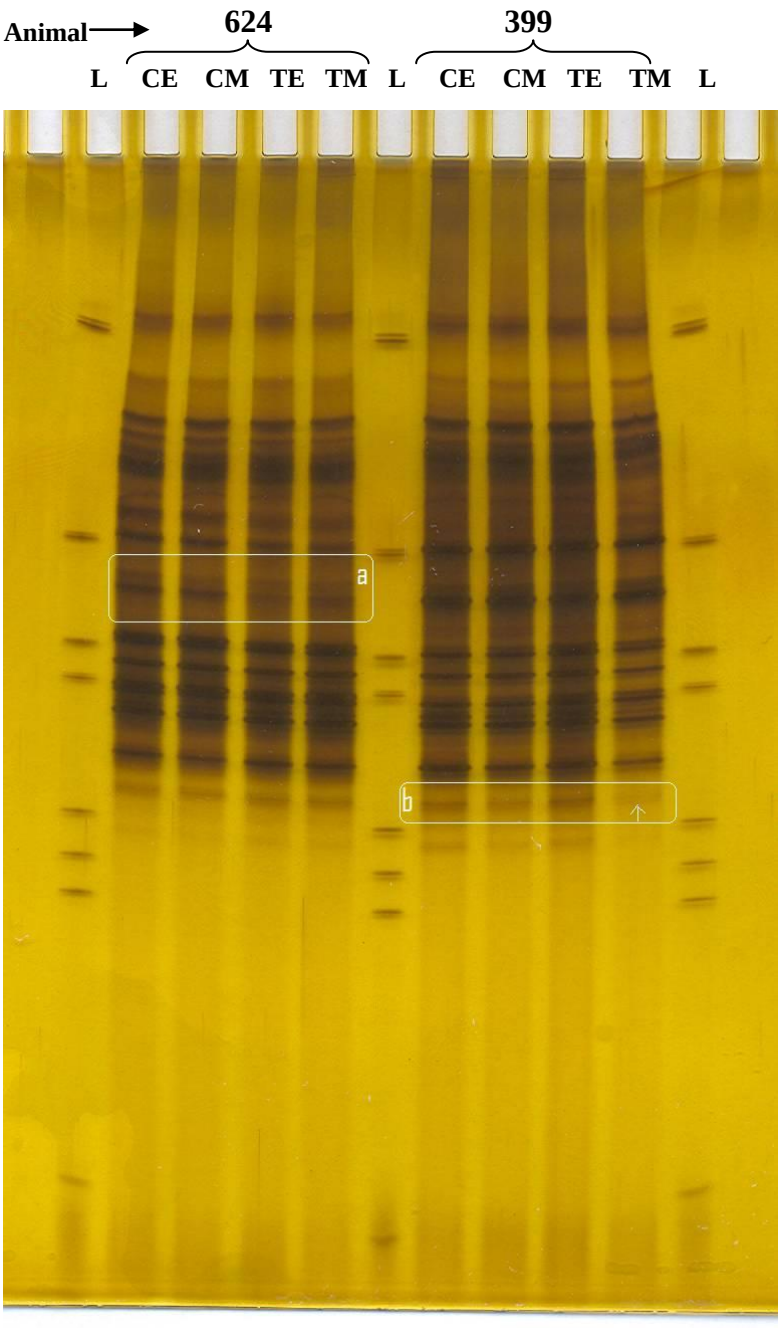



**Figure 3.5 DGGE of animals 624 and 399.**

***Arrows and boxes represent changes detected in gels. L- ladder, CE- control evening (3pm), CM- control morning (9am), TE- treatment evening (3pm), TM- treatment morning (9am).**

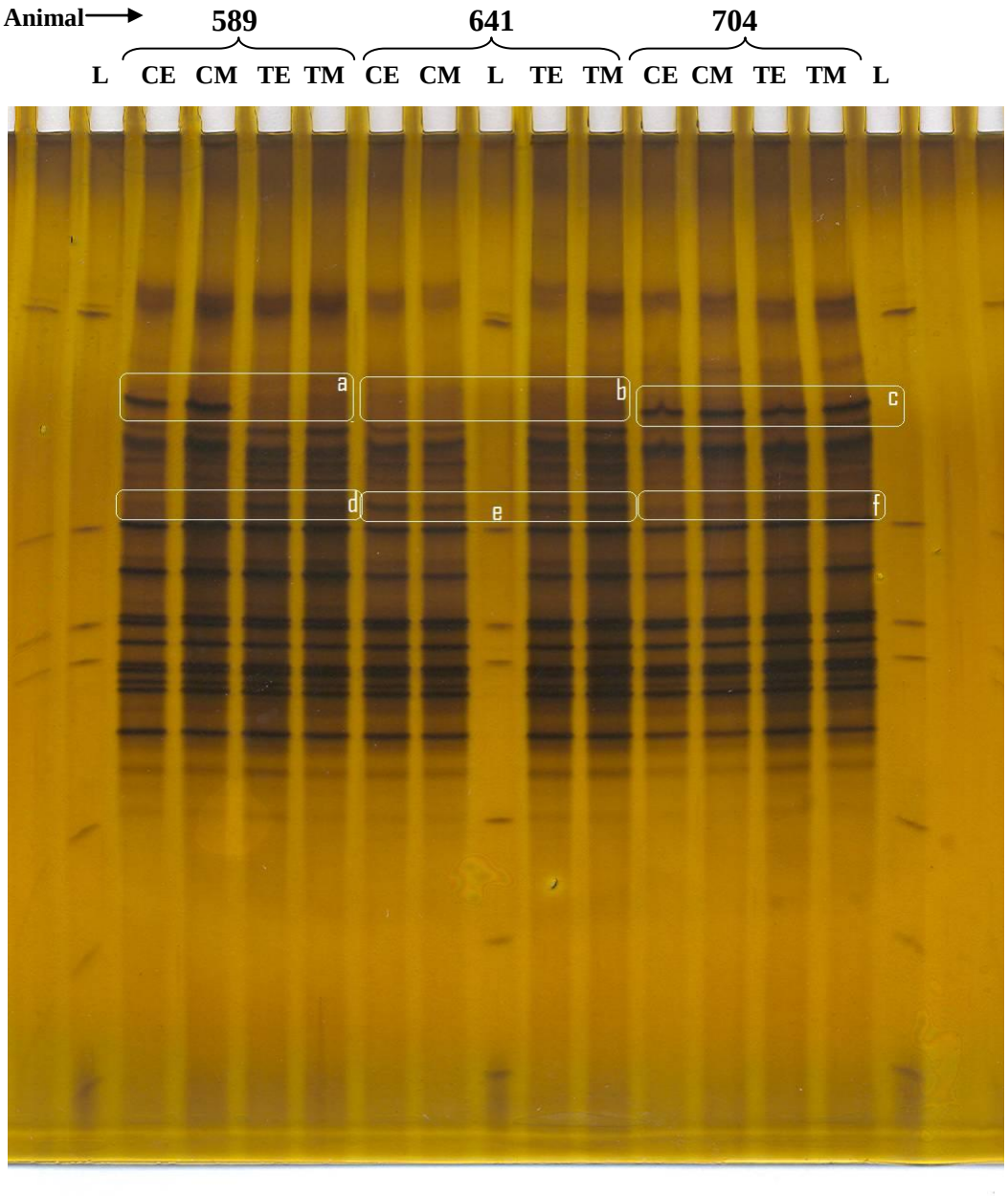



**Figure 3.6 DGGE analysis of animals 589, 641 and 704.**

***Arrows and boxes represent changes detected in gels. L- ladder, CE- control evening (3pm), CM- control morning (9am), TE- treatment evening (3pm), TM- treatment morning (9am).**

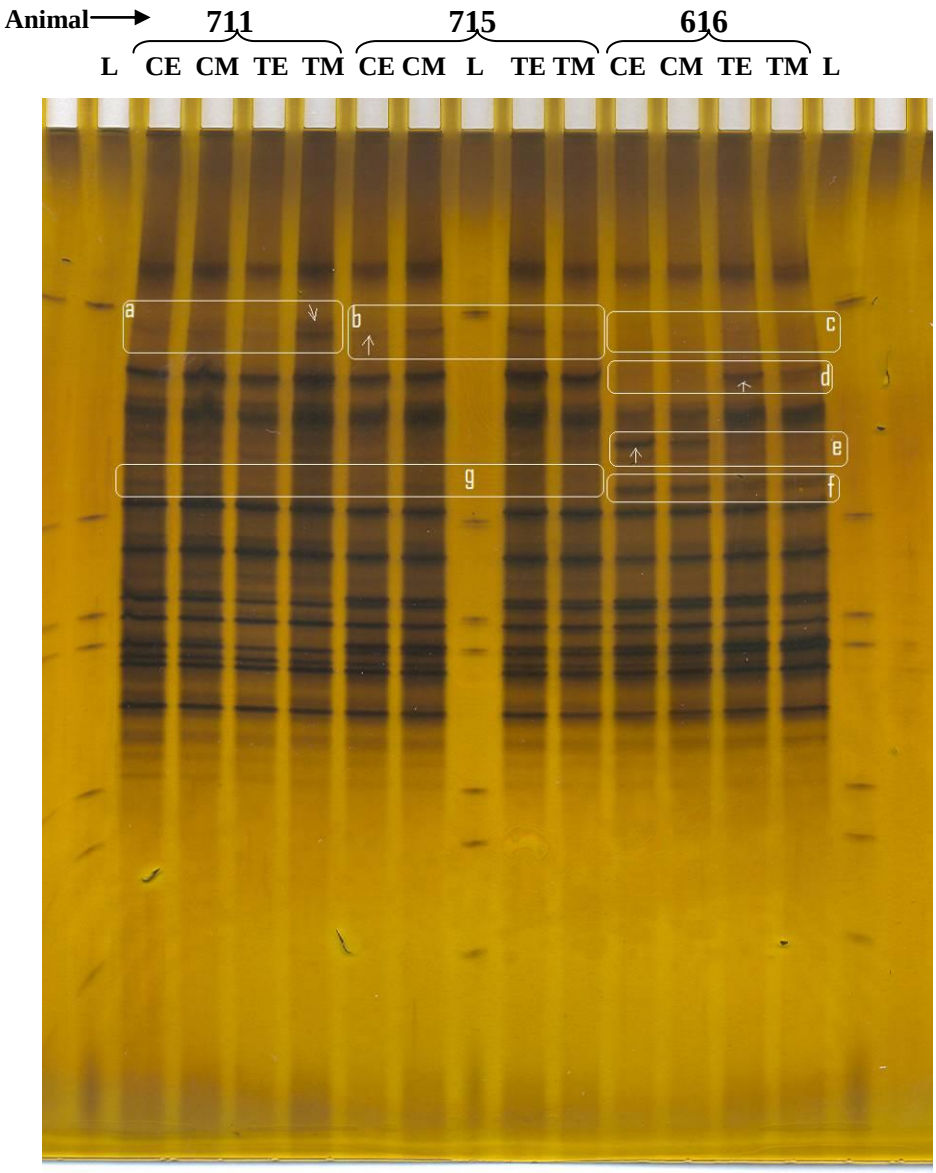



**Figure 3.7 DGGE analysis of animals 711,715 and 616.**

***Arrows and boxes represent changes detected in gels. L- ladder, CE- control evening (3pm), CM- control morning (9am), TE- treatment evening (3pm), TM- treatment morning (9am).**

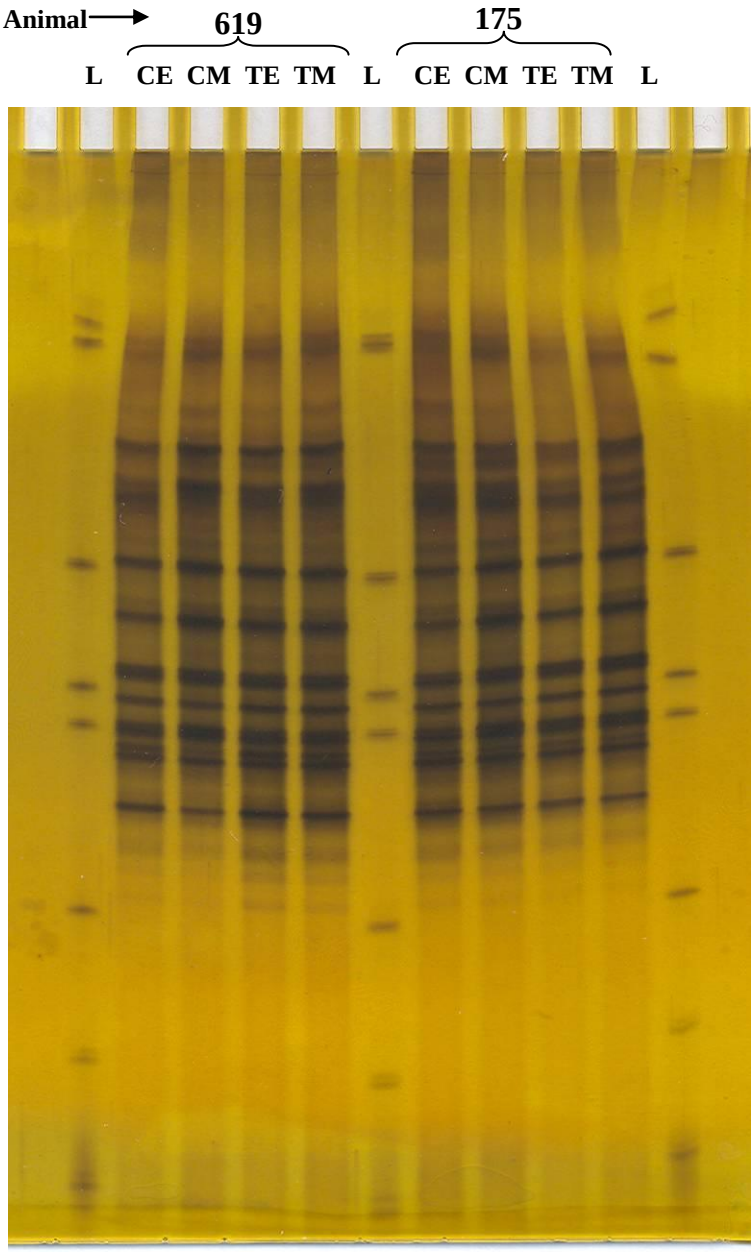



**Figure 3.8 DGGE analysis of animals 619 and 175.**

***L- ladder, CE- control evening (3pm), CM- control morning (9am), TE- treatment evening (3pm), TM- treatment morning (9am).**

### 3.2.1.2 Fat supplementation

In the DGGE gels for animals supplemented with fat, approximately 25 bands were detected. No difference in any banding pattern was observed for all 10 animals across both treatment and control periods at morning as well as evening times (figure 3.9 to figure 3.12).

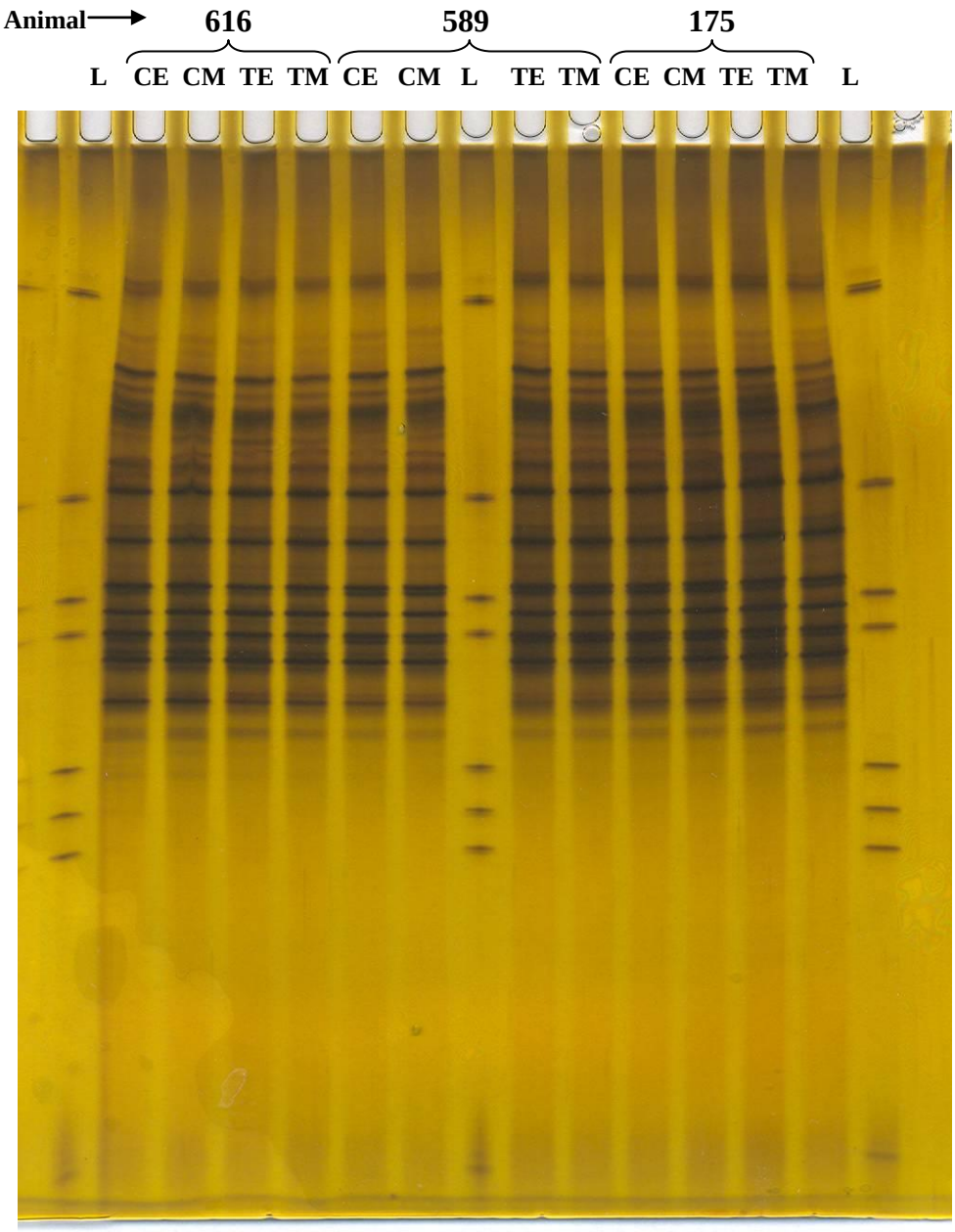



**Figure 3.9 DGGE analysis of animals 616, 589 and 175.**

***L- ladder, CE- control evening (3pm), CM- control morning (9am), TE- treatment evening (3pm), TM- treatment morning (9am).**

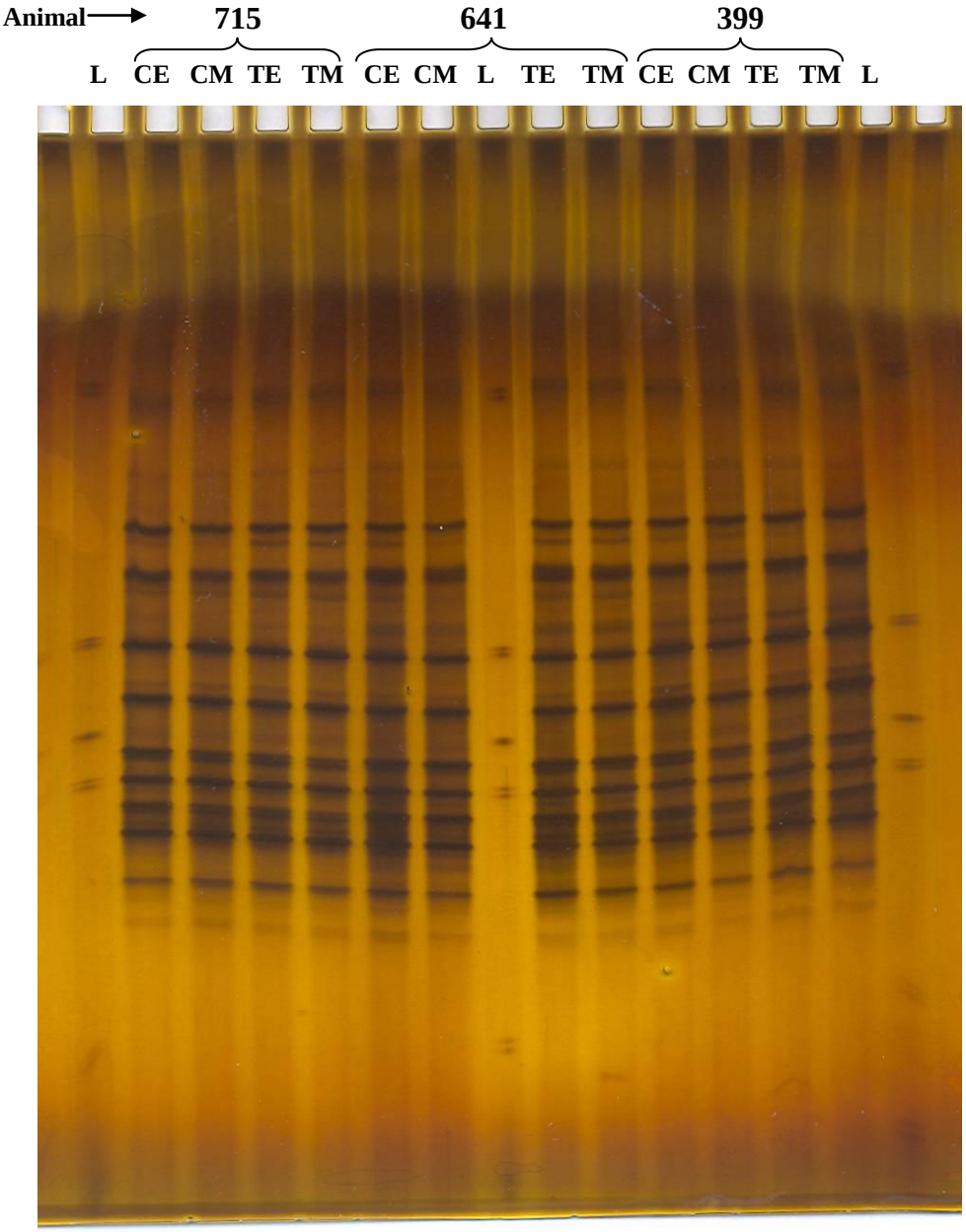



**Figure 3.10 DGGE analysis of animals 715, 641 and 399.**

*** L- ladder, CE- control evening (3pm), CM- control morning (9am), TE- treatment evening (3pm), TM- treatment morning (9am).**

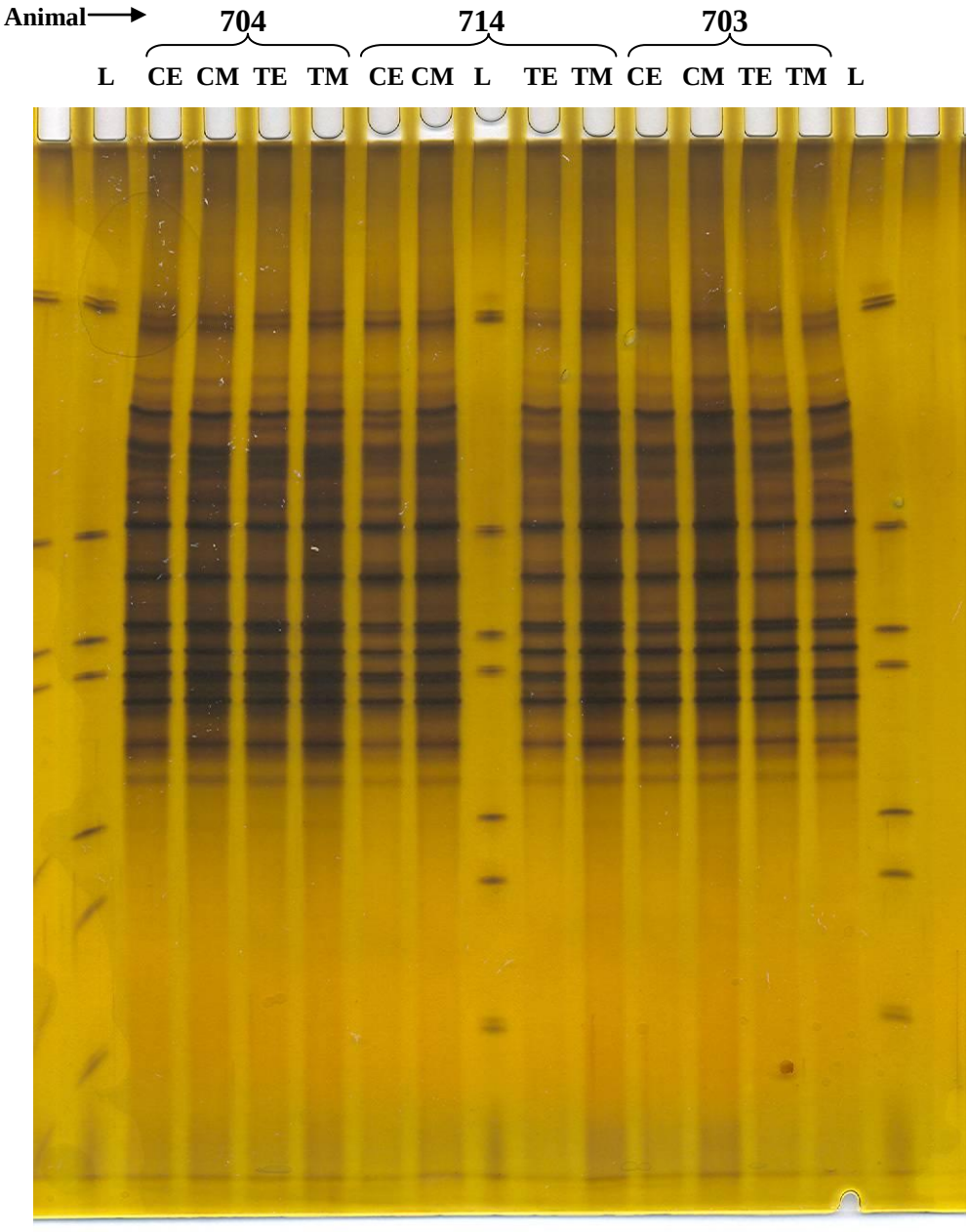



**Figure 3.11 DGGE analysis of animals 704, 714 and 703.**

*** L- ladder, CE- control evening (3pm), CM- control morning (9am), TE- treatment evening (3pm), TM- treatment morning (9am).**

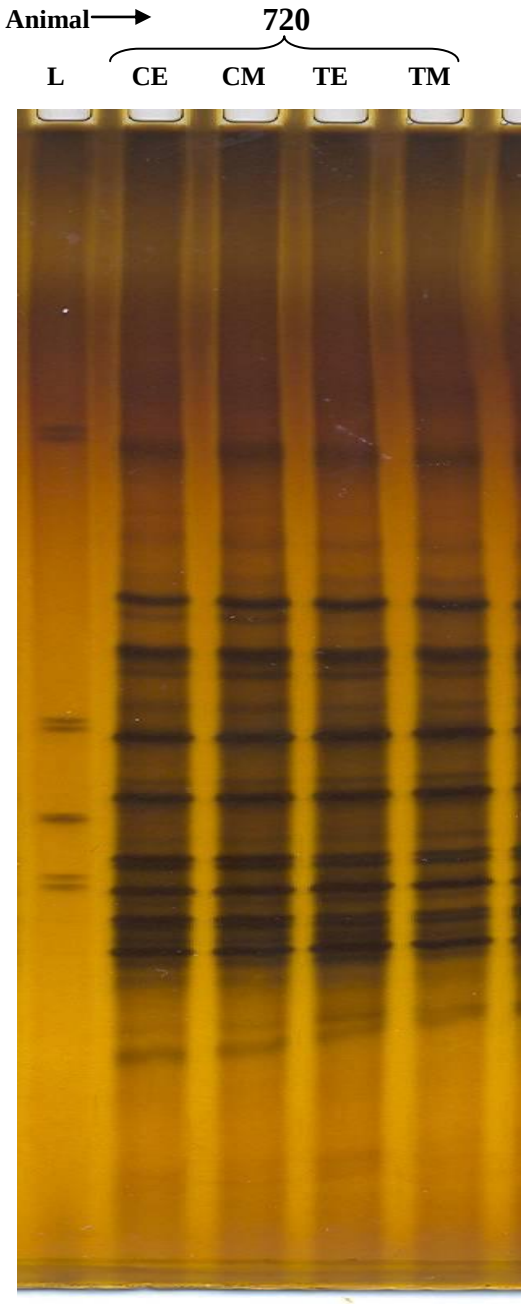



**Figure 3.12 DGGE analysis of animal 720.**

*** L- ladder, CE- control evening (3pm), CM- control morning (9am), TE- treatment evening (3pm), TM- treatment morning (9am).**

### 3.2.1.3 Monensin supplementation

Similar to the grain and fat supplementation experiments 25 bands were detected in the monensin addition experiment. But no change in any band profile could be detected across any of the samples in all 10 animals (figure 3.13 to figure 3.16).

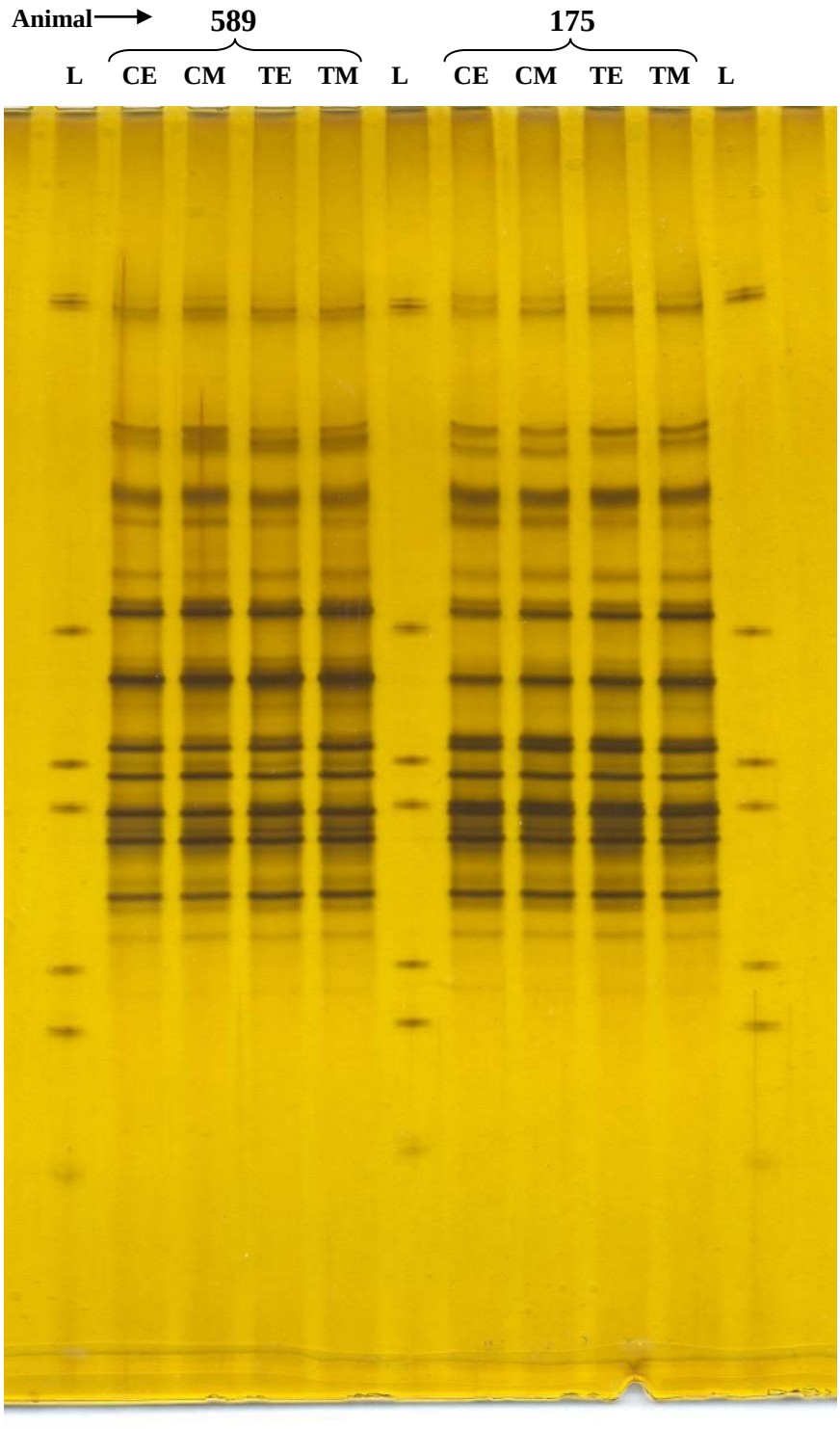



**Figure 3.13 DGGE analysis of animals 589 and 175.**

*** L- ladder, CE- control evening (3pm), CM- control morning (9am), TE- treatment evening (3pm), TM- treatment morning (9am).**

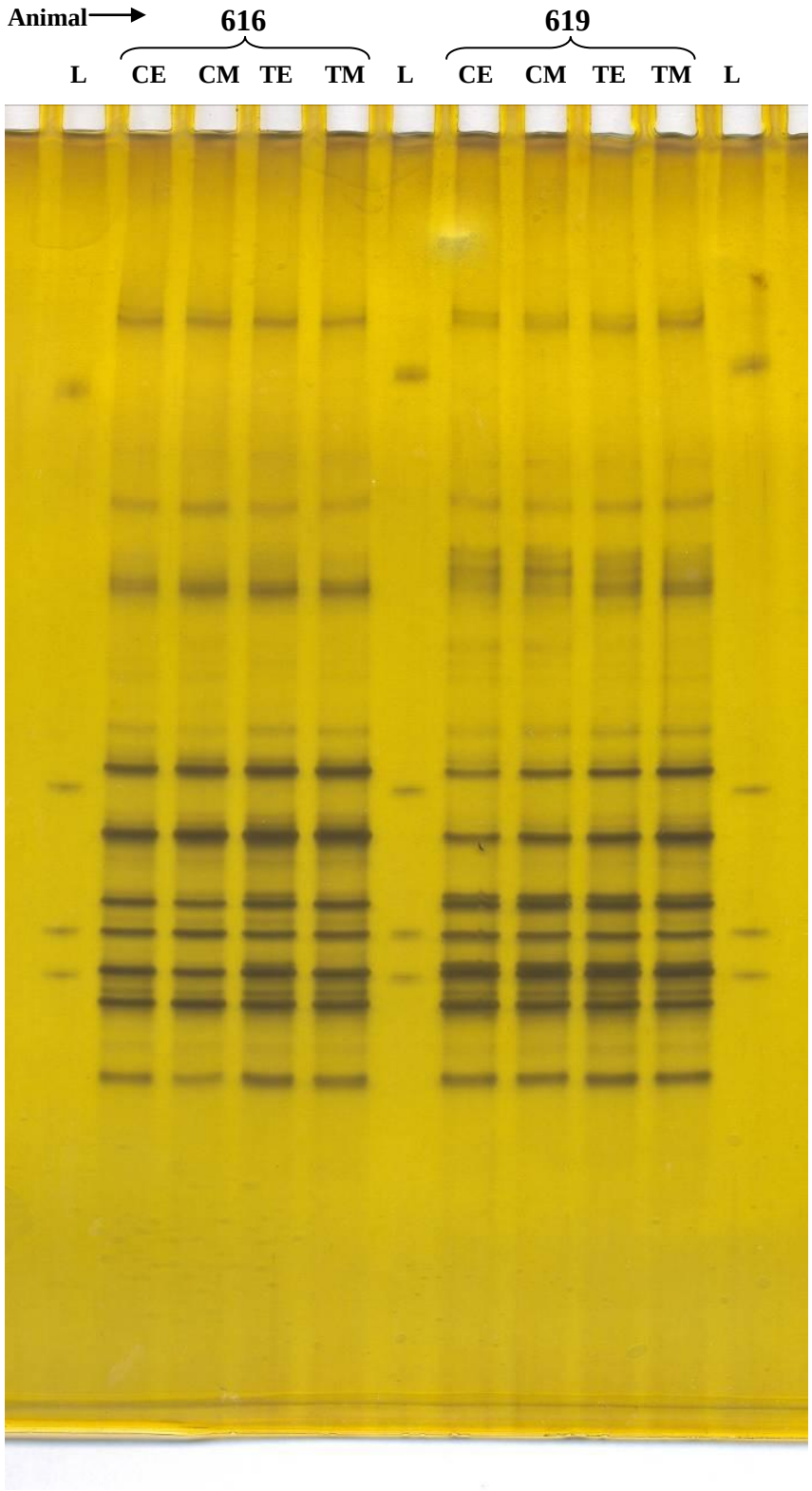



**Figure 3.14 DGGE analysis of animals 616 and 619.**

*** L- ladder, CE- control evening (3pm), CM- control morning (9am), TE- treatment evening (3pm), TM- treatment morning (9am).**

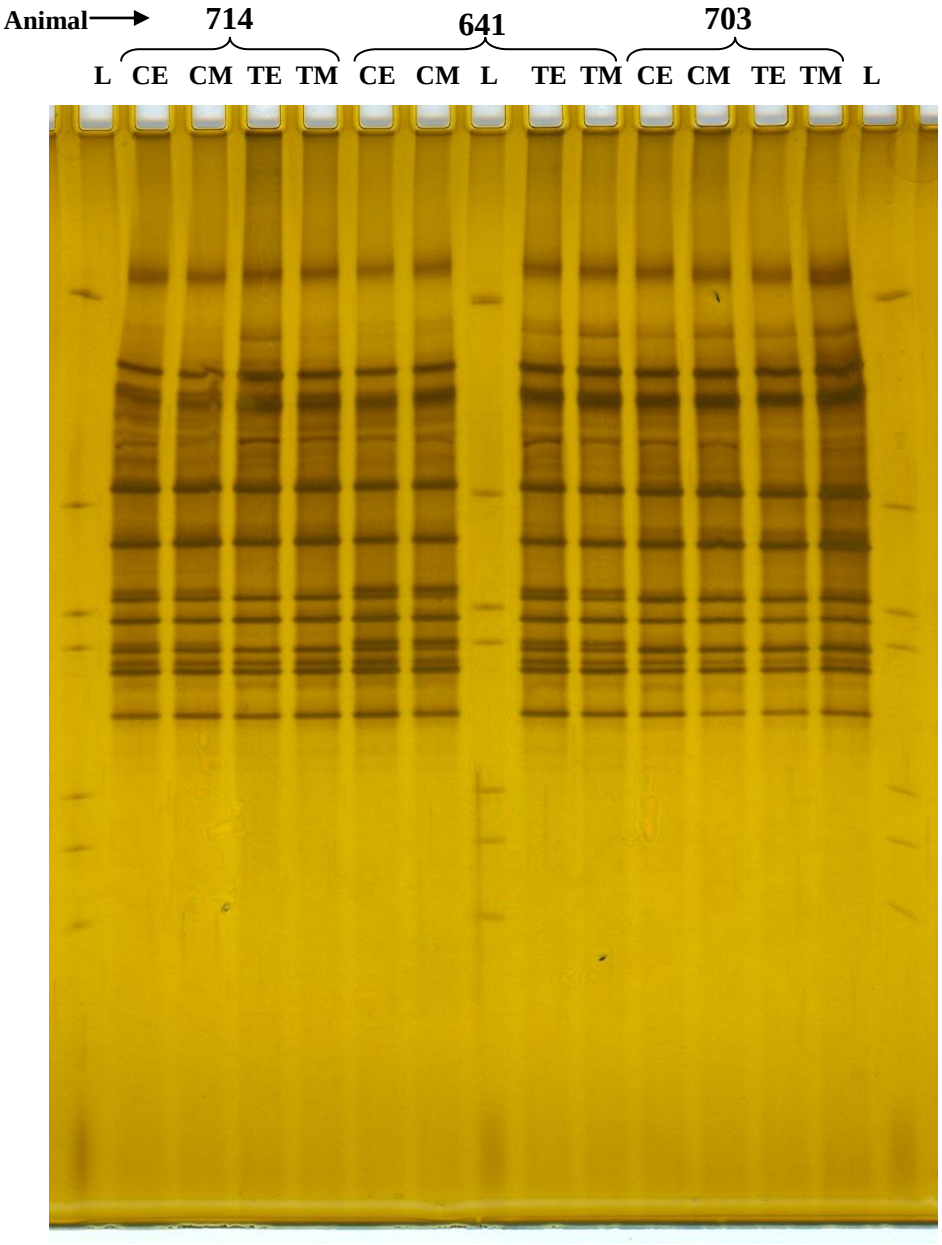



**Figure 3.15 DGGE analysis of animals 714, 641 and 703.**

*** L- ladder, CE- control evening (3pm), CM- control morning (9am), TE- treatment evening (3pm), TM- treatment morning (9am).**

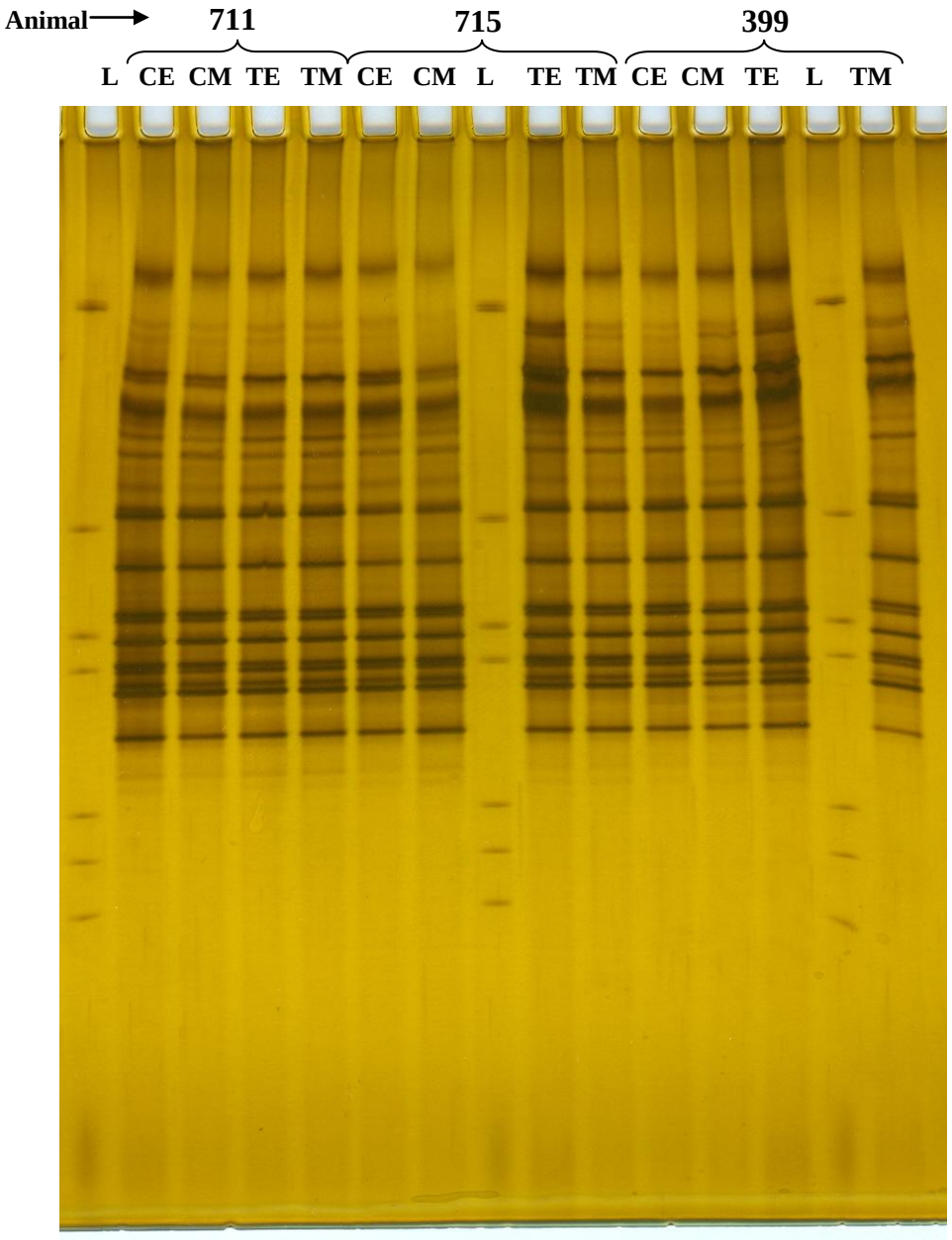



**Figure 3.16 DGGE analysis of animals 711, 715 and 399.**

*** L- ladder, CE- control evening (3pm), CM- control morning (9am), TE- treatment evening (3pm), TM- treatment morning (9am).**

## 3.3 Discussion

Approximately, 22-25 major bands were detected across all experiments in all animals. The bands showing closest match (100%) to *Methanosphaera stadtmanae* and species from family Methanobacteriacae were detected in the upper domain of the gels suggesting that the DNA from these species has a comparatively higher A: T content whereas the bands closest to

*Methanobrevibacter* sp. occupied the lower domain of the gel suggesting presence of a higher G: C content in these species and more stability towards denaturation.

The detection of between 22-25 bands in DGGE across all diets indicated that a very diverse methanogen population is present in pasture fed animals from South Island of New Zealand. A high diversity of methanogens in grazing cows of North Island, New Zealand had earlier been recognized by Skillman *et al*., (2006). Zhou *et al*., (2010) and Mohammed *et al*., (2011) using similar primer pairs, had also detected similar number of bands in their DGGE gels though the experimental animals were fed TMR based diets in their experiments. But in the experiment of Ouwerkerk *et al*., (2008) using the same protocol and primers, only 6-8 bands were detected for cattle fed tropical forages, and 3-4 bands for cattle fed on concentrate based diets. This may suggest a comparatively higher diversity of methanogens in cattle fed pastures of very high energy density and crude protein content.

The identification of DGGE bands through phylogenetic analysis and comparison against standards in DGGE bands indicates that the genus *Methanobrevibacter*, a member of order Methanobacteriales is the most prevalent followed by *Methanosphaera stadtmanae*, a member of family Methanobacteriacae, which is in accord with many earlier bovine and ovine studies across the world over different diet constitutions (Ouwerkerk *et al.* 2008; Wright *et al.* 2007; Wright *et al.* 2008; Wright *et al.* 2006; Zhou *et al.* 2010). Due to the relatively short size of sequences (190bp), identification to the species/ strain level could not be reported in some bands. But the results from the phylogenetic analysis and banding pattern of different species as obtained in the DGGE gels and compared against standards, was quite similar to other studies such as by Mohammed *et al*., (2011) and Zhou *et al*., (2010) in cattle where similar primer pairs had been used for PCR amplification and DGGE. Mohammed *et al*., (2011) had been able to compare the bands till species level because they had a bigger product size which was obtained by using a different reverse primer (915r) for sequencing the excised DGGE bands. Zhou *et al*., (2010) used similar primer pair for DGGE and sequencing and compared their products against a ladder created by mixing plasmids obtained from earlier identification experiments. Other studies have used different primer pairs for sequencing which give a larger product size in which it is possible to differentiate species having minor differences in sequences.

The DGGE profiles were similar across all experiments in all animals suggesting presence of a similar methanogen community in all animals under all treatments. Further, it has been reported that methanogens are able to utilize only a very narrow range of substrates (Wolin *et al.* 1997) and thus the composition of a particular methanogen community is largely

dependent on the substrate availability (Ouwerkerk *et al.* 2008). Lower methanogen numbers in concentrate supplemented weaned lambs (Chandramoni *et al.* 1999) and a lower diversity of methanogens upon grain supplementation as compared to hay fed animals has been reported (Ouwerkerk *et al.* 2008). Previous reports (Okine *et al.* 1989; Yan *et al.* 2000) have also reported a significant decrease in methane production upon concentrate supplementation. In the present grain supplementation experiment, some changes in band intensity or disappearance of bands were observed but no uniform pattern was detected upon supplementation amongst all the animals. The bands representing a species of *Methanobrevibacter* showed decreased intensity upon grain supplementation in five animals while in one animal the decreased intensity was observed in a different band or species of *Methanobrevibacter*. The bands representing *Methanosphaera stadtmanae* and family Methanobacteriacae were absent in some and present in some animals without any control or treatment effect, suggesting more effect of an animal to animal variation i.e. the variable banding pattern could be a result of varied animal response to a given treatment which may further be dependent on its relative feed efficiency for different substrates.

In the present study no observed differences were present in grain supplemented cattle compared to the pasture only control group. One explanation could be the difference in level of grain supplementation, as a total of 3 kg of cracked barley grain was fed in this experiment (i.e.16% of the total DMI). Johnson and Johnson (1995) had reported an increase in methane production when grain concentrate was fed at maintenance levels as compared to a decrease when fed at *ad lib* levels. Also, an increase in methane production was measured in another study up to 20% concentrate addition which was followed by a decrease in methane production at higher levels of supplementation (Benchaar *et al.* 2001). Further, Boadi *et al*., (2002) had concluded that when animals were fed high quality pastures, the quality of pasture had more significant effect on methane production than lower input grain supplementation. It may be that the grain supplementation rate used in this experiment, although industry standard in the South island, was simply too low to induce any major methanogen community changes, such as the complete elimination of certain species. It is also possible that subtle changes may have occurred in the methanogen population but these were not picked up by DGGE.

In another experiment, Zhou *et al*., (2009) had observed major pattern change from a community containing predominantly *Methanobrevibacter ruminantium* NT7 with the low-energy diet to a community containing predominantly *Methanobrevibacter smithii*, *Methanobrevibacter* sp. AbM4, and/or *M. ruminantium* NT7 with the high-energy diet. Also, for each diet, the methanogenic PCR-DGGE pattern was strongly associated with the feed

efficiency of the host**.** The presence or absence of different bands in this experiment could have been due to differences in diet and feeding level of concentrates (74% concentrate with 20% hay against 84% of concentrates with no hay).

For the monensin trial, no changes in banding pattern across all the animals were detected suggesting that monensin had no strong effect on the diversity or quantity of methanogens. This is a similar result to Karnati *et al*., (2009) using continuous culture fermenters, where no response of monensin supplementation to the DGGE banding patterns was detected. The lack of DGGE banding response to monensin supplementation was also detected by Hook *et al*., (2009) in cows fed a total mixed ration along with 24mg of monensin premix/kg of diet DM, which is a lower daily rate of administration than in the current experiment. However, monensin has been found to exert an inhibitory action on methane production even if with short term use (Guan *et al.* 2006), suggesting the possibility that monensin does not always strongly reduce methanogen populations but may decrease or reduce the activity, which was not detected by DGGE in this experiment.

Similarly, no effect on the methanogen community was observed upon palm oil granules fed at ≈2% of DMI though fats have been reported to exert a direct toxic effect on methanogens (Soliva *et al.* 2003) and decreasing their number. It is again possible that rather than a complete elimination of particular species, the effect is to reduce numbers and activity, which is not identified through DGGE.

The technical limitations to the community profiling by DGGE such as presence of more than one species in a single band or a single species being expressed in multiple bands which was detected in the present study as well as previous studies (Klieve *et al.* 2007; Muyzer and Smalla 1998) could also have interfered in analysis of community profile through DGGE.

Further, other studies have reported that methane production was not associated with methanogen diversity under the influence of inhibitors (Firkins and Yu 2006; Karnati *et al.* 2009) and it had also been suggested that when the sensitive species of methanogens are inhibited other less sensitive populations may take up the ecological niches left vacant by the inhibited populations. Therefore, at a given time some populations may be more active over the other. It is difficult to quantify such an effect through DGGE. DGGE is a technique more suitable to defining the existence or absence of a given species through the presence or absence of relative bands and poorly indicates any changes in population numbers via the intensity of band. It does not provide any information on the relative methanogenesis activity. Also, any changes in the methanogen community if occurring might be effected at different

times in different animals depending upon their own grazing pattern, feed efficiency, metabolism, etc. and spot sampling may not be an accurate way of depicting these changes. Therefore, a significant observation from this experiment was that, in order to fully describe the effect of any mitigation agent in terms of its effect on methane production, a profile study of the methanogen community alone is not sufficient but also requires quantification of relative methanogen activity.

To conclude, in this study a diverse methanogen community was found in pasture fed animals. *Methanobrevibacter sp*. was found to be the predominant population in all animals irrespective of any treatment along with *Methanosphaera stadtmanae* and members of family Methanobacteriacae and order Methanobacteriales along with some uncultured archaea or methanogen species. Animal to animal variation was visible in the composition of methanogen community. It was also concluded that when animals are fed a high quality pasture, it may override the effect of any dietary supplementations at low level such as grain supplementation at 16% of DMI or fat supplementation at 2% of DMI on methanogen diversity as is the practice in South Island conditions. But this statement cannot be supported from DGGE analysis alone and thus it is important to combine any study on the effect of methane mitigation strategy with other quantitative and qualitative techniques where the actual contribution of methanogen populations to methane production over different time periods and feeding can be accounted for.

# Chapter 4

# Quantification of *mcr*A RNA and DNA of Rumen Methanogens in Cattle Fed High Quality Forages

The identification of successful methane mitigation strategies requires accurate detection of its effect on methanogen community. An attempt to detect changes in the methanogen population by supplementing with methanogen inhibiting supplements had been done using DGGE as reported in Chapter 3. This technique, though, useful in initial screening of the populations could not depict any subtle changes in methanogen population within treatments. As it is possible that the supplements caused a change in the activity of methanogen population rather than a simple linear reduction in methanogen populations, there was a requirement for a methodology which was sensitive enough to estimate methanogen quantity and activity under the influence of rumen modification.

As discussed in section 2.1 of Chapter 2, the techniques generally used to measure methane production such as respiration calorimetry, $SF_6$ technique, inverse dispersion-laser technique, the tunnel method and *in vitro* technique are not suitable to measure rumen methanogenesis in individual animals under actual grazing conditions within a diurnal period. As an objective of this study was to characterise any diurnal variation in rumen methanogenesis or methanogen community structure in cattle grazing under the typical South Island production system, a more suitable methodology was required. Additionally, it is also possible that the methane emission at any given point in the diurnal cycle is a poor representative of methanogenesis at that time due to the size of the rumen and thus the lag period between the production of gas and its gathering at the oesophageal orifice for eructation. It has been suggested that performing qPCR and qRT-PCR targeting *mcr*A gene which is present exclusively in methanogens and is unlikely to have more than a single copy can provide a sensitive detection of methanogen numbers and activity at a particular time (Denman *et al.* 2007; Guo *et al.* 2008; Luton *et al.* 2002). Thus, the qPCR and qRT-PCR techniques offered a viable approach to analyse the rumen methanogen population and activity.

The efficient molecular analysis of any biological sample using these techniques relies heavily on the optimal qualitative as well as quantitative extraction of nucleic acids (DNA and RNA) (Fleige and Pfaffl 2006). In rumen, the efficient and unbiased extraction of undegraded nucleic acids is hampered by the relatively robust microbial cell walls and contamination of extracted material with proteins and phenolic compounds (Yu and Morrison 2004). Recently,

procedures have been reported for extracting RNA and DNA from rumen microbes that use commercial, column based kits for purifying the extracted nucleic acids. The use of these kits is limited by their cost, their ability to extract RNA and DNA efficiently from diverse samples, and the limitation of nucleic acid yield by the absorptive capacity of the column. The yield and quality of both RNA and DNA assumes greater significance in analysis where quantification has to be carried out (Fleige and Pfaffl 2006). In particular high quality RNA is required for reverse transcriptase quantitative polymerase chain reaction (qRT-PCR), the process used to quantify target gene expression and which requires the extracted RNA to be used in two linked enzymatic processes (reverse transcription and qPCR) (Fleige and Pfaffl 2006; Popova *et al.* 2010).

In order to analyze the methanogen DNA and RNA changes over the diurnal period, there was a requirement for a method that could yield high quality RNA from large numbers of trial samples, was simple, efficient, robust, inexpensive and could simultaneously extract DNA from the same sample. Also, existing methods, do not satisfactorily extract good quality and quantity RNA from rumen samples of kale fed cattle, perhaps due to higher concentration of some metabolites such as phenols which may interfere in the nucleic acid extraction. It was therefore important to overcome problems of low yield and poor RNA quality as well as denaturation of RNA extracted from rumen samples derived from kale fed animals along with the grass fed animals.

After experimenting with different methods used for RNA extraction, a method was developed from modification of two existing methods for nucleic acid extraction, the conventional phenol: chloroform extraction method (Whitford *et al.* 1998) incorporated with few steps from the method of Gambino *et al*., (2008). In order to check the efficacy of this modified method, it was tested against two published methods for RNA yield and quality.

This study compares our modified method with RNA extraction methods using commercially available RNA extraction kits from MOBIO$^{TM}$ and the method of Kang *et al*., (2009) which also utilizes a commercial kit to extract RNA from whole rumen contents and rumen fluid in New Zealand Holstein Friesian cattle grazing two different diets.

## 4.1 Materials and Methods

### *4.1.1 Sample collection and preparation*

Two multiparous, ruminally fistulated Holstein Friesian crossbred cows (average 490kg) were used. They were first grazed on kale *(Brassica oleracea)* with barley straw for 40d at an

approximate daily intake of 10 and 2.5kg DM, respectively. They were then grazed for 40d on a mixed ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) pasture at a daily allocation of approximately 16kg DM. At day 40 and day 80 both whole rumen contents (WRC) and rumen fluid (RF) were collected from the rumen of both cows. 400g of WRC was collected from the ventral sac of the rumen via the fistulas, and 200g was placed in an ice bath for 5min, then aliquoted to microtubes. RF was obtained by squeezing the remaining 200g of WRC through four layers of cheese cloth, and then placed in an ice bath for 5min and aliquoted into microtubes. The microtubes were then centrifuged under refrigeration (4°C) at 13200rpm for 5min. The supernatant was discarded and pellet was resuspended in 500µl of RNA protectant (RNAprotect® Bacteria Reagent, Qiagen, Bio-Strategy, Auckland, New Zealand) followed by incubation at room temperature for 5min, vortexing and finally centrifugation at 13200rpm for 10min. Supernatant was again discarded and tubes containing pellets were snap frozen under liquid nitrogen and stored at -80°C for further analysis.

### 4.1.2 RNA Extraction

The modified method (method 1) was evolved as a combination of the conventional phenol and chloroform extraction (Whitford *et al.* 1998) and the incorporation of 5M lithium chloride (LiCl) solution for RNA precipitation as adapted from Gambino *et al*., (2008). The method is detailed in appendix A.7. Briefly, 0.5g of WRC or 200µl of RF were added to bead beating tubes containing 0.5g of 0.1mm zirconia beads (Daintree Scientific, St. Helens, Tasmania) along with 500µl of TE buffer (pH 7.2, 10mM Tris-HCl, 1mM EDTA), 500µl of phenol (pH of phenol phase 6.7±0.2, Sigma-Aldrich, Auckland, New Zealand) and 20% sodium dodecyl sulfate (SDS) solution. The samples were then subjected to three bead beatings of 2min each in a Mini-Beadbeater-8™ (Biospec Products, BioLab, Auckland, New Zealand) with an intervening 2min interval on ice for all samples. In case of RF (200µl), the samples were treated the same way except that they were subjected to only two bead beatings of 2min each. This was followed by additional equal volume (700µl) phenol and chloroform extractions at 4°C. The RNA in aqueous supernatant was divided into two tubes (350µl each) and precipitated by addition of 2.5 volumes of 5M LiCl and incubation on ice for 30min followed by centrifugation at 13200rpm for 15min. The supernatant from this step was pooled and used for DNA precipitation facilitated by addition of equal volume (2ml) of isopropanol. Following precipitation the resulting pellet containing RNA was washed with 70% ethanol and air dried. Precipitated RNA from both tubes were reconstituted with 50µl of TE buffer (pH 7.2) and pooled to a net volume of 100µl. To remove contaminating DNA, the samples were treated with Turbo DNA-free™ DNase (Ambion, Applied Biosystems, Auckland, New

Zealand). To remove DNase and any residual contaminants, RNA samples (volume made up to 500µl with TE buffer pH 7.2) were purified with an equal volume phenol and chloroform (250µl:250µl) followed by a chloroform (500µl) extraction in 1.5ml light Phase Lock Gel (light) tubes (5 PRIME, Eppendorf, Global Sciences, Auckland, New Zealand) to facilitate phase separation. The aqueous supernatant from this extraction was incubated at -20°C for 1h after addition of $1/10^{th}$ volume of 3M sodium acetate (pH 5.4) and 2.5X volume of absolute ethanol. RNA was pelleted by centrifugation at 13200rpm for 15min and was washed in 70% alcohol. Pellet was air dried and reconstituted in TE buffer (pH 7.2) and stored at -80°C until further analysis. In order to recover DNA, supernatant from $LiCl_2$ precipitation was transferred to a 15ml tube and precipitated with equal volume (2ml) of cold isopropanol followed by incubation at -20°C for 1h. DNA was pelleted by centrifugation at maximum speed (4000g) for 30min. The pellet containing DNA was reconstituted in 500µl of TE buffer (pH 7.2) and followed by further phenol and chloroform (250µl:250µl) extraction and a single chloroform only (500µl) extraction in 1.5ml Phase Lock gel (light) tubes. The remaining procedure for DNA purification is exactly as previously described for RNA.

The second method (method 2) used for RNA extraction was the optimized method of Kang *et al*., (2009) which is a combination of enzymatic lysis, bead beating, TRIZOL™ reagent (Invitrogen) and cold chloroform followed by precipitation with isopropanol. The contaminating DNA was removed with Turbo DNA-free™ DNase. The DNA free RNA was then purified using column from the RNeasy mini kit from Qiagen™ (Haldane, Germany) following instructions provided with the kit.

The third method (method 3) examined as a possible alternative for obtaining RNA high in quantity and quality was using RNA PowerSoil™ Total RNA Isolation Kit (MOBIO, GeneWorks, Auckland, New Zealand) kit which has been optimized for extraction of RNA from all kinds of soil including manure rich soils containing high humic acid and phenolic acid contents. 2g of WRC were used for RNA extraction from this method following the protocol provided with the kit. The only modification in accordance with the manufacturers' suggestion was the addition of an extra chloroform (5ml) extraction of the supernatant obtained after bead beating to remove any excess protein that may contaminate the samples.

### *4.1.3 RNA yield and integrity assessment*

The quantification of RNA extracted using the three methods was done using fluorometry (Qubit™, Invitrogen, Auckland, New Zealand) and spectrophotometry (NanoDrop Technologies, Thermo Fisher Scientific, Auckland, New Zealand). Spectrophotometry was

used to calculate $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios as indices of protein and phenolic compound contamination (Teare *et al.* 1997). Sample integrity was determined by analysis of RNA samples separated using denaturing agarose gel electrophoresis (Sambrook and Russell 2001) where an equal amount of RNA based upon fluorometric estimation was loaded into the gel (figure 4.1, appendix A.7).

### *4.1.4 Reverse transcription and PCR amplification*

RNA samples were checked for DNA contamination by performing a PCR using archaeal 344f-GC and 522r primers targeting archaeal V2V3 region (as described earlier in section 3.1.1.1.3). Complementary DNA (cDNA) was synthesized from 1µg of RNA using reverse transcriptase enzyme (TaKaRa, BluePrint™ RT reagent kit for Real Time, Norrie Biotech, Auckland, New Zealand). A 20µl reaction was performed for the synthesis of cDNA from RNA as per manufacturers' protocol. This included incubation with the reverse transcriptase enzyme at 37°C for 15min. and inactivation of enzyme by heating at 85°C for 5sec. The cDNA obtained was stored at -80°C until further use. The synthesized cDNA from all extraction methods was used in PCR amplification tests targeting the following gene targets: protozoal 16S rRNA (V2V3 region) (Sylvester *et al.* 2004), bacterial universal 16S rRNA gene (Miller *et al.* 1995) and bacterial 16S rRNA (V2V3 region) (Muyzer *et al.* 1993a). A nested PCR approach was used to detect methanogen 16S rRNA (Barns *et al.* 1994; Ovreas *et al.* 1997) and the methanogen 16S rRNA (V2V3 region) (Amann *et al.* 1995; Raskin *et al.* 1994). PCR amplification conditions were optimized previously for each target. Primer sets are described in table 4.1.

In order to test the quality of cDNA obtained from RNA extracted by different methods, quantitative reverse transcriptase PCR (qRT-PCR) reactions were setup containing undiluted, 2 fold and 4 fold dilutions of the cDNA sample respectively using primers against *mcrA* (methanogen specific) gene. Primers *mcr*-f and *mcr*-r (Luton *et al.* 2002) were used to quantify the relative detection of *mcr*A transcript abundance. Similarly, to check any inhibition of LiCl in efficient synthesis of cDNA from RNA, serial dilutions of RNA (1, ½, ¼) extracted from different methods were used to synthesize cDNA and quantified by qRT-PCR. The estimation of *mcr*A transcript abundance in cDNA was done by real time PCR (qPCR) with SYBR Premix Ex Taq™ - Perfect Real Time (TaKaRa, Norrie Biotech, Auckland, NZ) in Applied Biosystems 7000 Real-Time PCR system. A standard curve was prepared by serial dilution of cloned amplicon of *Methanobrevibacter smithi* and subjected to qPCR along with the test samples. Dissociation curve analysis was performed at the end of quantification to check for the specificity of products obtained. The CT values were then

extrapolated against the standard curve to obtain relative quantities of *mcr*A transcript in the starting material. A minimum reaction efficiency of 99-100% and $R^2$ value of minimum 0.900 was considered for analysis.

**Table 4.1 Primer sets used for amplification of PCR cDNA.**

| Amplification target | Primer pair used | Sequence | Expected product size | Reference |
|---|---|---|---|---|
| Protozoal 16SrRNA V2V3 | 316F<br>539R-GC | GCGCTTTCGWTGGTAGTGTATT<br>CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG<br>GCACGGGGGACTTGCCCTCYAATCGTWCT | 223 | Sylvester *et al*., (2004) |
| Bacterial 16SrRNA (V2V3) | 341f-GC<br><br>34r | CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG<br>GCA CGG GGGGCCTACGGGAGGCAGCAG<br>ATTACCGCGGCTGCTGG | 200 | Muyzer *et al*., (1993a) |
| Bacterial 16SrRNA (universal) | 27f<br>1494r | AGAGTTTGATCMTGGCTCAG<br>CCCCTACGGTTACCTTGTTACGAC | 1470 | Miller *et al*., (1995) |
| Archaeal 16SrRNA (universal) | 46f<br>1017r | YTAAGCCATGCRAGT<br>GGCCATGCACCWCCTCTC | 970 | Barns *et al*., (1994)<br>Ovreas *et al*., (1997) |
| Archaeal 16SrRNA (V2V3) | 344f-GC<br><br>522r | CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG<br>GCACGGGGGGACGGGGHGCAGCAGGCGCGA<br>GWA TTACCGCGGCKGCTG | 180 | Akarsubasi *et al*.,(2005) |
| *mcr*A (RT-qPCR) | *mcr*-f<br>*mcr*-r | GGTGGTGTMGGATTCACACARTAYGCWACAGC<br>TTCATTGCRTAGTTWGGRTAGTT | 464-491 | Luton *et al*., (2002) |

## 4.2 Results and discussion

Fluorometry and spectrophotometry estimates of nucleic acid recoveries and sample purity obtained with all three methods is presented in table 4.2. Maximum yield of RNA across all the samples in both diets was obtained with the modified method (method 1), followed by the method of Kang *et al*., (2009) (method 2), while the lowest yields were obtained using the MOBIO method (method 3). The absorbance ratios of 1.94- 2.1 ($A_{260}/A_{280}$) and 2.16-2.45 ($A_{260}/A_{230}$) also suggest that RNA extracted using method 1 is clean. It has been reported that for a pure RNA the absorbance ratio of $A_{260}/A_{280}$ should be ≈2.0 and the $A_{260}/A_{230}$ ratio should be in the range of 2.0-2.2 (Anonymous 2012; Teare *et al.* 1997). The $A_{260}/A_{280}$ ratios between the other methods were lower but comparable, while the $A_{260}/A_{230}$ ratios were lower (1.10, 1.50) for other methods in comparison to the method 1 suggesting an interference from protein, phenol or other contaminants in these samples.

The denaturing agarose gel electrophoresis (figure 4.1) revealed that RNA extracted using the method 1 and method 2 was visibly intact, showing the characteristic RNA subunits. No visibly intact RNA was observed for the method 3 and the results are therefore not shown in figure 4.1.

Fluorometric quantification of RNA was used for loading samples in the gel because the dye binds specifically to RNA during fluorometry and reduces the possibility of error which can occur through spectrophotometry if the samples have significant phenol or trizol reagent contamination (Sambrook and Russell 2001). Though an equal amount of RNA (2µg) was loaded into all wells, the relative intensity of bands depicting RNA quantity was variable for the samples extracted with method 2 and none visible with the method 3. This could be due to the difference in amount of degraded RNA present in samples which would be registered by fluorometry (or spectrophotometry) irrespective of its quality.

**Table 4.2 Total yield and purity of RNA extracted by different methods.**

| Diet | Method | Type of sample used | Amount of sample | Total yield of of RNA in µg Spectrophotometry (Nanodrop) | Total yield of RNA in µg Fluorometry (Qubit) | Absorbance ratio | |
|---|---|---|---|---|---|---|---|
| | | | | | | $A_{260}/A_{280}$ | $A_{260}/A_{230}$ |
| **Kale** | Method 1 | RF* | 200 µL | 51.0 | 41.0 | 2.00 | 2.45 |
| | Method 2 | RF | 200 µL | 6.0 | 5.58 | 2.06 | 1.50 |
| | Method 1 | WRC** | 500 mg | 60.1 | 65.0 | 1.94 | 2.30 |
| | Method 2 | WRC | 500 mg | 50.1 | 28.5 | 2.06 | 1.96 |
| | Method 3 | WRC | 2 g | 11.7 | 19.0 | 1.89 | 1.58 |
| **Grass** | Method 1 | RF | 200 µl | 66.5 | 65.0 | 2.09 | 2.16 |
| | Method 2 | RF | 200 µl | 6.4 | 6.42 | 2.06 | 1.10 |
| | Method 1 | WRC | 500 mg | 67.7 | 70.0 | 2.12 | 2.19 |
| | Method 2 | WRC | 500 mg | 28.6 | 22.5 | 2.03 | 1.80 |
| | Method 3 | WRC | 2 g | 24.2 | 37.9 | 2.00 | 1.91 |

***RF-Rumen Fluid, **WRC-Whole Rumen Contents. Method 1- Modified method, Method 2- Kang *et al*., 2009, Method 3- MOBIO™.**

The clear differences in comparative efficiency of these extraction methods may be explained by the requirement to lyse the relatively robust cell walls of rumen microorganisms. The rumen is a challenging environment for microorganisms, with significant flux of pH, redox, osmolarity, and temperature (Gibbs *et al.* 2007). The stable populations that inhabit the rumen are commonly characterized by physiological adaptations and a robust physical structure capable of withstanding this environment. Forage based diets in temperate systems typically have a higher K and P content that is reflected in the rumen environment, and there is some

evidence that these increased concentrations confer protection (e.g. $H^+$ / $K^+$ exchange to ameliorate impacts of low pH) to some populations (Dawson and Boling 1987). As a result, some cell lysis methods using chemical or enzymatic disruption of cell wall or reduced physical disruption alone may be less successful in extracting RNA. The high yields of RNA obtained using method 1 may be due to the combination of organic solvents, detergent and a vigorous physical disruption of cell walls using serial bead beating procedures.

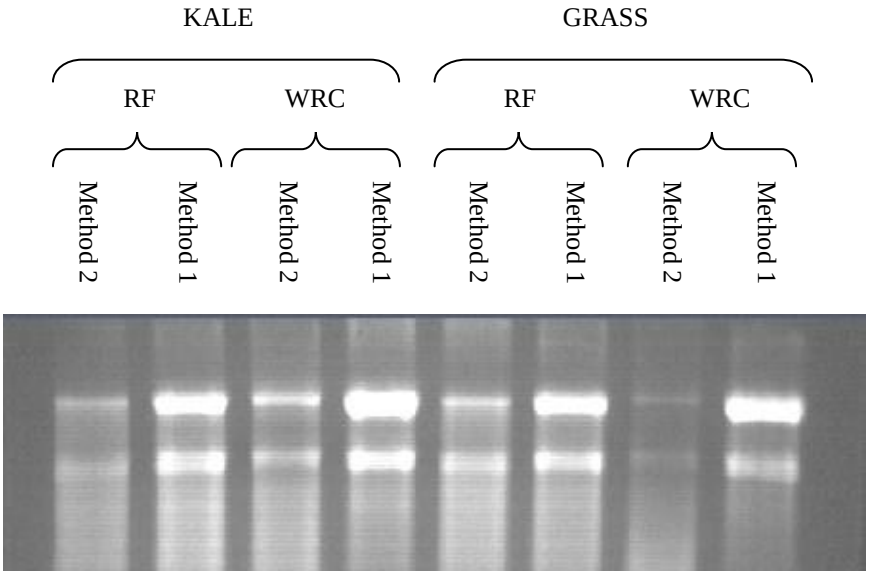



**Figure 4.1 Denaturing gel electrophoresis of RNA obtained with different methods of extraction.**

**Equal amounts of RNA (2µg) based upon fluorometric estimation was separated on a 1% denaturing agarose.gel. Method 1- modified method, method 2- Kang *et al*., (2009), RF-rumen fluid, WRC-whole rumen contents.**

Also, the specific ability of LiCl to precipitate intact RNA coupled with the use of phenol and chloroform extractions in phase lock gel (light) tubes promotes a more rigorous phase separation that may contribute to greater yields of clean and intact RNA. The repeated use of phenol and chloroform in method 1 may also be more effective in reducing protein contamination of samples, which may explain the improved purity of extracted RNA and DNA compared with the other methods (table 4.2).

Method 2, though including a combination of chemical and enzymatic disruption of cell walls and a single bead beating step, did not obtain as high a yield of RNA as method 1. This may reflect the less vigorous physical disruption of the microbial cell wall. Method 2 also requires the use of a column for purifying extracted RNA, and these necessarily limit the RNA to that which may be bound on the column, a limitation not present with the phase lock tubes used at the comparable step in method 1. Method 3 similarly uses a less vigorous cell lysis procedure, which may explain the comparably lower yield of RNA, and the method also lacks suitable protection against RNases during column separation, which could increase RNA degradation.

The range of target genes included (table 4.1) for PCRs was suitable to assess bias towards particular subpopulations, which is undesirable for studies of the rumen microbial ecosystem. The bands obtained from agarose gel electrophoresis of the PCR products of cDNA for all three methods are presented in figure 4.2. For both universal 16S primers (general and archaeal), there was a marked increase in the quantity of product obtained from cDNA synthesized from RNA isolated using method 1. However, the yield of PCR amplicons generated using V2V3 primers (bacterial, archaeal and protozoal) commonly used in PCR-DGGE analysis was broadly similar for cDNA from all methods (figure 4.2). No bias was observed with any of the three methods for V2V3 targeted amplifications.

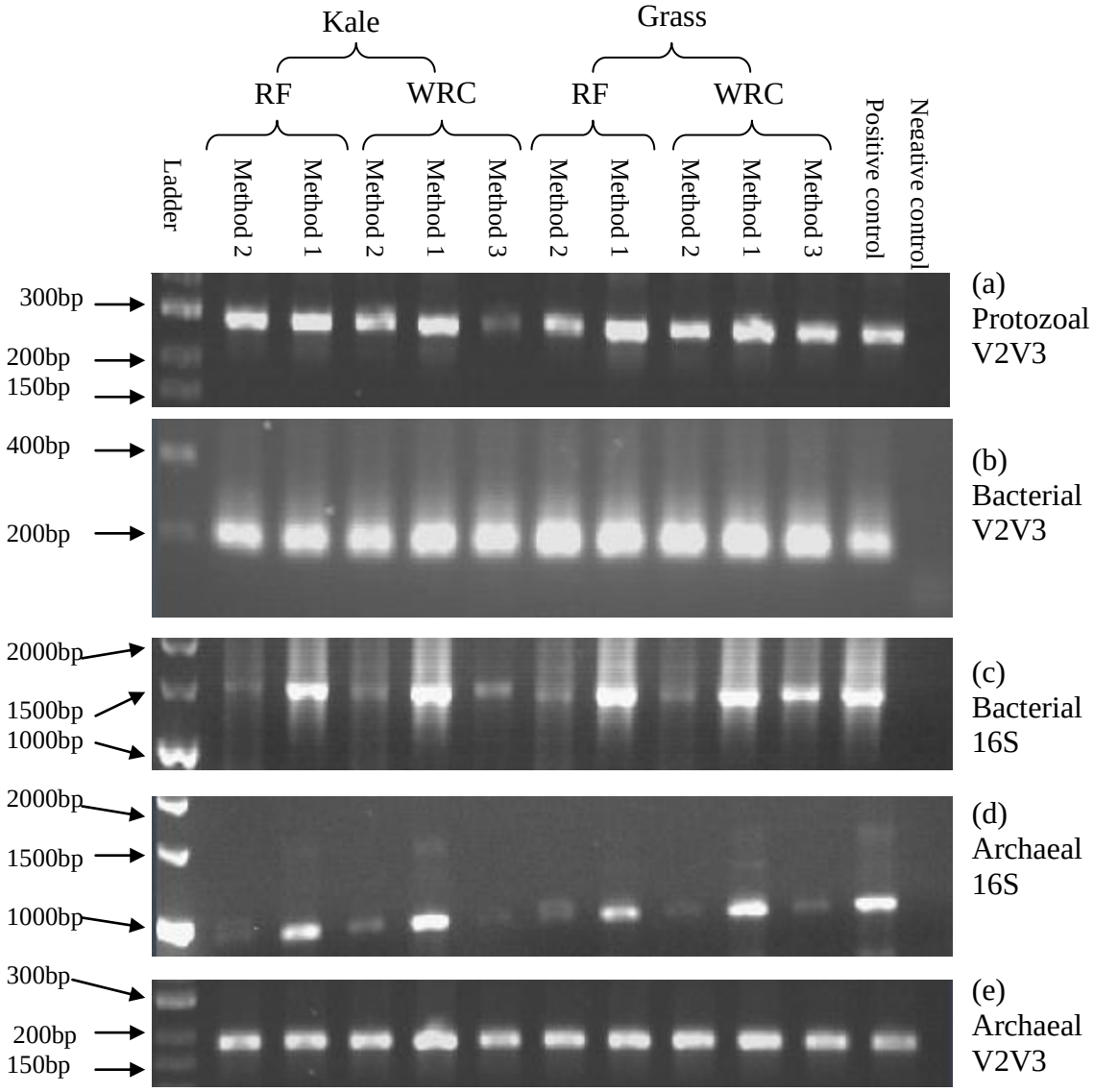



**Figure 4.2 PCR amplification of cDNA obtained with different methods of extraction using different primer sets.**

**Method 1- modified method, method 2- Kang *et al*., (2009), method 3-MOBIO™ kit, RF-rumen fluid, WRC-whole rumen contents. (a) Protozoal 16S V2V3 primers (P-SSU-316f/539r-GC). (b) Bacterial 16S V2V3 primers (341f-GC/534r). (c) Bacterial 16S universal primers (27f/1494r). (d) Archaeal 16S universal primers (47f/1017r). (e) Archaeal 16S V2V3 primers (344f-GC/522r).**

The respective CT values of the serial dilutions of synthesized cDNA set up as a check for quality of RNA from method 1 and method 2 are displayed in figure 4.3 and figure 4.4. Since, very low amount of intact RNA was extracted using method 3; the cDNA was not used for dilution test. Dilution of cDNA produced the expected increase in CT values for method 1

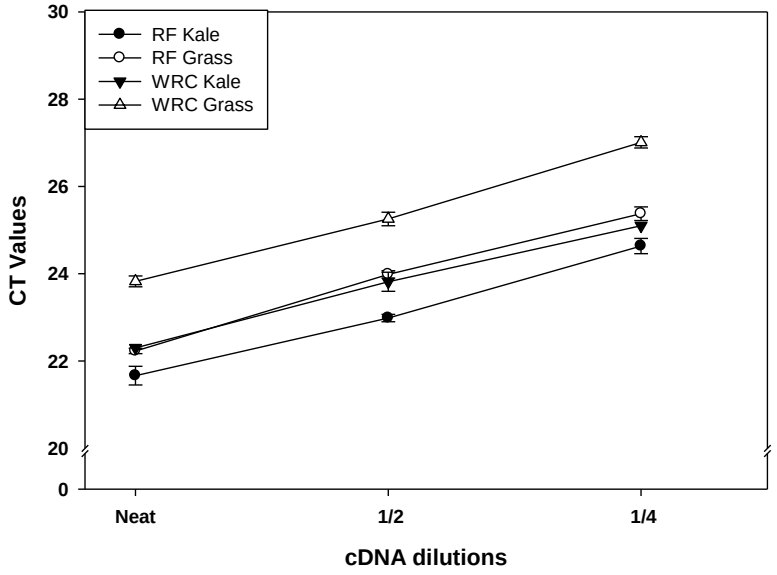



**Figure 4.3 Amplification of diluted cDNA made from RNA extracted by the modified method (amplified with *mcr*A forward and reverse primers).**

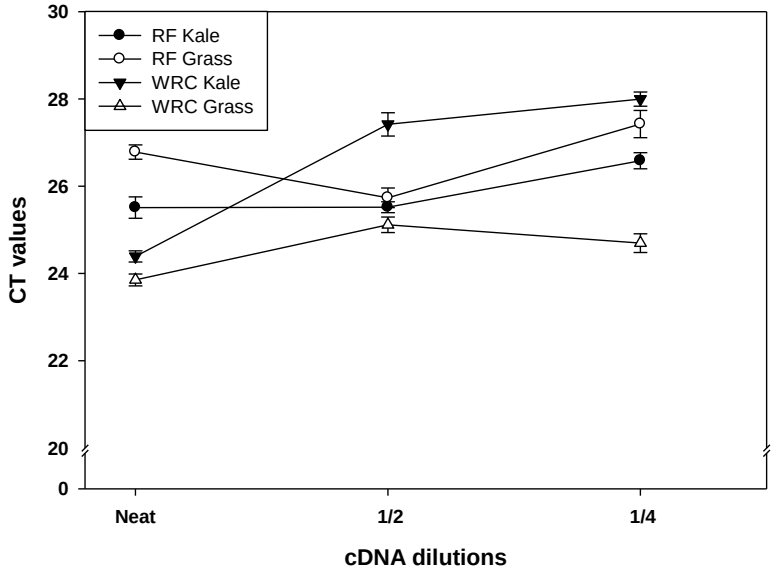



**Figure 4.4 Amplification of diluted cDNA made from RNA extracted by the method of Kang *et al*., (2009) (amplified with *mcr*A forward and reverse primers).**

implying a decreasing concentration whereas the increase in CT values was not consistent for all the cDNA obtained using method 2. The observed increase in CT values is consistent with

their being little or no inhibitors of the PCR reaction present in cDNA samples prepared using method 1.

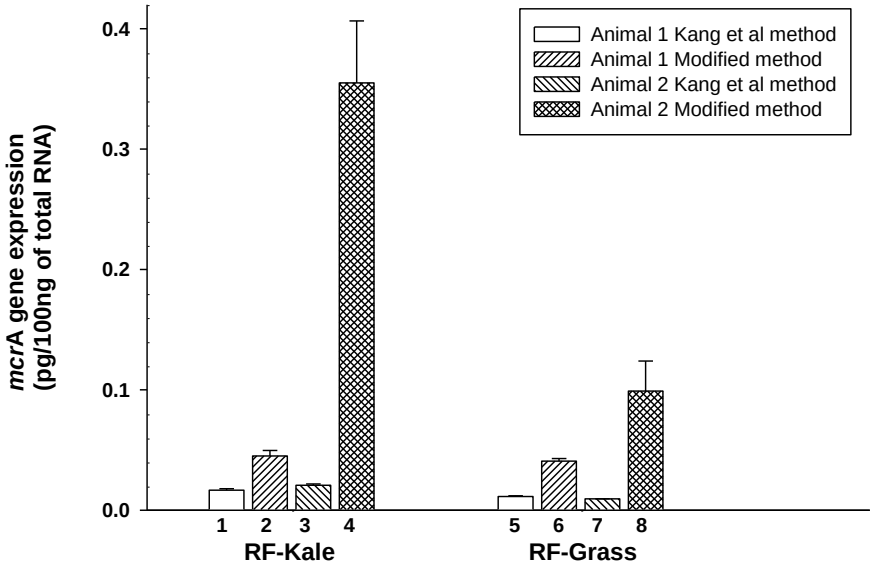



**Figure 4.5 *mcr*A transcript abundance in RNA samples extracted from rumen fluid of kale or grass fed animals using different methods.**

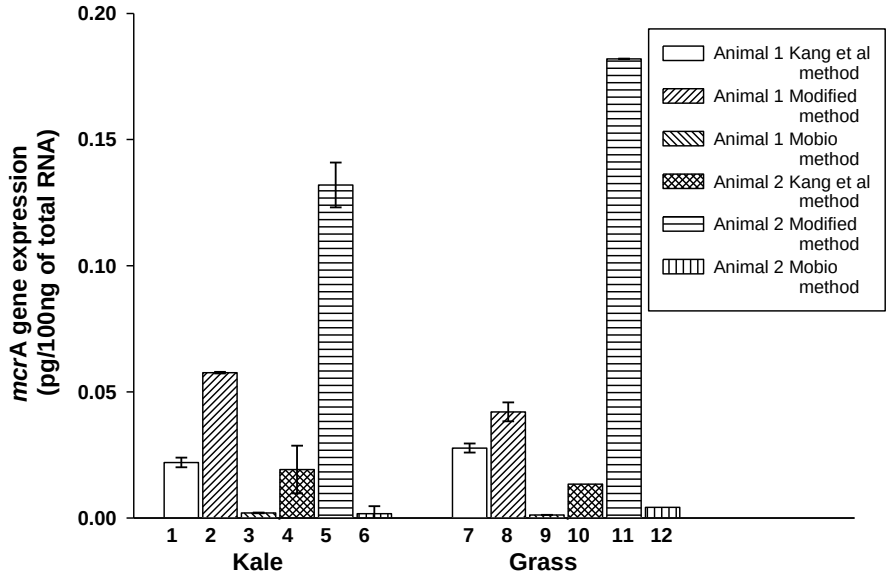



**Figure 4.6 *mcr*A transcript abundance in RNA samples extracted from WRC of kale or grass fed animals using different methods.**

While this analysis is useful for establishment of the presence or absence of PCR inhibitors it does not necessarily assist in determining the presence of inhibitors of the reverse transcription reaction. LiCl can inhibit reverse transcriptase and can cause interference in efficient synthesis of cDNA from RNA. However, when serial dilutions of RNA obtained

from methods 1 and 2 were used to synthesize cDNA from all samples, the decrease in *mcr*A transcription abundance was consistent with decreasing concentrations for all the samples obtained from both extraction methods, suggesting no interference from LiCl in method 1.

The *mcr*A transcript abundance from rumen fluid or WRC samples is displayed in figure 4.5 and figure 4.6. A very large difference in *mcr*A transcript yield was observed between methods. Method 1 returned between 100 to 1700 fold increases in transcript abundance compared to other methods across both types of samples in both diets. The highest difference was found for the rumen fluid samples from kale based diets (1700 fold) and lowest for rumen fluid samples from grass based diets (100 fold). If the methods were extracting the target mRNA equally, a similar abundance of the selected target gene would be expected when compared to the proportion of target within a given amount of total RNA. This suggests method 1 is extracting greater amounts of mRNA. Also, the amount of *mcr*A gene expression obtained using method 2 was similar for all types of samples, whereas method 1 revealed differences amongst different diets and different type of samples. It was also able to show between animal variance for same diet and sample. This finding is of significant importance in the qRT-PCR analysis of methanogen expression where an accurate determination of the methanogen gene expression within a given sample is required.

There are several possible explanations for these differences. The differing efficiency of the methods in extracting intact RNA could contribute these observed differences. Also, approximately 25% of methanogens are symbiotically attached with protozoa and many of the remainder are in close association (e.g. present on external surface of protozoa) (Moss *et al.* 2000; Newbold *et al.* 1995). Less vigorous physical separation may not be sufficient to obtain the RNA from these protozoal associated methanogens, and this may also help explain the clear differences in transcript abundance. Another possible explanation could be a reduction of PCR inhibitors in prepared cDNA with method 1, as a consequence of more numerous phenol and chloroform washing procedures, which would then increase detection of transcript abundance.

Comparison between methods of the time required for the procedures reveals the method 2 was the quickest with about 4 hours required for RNA extraction, DNase treatment and purification from 8 samples. Method 3 required 7h for RNA extraction and 5h for DNase treatment and purification, while method 1 required 5h for RNA extraction, DNase treatment and purification, and 1h for DNA recovery if desired, a total of 6h. The DNA extracted simultaneously with method 1 from the samples reported here was also similarly examined for quality, quantity and wide representation of microbial diversity and found to be delivering

the expected standard (unpublished data). Because there are many efficient methods available for good quality DNA extraction, DNA comparison results are not included.

## 4.3 Conclusions

The modified method presented in this study, when compared to the existing methods of Kang *et al*., (2009) and the MOBIO method, was demonstrated to confer advantages in procedural simplicity during extraction, the use of less expensive and more readily available chemicals, and the high quality and yield of both RNA and DNA recovered simultaneously from rumen contents. It also proved more sensitive in detecting *mcr*A gene expression which was a crucial requirement for this study. These key advantages make the method suitable for future use in this expanding field of investigation into the structure, diversity and function of the rumen milieu.

# Chapter 5

# Quantification of mcrA RNA and DNA of Rumen Methanogens in Cattle Fed High Quality Forages under Different Feeding Frequencies

In New Zealand, grazing of perennial pastures is the main source of nutrients for animals. Constraint in the amount of dry matter intake (DMI) is the main limiting factor to achieving high production from animals in forage based systems. Consequently, production strategies are targeted towards improving the metabolisable energy (ME) content of pastures, forages and supplements (Buddle *et al.* 2011). The animals grazed on pastures are generally given fresh breaks once daily which is more practical than a smaller pasture break twice a day. As a result animals consume the majority of the daily allocation of fresh, high quality forages within the first few hours and are on low intake of lower quality DM or negligible feeding during the rest of period, which establishes a diurnal pattern in the animals (Gibbs and Laporte 2009). It has also been hypothesized that large variations in rumen fill due to these diurnal grazing patterns can influence rumen function and physiological parameters in comparison to more frequent grazing bouts (Wales *et al.* 2004). But sub-optimal rumen function has not been observed in these high energy intake production systems of New Zealand (Gibbs and Laporte 2009). However, different feeding patterns and corresponding rumen fill fluctuations may influence the microbial community (Weimer 1998). A prolonged availability of high quality fodder has been associated with high availability of readily soluble carbohydrates resulting in a linear decrease in pH, shift of fermentation and VFA production towards more propionate production than acetate production, though, without much change in the total VFA production (Lee *et al.* 2003). A low pH has been reported to stop the activity of methanogens (Kessel and Russell 1997a) and lower acetate to propionate ratio has often been associated with reduced methane production (Lana *et al.* 1998).

An experiment was conducted to compare methanogen communities in cattle fed fresh forage once a day or twice a day to identify the effect of different feeding patterns on methanogen community profile, methanogen population number and methanogen activity. As it is possible that the effect of any external rumen modification may cause only a shift in fermentation or methane production pattern, spot samples may be unable to give a clear

picture of the methanogen community. Thus samples were collected over a diurnal period at 4h intervals.

Since, the expression level of the *mcr*A gene has been reported to be proportional to the methane production, an estimation of *mcr*A specific mRNA out of the total pool of RNA can give an idea of the methanogen activity within a sample (Denman *et al.* 2007). Therefore, to investigate the effect of different feeding frequencies on methanogens, *mcr*A RNA/cDNA and *mcr*A DNA were quantified as indicators of methanogen activity and numbers, respectively. DGGE analysis was also performed on the 24h samples to compare the methanogen populations with their activity profiles over the diurnal period.

## 5.1 Materials and methods

### *5.1.1 Animals, diets and experimental design*

Four ruminally fistulated Holstein Friesian steers (370kg live weight) were randomly assigned to two groups of two steers each. One group was fed 7kg DM of freshly cut kale along with 1.5kg DM of straw daily. The second group was allocated 3.5kg DM of freshly cut kale in the morning at 9am along with 0.75kg DM of straw and the remaining 3.5kg DM and 0.75kg straw at 3pm in the evening. Animals had free access to fresh water throughout the day. They were fed the respective diet for a 14d pre-trial period and then housed in pens for another 14d. After that they were kept in animal crates for another 7d period. After sample collection on day 35, the feeding pattern was switched over between two groups.

At day 35 and day 70, WRC were collected from the rumen of all four steers every 4h over a 24h period. Sample collection was done at 8.30am, 12.30pm, 4.30pm, 8.30pm, 12.30am and 4.30am i.e. 0, 4, 8, 12, 16 and 20h after first feeding. 400g of WRC was collected from the ventral sac of the rumen via the fistulas, placed in an ice bath for 5min and then aliquoted to microtubes. The microtubes were then centrifuged under refrigeration (4°C) at 13200rpm for 5min. The supernatant was discarded and pellet was resuspended in 500µl of RNA protectant (RNAprotect® Bacteria Reagent, Qiagen, Bio-Strategy, Auckland, New Zealand) followed by incubation at room temperature for 5min, vortexing at maximum speed and finally centrifugation at 13,200rpm for 10min. Supernatant was again discarded and tubes containing pellets were snap frozen under liquid nitrogen and stored at -80°C until further analysis.

### *5.1.2 Sample analysis*

Samples were taken out from -80°C and RNA & DNA were extracted using the procedure described in section 4.1.2 (appendix A.7) and quantified with fluorometry (Qubit™,

Invitrogen, Christchurch, New Zealand). RNA was also treated with Turbo DNA-free™ DNase (Ambion, Applied Biosystems, Auckland, New Zealand) to remove any contaminating DNA. RNA samples were checked for DNA contamination by performing a PCR using archaeal 344f-GC and 522r primers targeting archaeal V2V3 region and using 1µl (≈300ng) of RNA as the template. Complementary DNA (cDNA) was synthesized from 1µg of RNA using Blue Print ™ reverse transcriptase kit according to the manufacturers' instructions (TaKaRa, Norrie Biotech, Auckland, New Zealand).

#### 5.1.2.1 PCR amplification for DGGE

The cDNA & DNA were PCR amplified for DGGE using a nested PCR with universal 16S rRNA archaeal and archaeal 16SrRNA V2V3 primers 344f-GC and 522r (Akarsubasi *et al.* 2005). The procedure followed was the same as described in section 3.1.1.3.3 and appendix A.3.

#### 5.1.2.2 Denaturing gradient gel electrophoresis (DGGE) analysis

Both DNA and RNA extracted from samples collected every 4h during once a day vs. twice a day feeding were used for DGGE analysis. DGGE gels were constructed in such a way that any changes in methanogen community, its number and relative activity over 24h period for an animal could be detected from a single gel. One half of gel contained PCR amplified DNA samples and the other half contained PCR amplified cDNA (RNA) samples from 8.30am to 4.30am (0 to 20h after first feeding) for the same animal. DGGE was performed for PCR product analysis as described earlier in section 3.1.1.3.5 and appendix A.4.

#### 5.1.2.3 qPCR/ qRT-PCR design and analysis

Quantitative PCR (qPCR) or reverse transcriptase quantitative PCR (qRT-PCR) were performed on DNA/ cDNA obtained from rumen contents of animals to quantify the relative number of methanogens across different time periods (pre-prandial/ post-prandial) in animals fed once a day vs. twice a day. 20µl reactions were performed using 10µl (1X final concentration) of SYBR Premix Ex Taq™ - Perfect Real Time (TaKaRa, Norrie Biotech, Auckland, NZ) containing Hot start Taq polymerase, $MgCl_2$, dNTP mixture and SYBR Green, 0.4µl (1X) of ROX dye for background calibration (supplied with the kit), 0.4µl (0.2µM) each of *mcr*A forward (5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3') and *mcr*A reverse (5'-TTCATTGCRTAGTTWGGRTAGTT-3') primers , 0.8µl of sterile distilled water and 8µl (15-20ng) of DNA/cDNA template (concentration measured with Qubit, Invitrogen,Auckland, New Zealand) in Applied Biosystems 7000 Real-Time PCR system. The optimized reaction conditions involved initial denaturation for 10sec at 95°C followed by 35 cycles of denaturation at 95°C for 5sec, annealing at 60°C for 10sec and

extension at 72°C for 30sec. A standard curve was prepared by five point serial dilution of cloned amplicon of *Methanobrevibacter smithi* from a concentration of 1.87 x $10^{-1}$ng/µl to 2.99 x $10^{-4}$ng/µl and subjected to qPCR along with the test samples. The CT values were then extrapolated against the standard curve to obtain relative quantities of *mcr*A DNA/ *mcr*A cDNA in the starting material. The measured values were expressed in pg/100ng of total DNA or RNA, because this would express the amount of target *mcr*A DNA or mRNA within a fixed amount of total DNA or RNA. The products were also checked for specificity by performing a dissociation curve analysis each time with the qPCR. The reaction efficacy was also checked each time and only the standard curve with a slope of -3.3 to -3.5 or a reaction efficiency between 95-100% along with a $R^2$ value of between 0.90-0.99 was considered for analysis.

The amplification primers were tested for specificity by running the qPCR products in a 2% agarose gel containing ethidium bromide against a standard 100bp DNA ladder. The products matched the expected size of 414-438 bp. The bands were then excised and DNA isolated using gel extraction kit from Axygen™ (Catalogue no. AP-GX-250). Purified products were then cloned and sequenced as described in appendix A.6. The sequences obtained were specific to those of methanogen species.

### *5.1.3 Statistical analysis*

For each animal and variable, the data values were averaged for control (once a day feeding) and treated (twice a day feeding) separately, and the difference between control and treated was calculated. These differences were then statistically analysed using a two tailed paired samples t test in SPSS software (version 16.0, IBM SPSS statistics). The variables analysed were: *mcr*A gene expression, *mcr*A gene quantity within the diurnal period after treatment.

## 5.2 Results

### *5.2.1 PCR-DGGE*

Denaturing gradient gel electrophoresis (DGGE) was performed on PCR amplified products to determine community response to different feeding patterns. Approximately 21 to 25 bands were obtained upon DGGE analysis of DNA (DNA-PCR) and RNA (cDNA-PCR) samples obtained from whole rumen contents of experimented animals fed kale and silage once or twice a day. The bands obtained from DNA-PCR samples would indicate the relative contribution or proportion of a strain/ species in the methanogen community while the bands obtained from cDNA-PCR would indicate the relative activity of that strain/ species in the

population. When the animals were fed once a day, a predominant observation was the absence or lower intensity of some bands in DNA-PCR products and their considerable presence in cDNA-PCR products. As seen in figure 5.1 (animal no.1), the arrows ‘A’, ‘B’ and ‘C’, indicate bands which are low in intensity or absent in DNA-PCR products but are significantly visible in cDNA-PCR products.

The box ‘b1’ indicates a domain of the gel where bands are of low intensity in DNA-PCR products and considerably darker in cDNA-PCR products. As determined earlier by cloning and sequencing (section 3.2.2), arrow ‘A’ indicates band closest to *Methanobacteriacae* sp., arrow ‘B’ to *Methanosphaera stadtmanae* while the arrow ‘C’ is for band closest to *Methanobrevibacter* sp. The bands in box ‘b1’also represent different strains/ species closest to *Methanobrevibacter* sp. On the other hand, some bands mostly of *Methanobrevibacter* sp and one band of order Methanobacteriales were present in both DNA and cDNA samples and a similar DGGE profile was obtained when same animal was fed twice a day (figure 5.2).

Further, during once a day feeding two strains/ species closest to *Methanobrevibacter* sp (arrows ‘D’ and ‘E’ in figure 5.1) showed comparative lower intensity for cDNA samples at 12.30am and 4.30am i.e. 16h and 20h after feeding. This difference was not visible when the same animal was fed twice a day (figure 5.2).

Similar to animal 1, a relative difference between respective band intensities of DNA and cDNA samples was observed for animal 2 and a lighter intensity of few bands was observed at 12.30am and 4.30am i.e. 16h and 20h after feeding (figure 5.3). This difference was again absent when the animal was fed twice a day (figure 5.4). Also, the relative intensity of few cDNA bands closest to *Methanobrevibacter* sp. was lower in twice a day fed animals (arrow ‘A’ in figure 5.4) as compared to their intensity in once a day feeding. Further, among these three bands (figure 5.4), the intensity of band was highest at 12.30pm i.e. 4h after feeding.

Similar to animals 1 and 2, a marked difference in intensity of few cDNA bands closest to *Methanobrevibacter* sp. than their respective DNA bands was observed in animals 3 and 4 during both once a day and twice a day feeding (figure 5.5 to figure 5.8). A band representing a strain/ species closest to *Methanobrevibacter* marked by arrow ‘X' in all gels (figure 5.5 to 5.8) was typically absent in DNA profile and appeared in cDNA profile of all animals. Thus it appears from all the DGGE gels that some strains/ species may account for a very small proportion in methanogen population but are relatively active members of the population. Further, a relative low intensity of bands at certain times may signify a relative lower

population or activity of these strains/ species at that time which is more predominant in animals fed once a day.

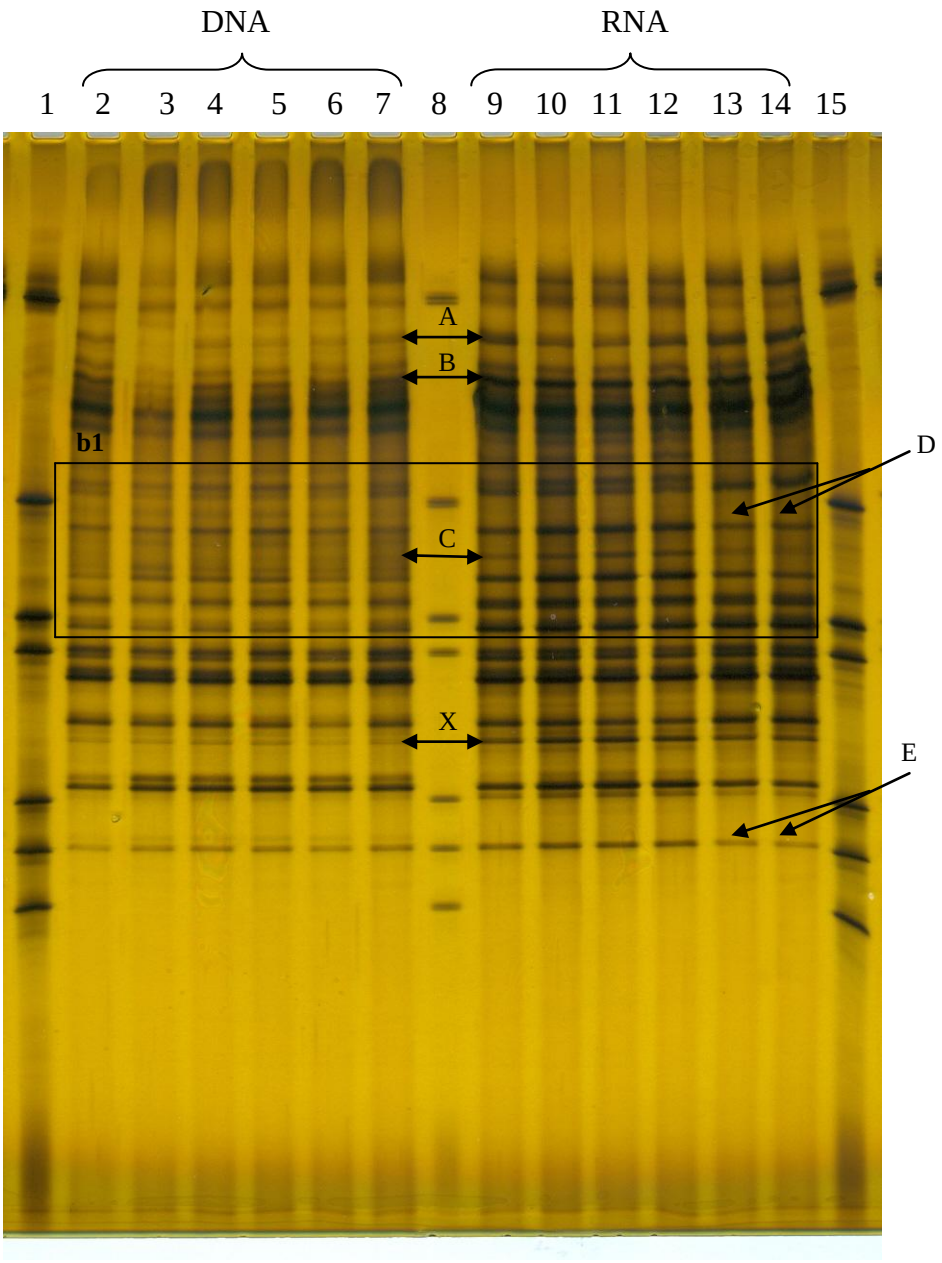



**Figure 5.1 DGGE analysis of DNA- cDNA/RNA in animal 1 fed once a day.**

***Arrows or box in the figure indicate the site of changes detected in gels.**
**Lane 1- standard, Lane 2- 8.30am, Lane 3- 12.30pm, Lane 4- 4.30pm, Lane 5- 8.30pm, Lane 6- 12.30am, Lane 7- 4.30am, Lane 8- standard, Lane 9- 8.30am, Lane 10-12.30pm, Lane 11- 4.30pm, Lane 12- 8.30pm, Lane 13- 12.30am, Lane 14- 4.30am, Lane 15- standard.**

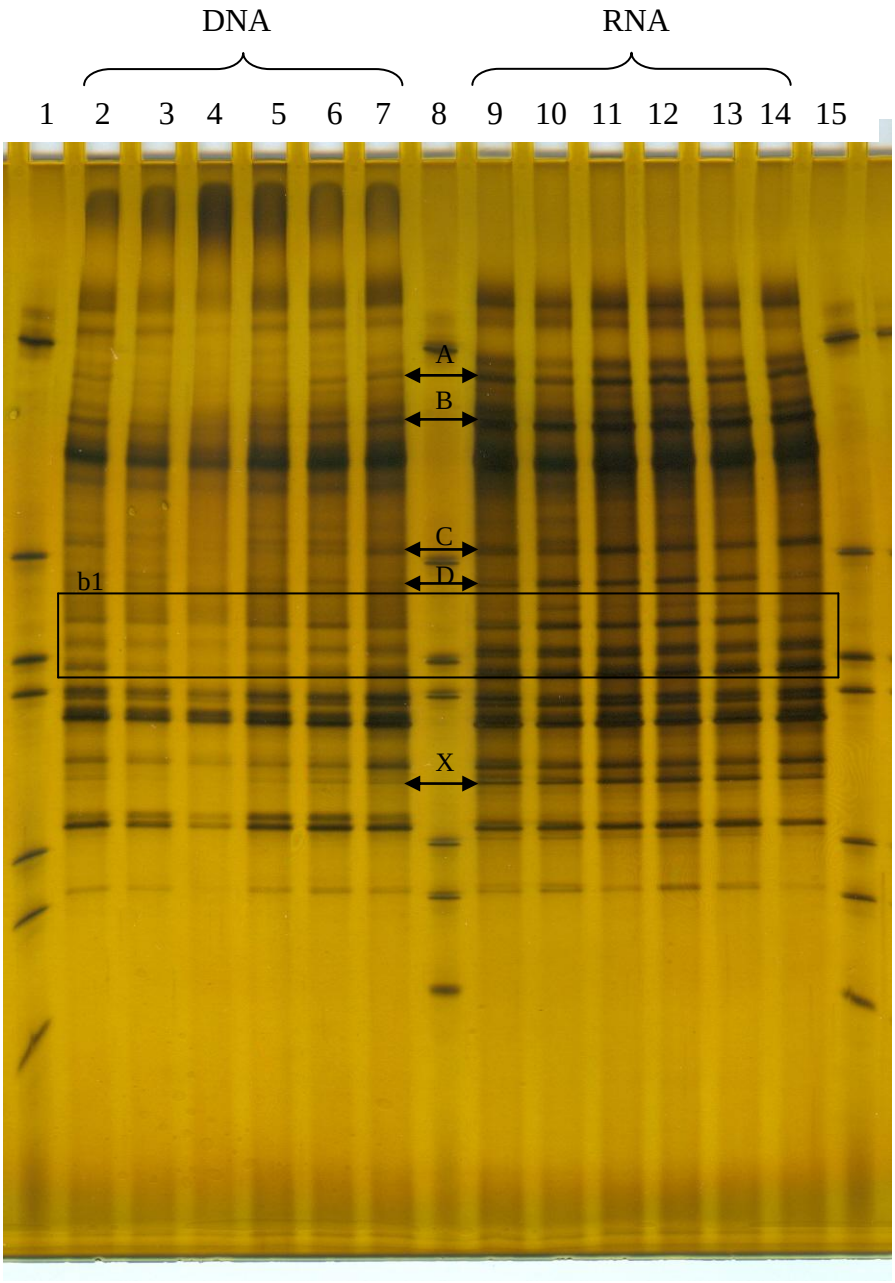



**Figure 5.2 DGGE analysis of DNA- cDNA/RNA in animal 1 fed twice a day.**

***Arrows or box in the figure indicate the site of changes detected in gels.**
**Lane 1- standard, Lane 2- 8.30am, Lane 3- 12.30pm, Lane 4- 4.30pm, Lane 5- 8.30pm, Lane 6- 12.30am, Lane 7- 4.30am, Lane 8- standard, Lane 9- 8.30am, Lane 10-12.30pm, Lane 11- 4.30pm, Lane 12- 8.30pm, Lane 13- 12.30am, Lane 14- 4.30am, Lane 15- standard.**

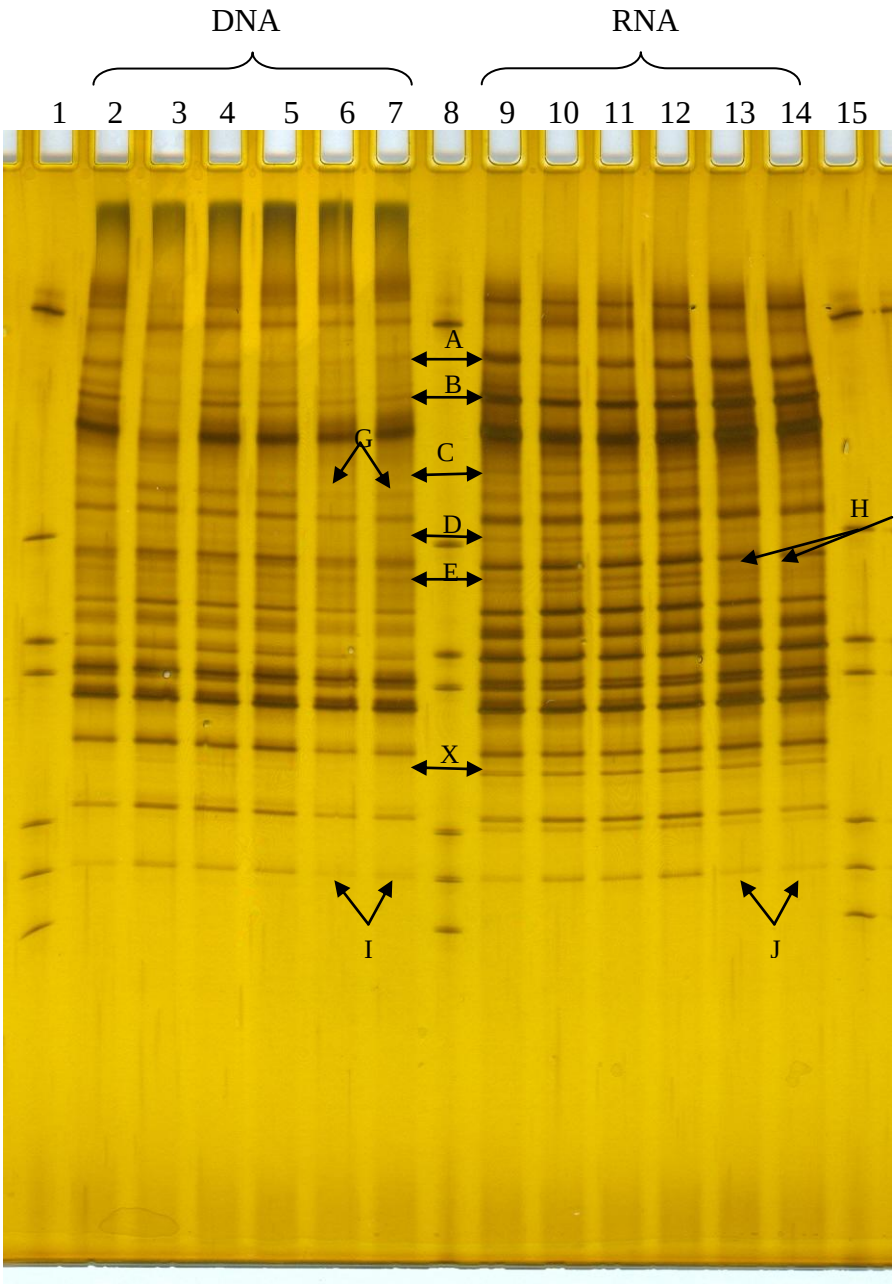



**Figure 5.3 DGGE analysis of DNA- cDNA/RNA in animal 2 fed once a day.**

***Arrows or box in the figure indicate the site of changes detected in gels.**
**Lane 1- standard, Lane 2- 8.30am, Lane 3- 12.30pm, Lane 4- 4.30pm, Lane 5- 8.30pm, Lane 6- 12.30am, Lane 7- 4.30am, Lane 8- standard, Lane 9- 8.30am, Lane 10-12.30pm, Lane 11- 4.30pm, Lane 12- 8.30pm, Lane 13- 12.30am, Lane 14- 4.30am, Lane 15- standard.**

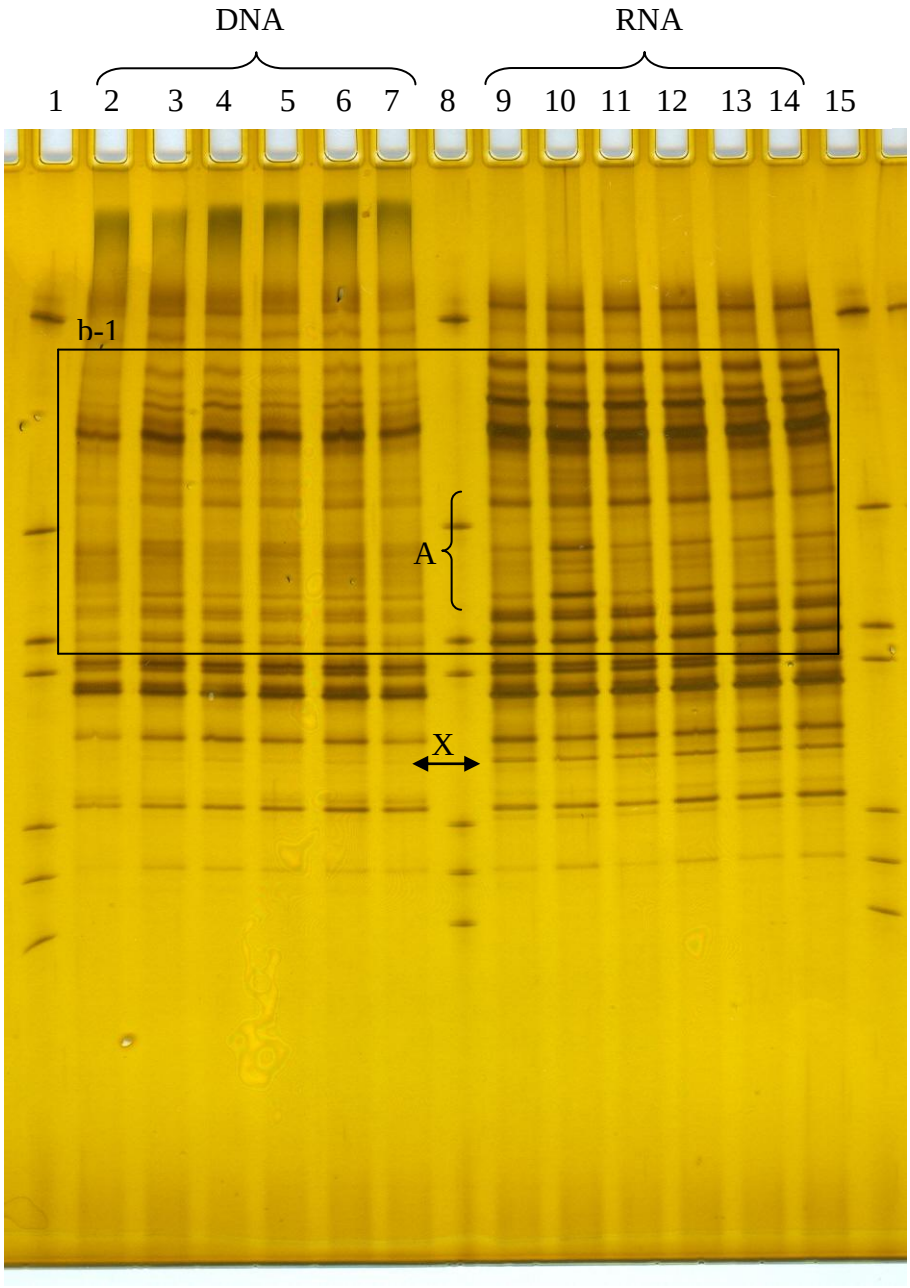



**Figure 5.4 DGGE analysis of DNA- cDNA/RNA in animal 2 fed twice a day.**

***Arrows or box in the figure indicate the site of changes detected in gels.**
**Lane 1- standard, Lane 2- 8.30am, Lane 3- 12.30pm, Lane 4- 4.30pm, Lane 5- 8.30pm, Lane 6- 12.30am, Lane 7- 4.30am, Lane 8- standard, Lane 9- 8.30am, Lane 10-12.30pm, Lane 11- 4.30pm, Lane 12- 8.30pm, Lane 13- 12.30am, Lane 14- 4.30am, Lane 15- standard.**

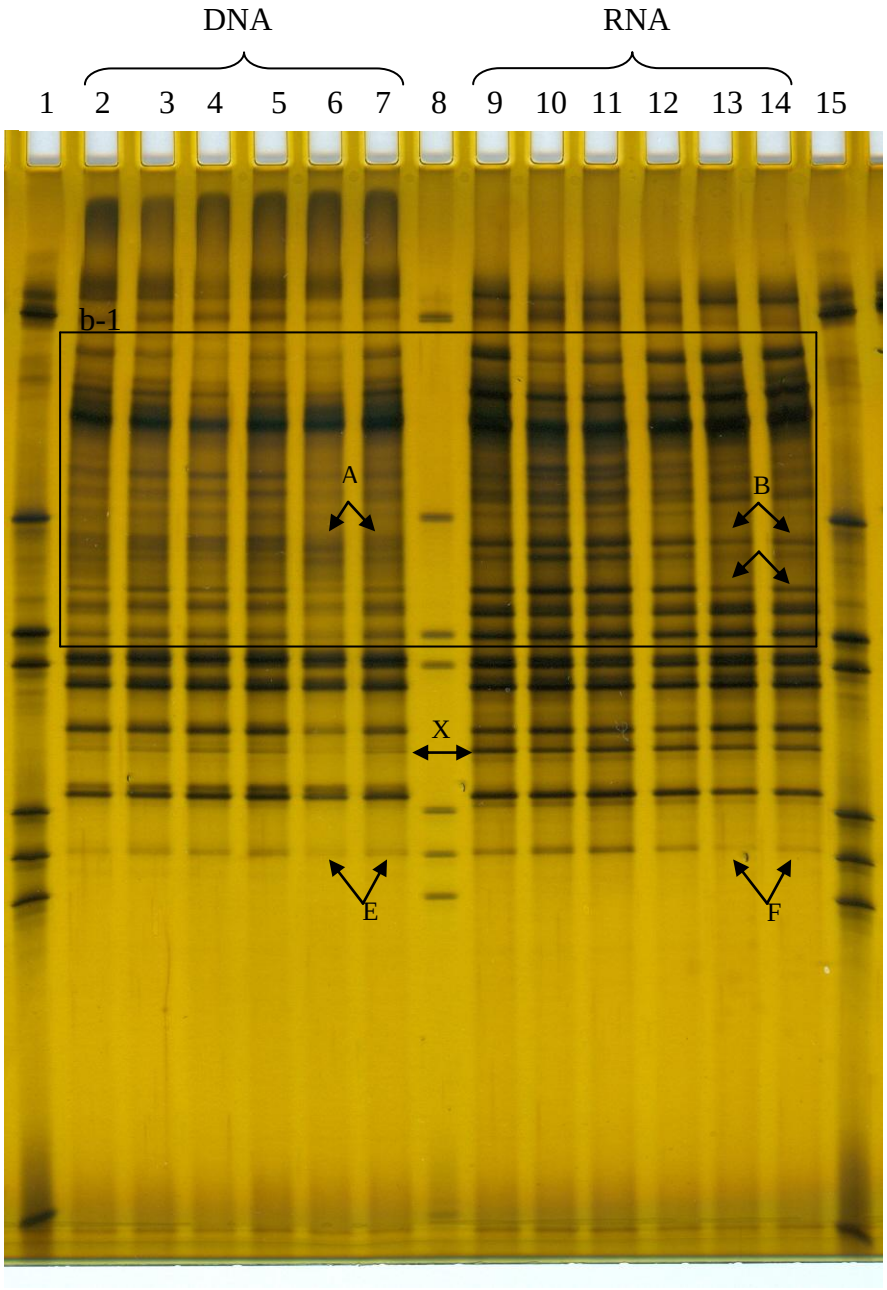



**Figure 5.5 DGGE analysis of DNA- cDNA/RNA in animal 3 fed once a day.**

***Arrows or box in the figure indicate the site of changes detected in gels.**
**Lane 1- standard, Lane 2- 8.30am, Lane 3- 12.30pm, Lane 4- 4.30pm, Lane 5- 8.30pm, Lane 6- 12.30am, Lane 7- 4.30am, Lane 8- standard, Lane 9- 8.30am, Lane 10-12.30pm, Lane 11- 4.30pm, Lane 12- 8.30pm, Lane 13- 12.30am, Lane 14- 4.30am, Lane 15- standard.**

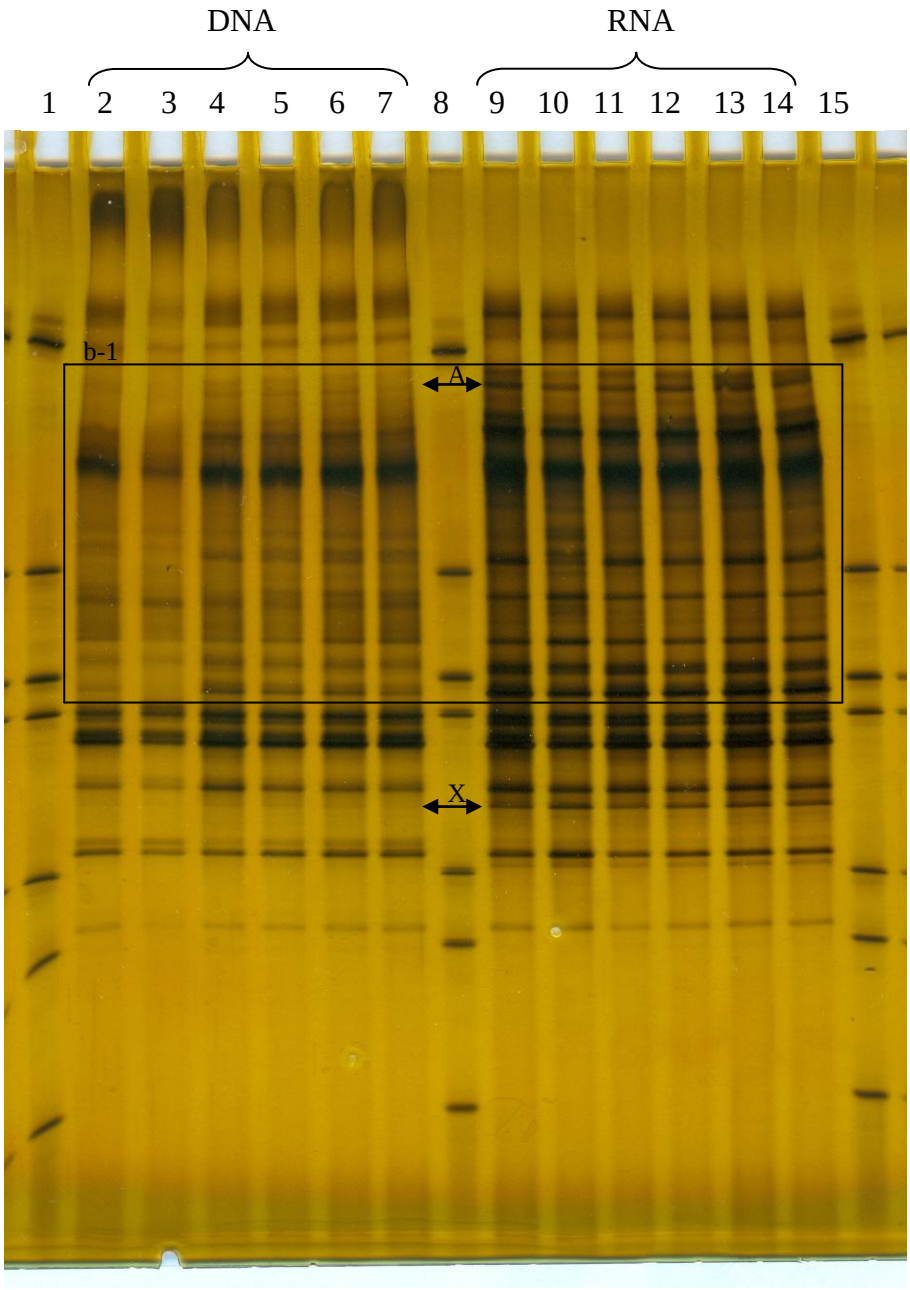



**Figure 5.6 DGGE analysis of DNA- cDNA in animal 3 fed twice a day.**

***Arrows or box in the figure indicate the site of changes detected in gels.**
**Lane 1- standard, Lane 2- 8.30am, Lane 3- 12.30pm, Lane 4- 4.30pm, Lane 5- 8.30pm, Lane 6- 12.30am, Lane 7- 4.30am, Lane 8- standard, Lane 9- 8.30am, Lane 10-12.30pm, Lane 11- 4.30pm, Lane 12- 8.30pm, Lane 13- 12.30am, Lane 14- 4.30am, Lane 15- standard.**

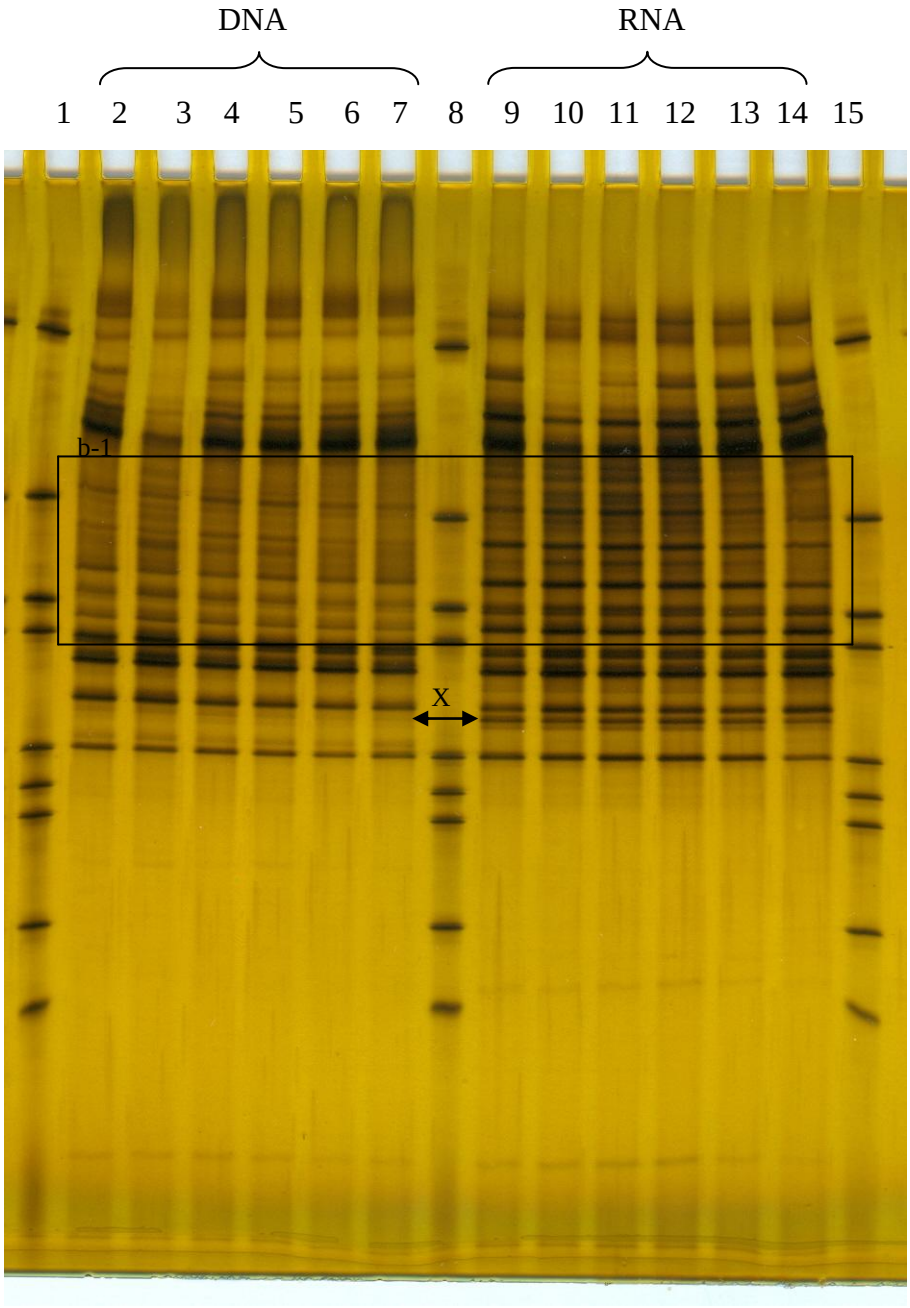



**Figure 5.7 DGGE analysis of DNA- cDNA in animal 4 fed once a day.**

***Arrows or box in the figure indicate the site of changes detected in gels.**
**Lane 1- standard, Lane 2- 8.30am, Lane 3- 12.30pm, Lane 4- 4.30pm, Lane 5- 8.30pm, Lane 6- 12.30am, Lane 7- 4.30am, Lane 8- standard, Lane 9- 8.30am, Lane 10-12.30pm, Lane 11- 4.30pm, Lane 12- 8.30pm, Lane 13- 12.30am, Lane 14- 4.30am, Lane 15- standard.**

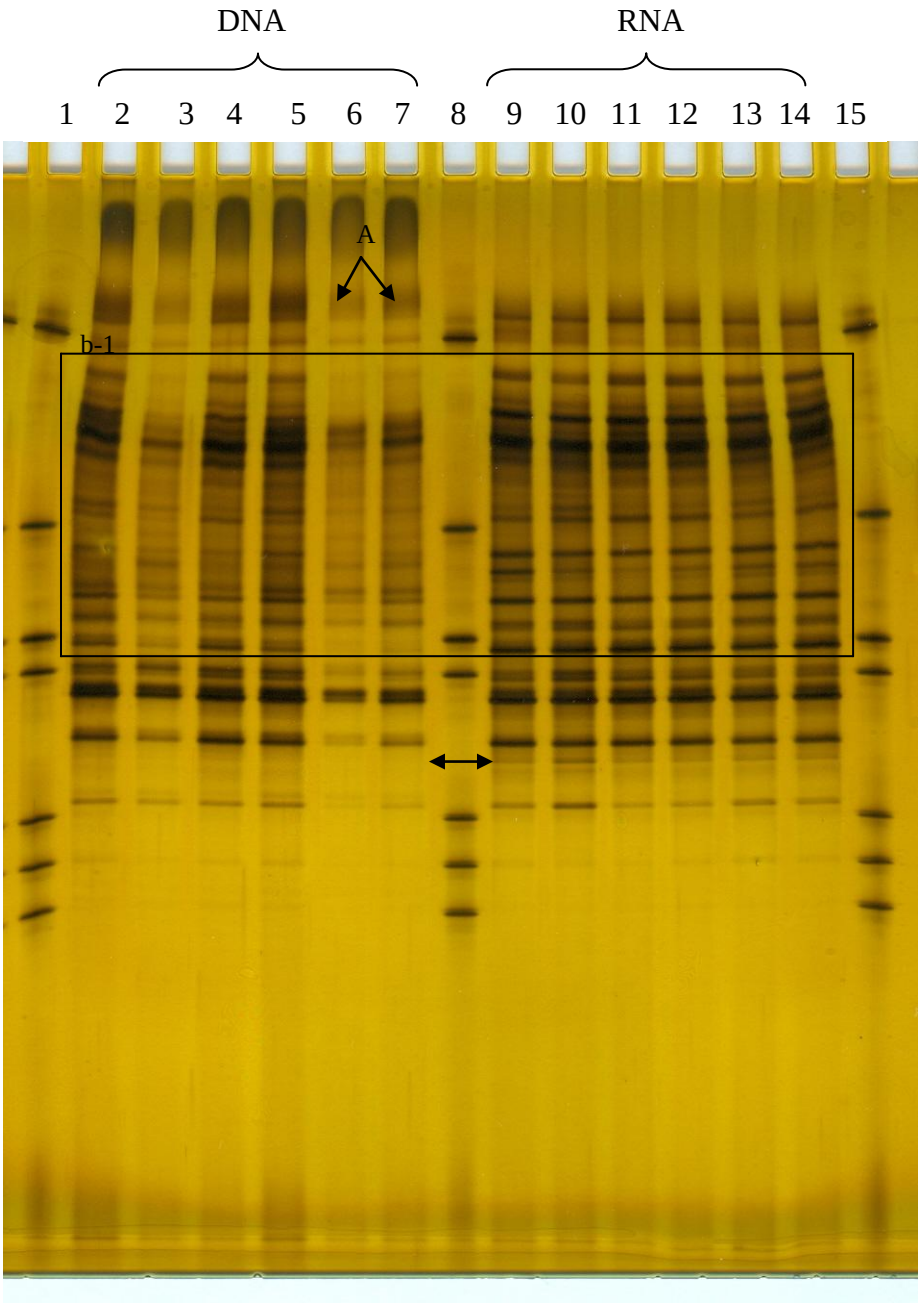



**Figure 5.8 DGGE analysis of DNA- cDNA in animal 4 fed twice a day.**

***Arrows or box in the figure indicate the site of changes detected in gels.**
**Lane 1- standard, Lane 2- 8.30am, Lane 3- 12.30pm, Lane 4- 4.30pm, Lane 5- 8.30pm, Lane 6- 12.30am, Lane 7- 4.30am, Lane 8- standard, Lane 9- 8.30am, Lane 10-12.30pm, Lane 11- 4.30pm, Lane 12- 8.30pm, Lane 13- 12.30am, Lane 14- 4.30am, Lane 15- standard.**

### *5.2.2 qPCR/ qRT-PCR*

The average *mcr*A gene quantity varied diurnally in once a day fed animals (table 5.2, figure 5.9). It was maximum (2.121pg/100ng of total DNA) at 8h (4.30pm) after feeding and minimum (0.527pg/100ng of total DNA) at 20h (4.30am) after feeding or just before the next feeding. In animals fed twice a day the maximum (1.095pg/100ng of total DNA) average *mcr*A gene quantity was again measured at 8h (4.30pm) after morning feeding and the

minimum (0.274pg/100ng of total DNA) was measured 16h (12.30am) after morning feeding (figure 5.3). The only significant ($p \leq 0.05$, $t=4.299$) change in average *mcr*A gene quantity was between the measurements at 8h and 16h after morning feeding in twice a day fed animals and there were no significant changes between the other times measured (table 5.3). The mean *mcr*A DNA values or the methanogen numbers as enumerated by real time PCR (qPCR) did not vary significantly ($p \leq 0.05$) between animals fed once a day or twice a day (figure 5.9, table 5.1).

**Table 5.1 *mcr*A gene quantity upon once vs. twice a day feeding.**

| Time | *mcr*A quantity upon once a day feeding (pg/100ng of total DNA) | | | | | *mcr*A quantity upon twice a day feeding (pg/100ng of total DNA) | | | | | Paired t-test | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Mean | 1 | 2 | 3 | 4 | Mean | LSD 5% | t value |
| **8.30 am** | 0.332 | 2.769 | 1.124 | 0.974 | 1.300 | 0.386 | 0.528 | 0.811 | 0.254 | 0.495 | 1.604 | 1.59 |
| **12.30 pm** | 0.946 | 2.188 | 0.854 | 0.628 | 1.154 | 0.161 | 0.658 | 1.026 | 0.908 | 0.688 | 1.362 | 1.09 |
| **4.30 pm** | 1.824 | 3.445 | 1.484 | 1.731 | 2.121 | 0.101 | 1.038 | 1.859 | 1.381 | 1.095 | 2.015 | 1.62 |
| **8.30 pm** | 0.552 | 1.301 | 0.366 | 0.250 | 0.617 | 0.053 | 0.553 | 0.716 | 0.137 | 0.365 | 0.762 | 1.06 |
| **12.30 am** | 0.288 | 1.333 | 0.669 | 0.219 | 0.627 | 0.134 | 0.304 | 0.423 | 0.237 | 0.274 | 0.738 | 1.52 |
| **4.30 am** | 0.445 | 0.924 | 0.560 | 0.180 | 0.527 | 0.041 | 0.254 | 0.621 | 0.400 | 0.329 | 0.654 | 0.96 |
| **MAUC** | 0.800 | 2.022 | 0.843 | 0.681 | 1.087 | 0.132 | 0.589 | 0.948 | 0.598 | 0.567 | 1.102 | 1.50 |

**MAUC- Mean area under curve for 20h.**

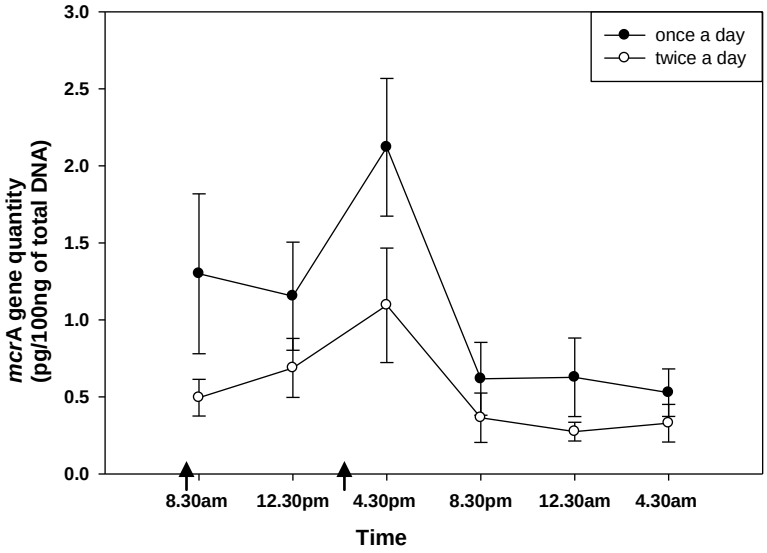



**Figure 5.9 *mcr*A gene quantity in animals fed once or twice a day.**

***Values plotted are average of data in table 5.1, over 20h diurnal period, error bars represent SE, n=4. Arrows on the time axis indicate time of feeding.**

**Table 5.2 Raw differences in *mcr*A gene quantity between times within once a day fed animals.**

| Time (Time 1-Time 2) | Raw differences between times within treatment (once a day) paired t- test | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Mean | LSD (5%) | t value |
| **12.30pm-8.30am** | 0.614 | -0.581 | -0.270 | -0.346 | -0.146 | 0.833 | 0.58 |
| **4.30pm-8.30am** | 1.492 | 0.676 | 0.360 | 0.757 | 0.821 | 0.762 | 3.43* |
| **8.30pm-8.30am** | 0.220 | -1.467 | -0.758 | -0.724 | -0.682 | 1.102 | 1.97 |
| **12.30am-8.30am** | -0.044 | -1.436 | -0.455 | -0.755 | -0.672 | 0.933 | 2.29 |
| **4.30am-8.30am** | 0.113 | -1.844 | -0.564 | -0.794 | -0.772 | 1.292 | 1.90 |
| **4.30pm-12.30pm** | 0.878 | 1.257 | 0.630 | 1.102 | 0.967 | 0.435 | 7.08* |
| **8.30pm-12.30pm** | -0.394 | -0.886 | -0.487 | -0.378 | -0.536 | 0.379 | 4.51* |
| **12.30am-12.30pm** | -0.658 | -0.855 | -0.185 | -0.409 | -0.527 | 0.464 | 3.61* |
| **4.30am-12.30pm** | -0.501 | -1.263 | -0.294 | -0.448 | -0.626 | 0.690 | 2.89 |
| **8.30pm-4.30pm** | -1.272 | -2.143 | -1.118 | -1.481 | -1.503 | 0.719 | 6.65* |
| **12.30am-4.30pm** | -1.536 | -2.112 | -0.815 | -1.512 | -1.494 | 0.844 | 5.63* |
| **4.30am-4.30pm** | -1.379 | -2.520 | -0.924 | -1.551 | -1.593 | 1.070 | 4.74* |
| **12.30am-8.30pm** | -0.264 | 0.031 | 0.302 | -0.031 | 0.010 | 0.371 | 0.08 |
| **4.30am-8.30pm** | -0.107 | -0.377 | 0.194 | -0.070 | -0.090 | 0.371 | 0.77 |
| **4.30am-12.30am** | 0.157 | -0.408 | -0.109 | -0.039 | -0.100 | 0.373 | 0.85 |

*** values are significant at p≤ 0.05, individual values are calculated by subtracting the value of time 2 from time 1, -ve value represents a decrease in gene quantity.**

**Table 5.3 Raw differences in *mcr*A gene quantity between times within twice a day fed animals.**

| Time (Time 1-Time 2) | Raw differences between times within treatment (twice a day) paired t test | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Mean | LSD (5%) | t value |
| **12.30pm-8.30am** | -0.225 | 0.130 | 0.216 | 0.653 | 0.194 | 0.574 | 1.07 |
| **4.30pm-8.30am** | -0.285 | 0.511 | 1.048 | 1.127 | 0.600 | 1.035 | 1.85 |
| **8.30pm-8.30am** | -0.333 | 0.025 | -0.094 | -0.118 | -0.130 | 0.237 | 1.74 |
| **12.30am-8.30am** | -0.252 | -0.224 | -0.387 | -0.017 | -0.220 | 0.243 | 2.88 |
| **4.30am-8.30am** | -0.344 | -0.274 | -0.189 | 0.145 | -0.165 | 0.345 | 1.53 |
| **4.30pm-12.30pm** | -0.060 | 0.380 | 0.832 | 0.474 | 0.407 | 0.584 | 2.22 |
| **8.30pm-12.30pm** | -0.108 | -0.105 | -0.310 | -0.771 | -0.323 | 0.499 | 2.06 |
| **12.30am-12.30pm** | -0.027 | -0.354 | -0.603 | -0.671 | -0.414 | 0.464 | 2.84 |
| **4.30am-12.30pm** | -0.119 | -0.404 | -0.405 | -0.508 | -0.359 | 0.266 | 4.29* |
| **8.30pm-4.30pm** | -0.048 | -0.485 | -1.142 | -1.244 | -0.730 | 0.900 | 2.58 |
| **12.30am-4.30pm** | 0.033 | -0.734 | -1.435 | -1.144 | -0.820 | 1.014 | 2.58 |
| **4.30am-4.30pm** | -0.060 | -0.784 | -1.237 | -0.982 | -0.766 | 0.805 | 3.03 |
| **12.30am-8.30pm** | 0.081 | -0.249 | -0.293 | 0.100 | -0.090 | 0.333 | 0.86 |
| **4.30am-8.30pm** | -0.012 | -0.299 | -0.095 | 0.263 | -0.036 | 0.370 | 0.31 |
| **4.30am-12.30am** | -0.092 | -0.050 | 0.198 | 0.163 | 0.055 | 0.234 | 0.74 |

*** values are significant at p≤ 0.05, individual values are calculated by subtracting the value of time 2 from time 1, -ve value represents a decrease in gene quantity.**

**Table 5.4 *mcr*A gene expression levels upon once vs. twice a day feeding.**

| Time | *mcr*A gene expression upon once a day feeding (pg/100ng of total RNA) | | | | | *mcr*A gene expression upon twice a day feeding (pg/100ng of total RNA) | | | | | Paired t test | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Mean | 1 | 2 | 3 | 4 | Mean | LSD 5% | t value |
| **8.30 am** | 0.332 | 2.769 | 1.124 | 0.974 | 1.300 | 0.386 | 0.528 | 0.811 | 0.254 | 0.495 | 0.244 | 1.34 |
| **12.30 pm** | 0.946 | 2.188 | 0.854 | 0.628 | 1.154 | 0.161 | 0.658 | 1.026 | 0.908 | 0.688 | 0.257 | 3.60* |
| **4.30 pm** | 1.824 | 3.445 | 1.484 | 1.731 | 2.121 | 0.101 | 1.038 | 1.859 | 1.381 | 1.095 | 0.308 | 4.90* |
| **8.30 pm** | 0.552 | 1.301 | 0.366 | 0.250 | 0.617 | 0.053 | 0.553 | 0.716 | 0.137 | 0.365 | 0.253 | 2.89 |
| **12.30 am** | 0.288 | 1.333 | 0.669 | 0.219 | 0.627 | 0.134 | 0.304 | 0.423 | 0.237 | 0.274 | 0.276 | 2.85 |
| **4.30 am** | 0.445 | 0.924 | 0.560 | 0.180 | 0.527 | 0.041 | 0.254 | 0.621 | 0.400 | 0.329 | 0.140 | 0.62 |
| **MAUC** | 0.254 | 0.346 | 0.435 | 0.421 | 0.364 | 0.113 | 0.073 | 0.081 | 0.144 | 0.103 | 0.140 | 5.92* |

***values are significant at p≤ 0.05. MAUC- Mean area under curve over 20h**.

**Table 5.5 Raw differences in *mcr*A gene expression between times within once a day fed animals.**

| Time (Time 1-Time 2) | Raw differences between times within treatment (twice a day) paired t test | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Mean | LSD (5%) | t value |
| **12.30pm-8.30am** | 0.073 | 0.344 | 0.370 | 0.176 | 0.241 | 0.224 | 3.42* |
| **4.30pm-8.30am** | 0.574 | 0.482 | 0.708 | 0.207 | 0.493 | 0.337 | 4.65* |
| **8.30pm-8.30am** | -0.036 | 0.287 | 0.385 | -0.039 | 0.149 | 0.349 | 1.36 |
| **12.30am-8.30am** | -0.083 | 0.194 | 0.378 | 0.010 | 0.124 | 0.325 | 1.22 |
| **4.30am-8.30am** | -0.024 | -0.031 | 0.051 | -0.175 | -0.045 | 0.150 | 0.95 |
| **4.30pm-12.30pm** | 0.501 | 0.138 | 0.338 | 0.031 | 0.252 | 0.333 | 2.41 |
| **8.30pm-12.30pm** | -0.110 | -0.057 | 0.015 | -0.215 | -0.092 | 0.154 | 1.99 |
| **12.30am-12.30pm** | -0.156 | -0.150 | 0.007 | -0.166 | -0.116 | 0.132 | 2.81 |
| **4.30am-12.30pm** | -0.097 | -0.375 | -0.320 | -0.351 | -0.286 | 0.203 | 4.47* |
| **8.30pm-4.30pm** | -0.611 | -0.195 | -0.324 | -0.246 | -0.344 | 0.295 | 3.71* |
| **12.30am-4.30pm** | -0.657 | -0.288 | -0.331 | -0.198 | -0.368 | 0.319 | 3.68* |
| **4.30am-4.30pm** | -0.598 | -0.513 | -0.658 | -0.382 | -0.538 | 0.190 | 8.99* |
| **12.30am-8.30pm** | -0.047 | -0.093 | -0.007 | 0.049 | -0.024 | 0.095 | 0.82 |
| **4.30am-8.30pm** | 0.013 | -0.317 | -0.334 | -0.136 | -0.194 | 0.262 | 2.36 |
| **4.30am-12.30am** | 0.059 | -0.225 | -0.327 | -0.185 | -0.169 | 0.261 | 2.07 |

***values are significant at p≤ 0.05, individual values are calculated by subtracting the value of time 2 from time 1, -ve value represents a decrease in expression**.

**Table 5.6 Raw differences in *mcr*A gene expression between times within twice a day fed animals.**

| Time (Time 1-Time 2) | Raw differences between times within treatment (twice a day) paired t test | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Mean | LSD (5%) | t value |
| **12.30pm-8.30am** | 0.088 | -0.010 | 0.062 | 0.075 | 0.054 | 0.069 | 2.47 |
| **4.30pm-8.30am** | 0.037 | -0.071 | 0.176 | 0.344 | 0.121 | 0.286 | 1.35 |
| **8.30pm-8.30am** | 0.011 | -0.039 | 0.032 | 0.088 | 0.023 | 0.084 | 0.87 |
| **12.30am-8.30am** | -0.017 | -0.073 | 0.024 | -0.013 | -0.020 | 0.064 | 0.99 |
| **4.30am-8.30am** | 0.093 | -0.007 | 0.015 | 0.023 | 0.031 | 0.069 | 1.43 |
| **4.30pm-12.30pm** | -0.051 | -0.062 | 0.114 | 0.269 | 0.068 | 0.249 | 0.86 |
| **8.30pm-12.30pm** | -0.077 | -0.030 | -0.030 | 0.013 | -0.031 | 0.058 | 1.67 |
| **12.30am-12.30pm** | -0.105 | -0.064 | -0.038 | -0.088 | -0.074 | 0.046 | 5.07* |
| **4.30am-12.30pm** | 0.005 | 0.002 | -0.046 | -0.052 | -0.023 | 0.049 | 1.49 |
| **8.30pm-4.30pm** | -0.026 | 0.032 | -0.144 | -0.256 | -0.098 | 0.204 | 1.54 |
| **12.30am-4.30pm** | -0.054 | -0.002 | -0.152 | -0.357 | -0.141 | 0.249 | 1.81 |
| **4.30am-4.30pm** | 0.056 | 0.064 | -0.161 | -0.321 | -0.090 | 0.295 | 0.97 |
| **12.30am-8.30pm** | -0.028 | -0.034 | -0.008 | -0.101 | -0.043 | 0.064 | 2.14 |
| **4.30am-8.30pm** | 0.082 | 0.032 | -0.017 | -0.065 | 0.008 | 0.101 | 0.25 |
| **4.30am-12.30am** | 0.110 | 0.066 | -0.008 | 0.036 | 0.051 | 0.079 | 2.04 |

*** values are significant at p≤ 0.05, individual values are calculated by subtracting the value of time 2 from time 1, -ve value represents a decrease in expression.**

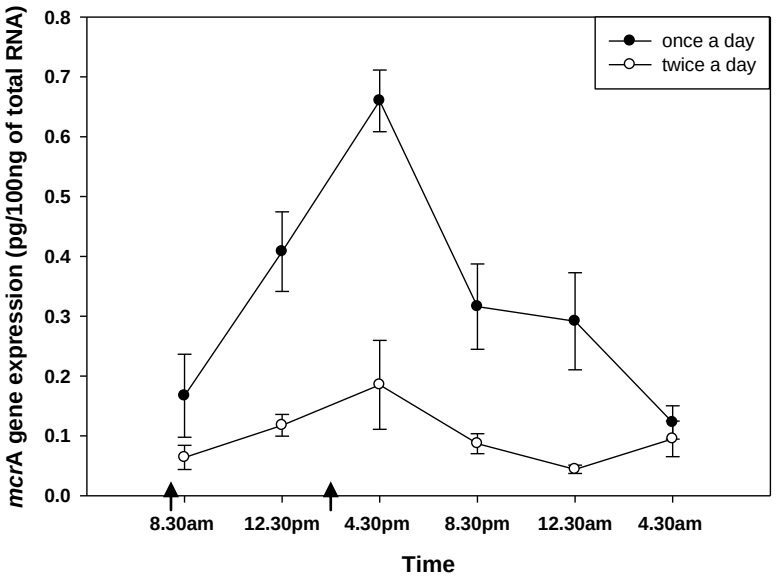



**Figure 5.10 *mcr*A gene expression in animals fed once or twice a day.**

***Values plotted are average of data in table 5.4, over 20h diurnal period, error bars represent SE, n=4. Arrows on the time axis indicate time of feeding.**

The average *mcr*A gene expression (table 5.4, figure 5.10) values of all four animals varied diurnally in once a day fed animals with a maximum (2.121pg/100ng of total RNA) at 8h (4.30pm) after feeding and a minimum value (0.527pg/100ng of total RNA) measured at 20h

(4.30am) after feeding or just before the next feeding. When the animals were fed twice a day the *mcr*A gene expression ranged from a maximum expression (1.095pg/100ng of total RNA) at 8h (4.30pm) after feeding to a minimum (0.274pg/100ng of total RNA) measured at 16h (12.30am) after feeding. Though there was a significant decrease in mean area under curve (MAUC) for 20h of *mcr*A gene expression for twice a day fed animals ($p< 0.05$, $t= 5.922$) than the once a day animals, this difference was significant only at 4h (12.30pm) and 8h (4.30pm) after morning feeding (table 5.4). The mean *mcr*A gene expression levels of all 4 animals estimated every 4h over a 24h period when fed once a day or twice a day are given in table 5.4.

On performing paired sample t test for the difference in means of *mcr*A gene expression across the time intervals in animals fed twice a day (table 5.6), apart from a significant difference between *mcr*A gene expression at 4h and 16h after morning feeding ($p< 0.05$, $t=5.077$), no significant changes in mean *mcr*A gene expression over the diurnal period could be detected. In contrast, when the animals were fed only once a day in the morning, marked differences were observed in *mcr*A gene expression levels across different time periods (table 5.5). The difference between the *mcr*A expression levels at peak i.e. 8h after feeding and at lowest i.e. 20h after feeding or 4h before feeding was the most significant ($p<0.01$, $t=8.991$). Thus, in once a day fed animals, the *mcr*A gene expression started increasing after feeding till 8h and then it started decreasing to its lowest at 20h after feeding or just before next feeding (figure 5.10) while in twice a day fed animals it was the lowest at 16h after morning feeding.

## 5.3 Discussion

The DGGE profile revealed the presence of 22 to 24 bands and it could be deduced from phylogenetic analysis that *Methanobrevibacter* sp. was the predominant methanogen along with few strains/ species closest to *Methanosphaera stadtmanae* and a few uncultured archaeal species. This finding is consistent with the earlier experiment (Chapter 3) and of other studies (Hook *et al.* 2009; Whitford *et al.* 2001; Wright *et al.* 2007; Zhou *et al.* 2009; 2010). A different feeding pattern may not necessarily be accompanied by a complete absence of an organism from the community (Hook *et al.* 2011). Accordingly, no effect of feeding frequency on DGGE profile was observed in this experiment on the presence or absence of bands. An interesting observation was the appearance of few bands phylogenetically closest to *Methanobrevibacter* sp. in the cDNA (RNA) samples while they were either absent or had very low intensity in corresponding DNA samples. This suggests that some strains/ species may not be of significant proportions in the DNA population but are actively participating in

methane production to warrant the appearance of corresponding bands in cDNA (RNA) profiles. It has also been suggested that organisms which might have been dormant earlier might become active under the influence of a new rumen physiological pattern or inhibitors and occupy the ecological niches left vacant by other populations (Attwood and McSweeney 2008). The bands representing species closest to *Methanosphaera stadtmanae* also showed variable changes in different animals suggesting the influence of animal to animal variation. But, it is a limitation of the DGGE method that only bands from dominant populations appear in the profiles and community members with lower proportion may not be expressed (Kocherginskaya *et al.* 2001). Thus any changes happening in low proportion strains/species may not have been detected. Also, the relative increase or decrease in number and activity of particular populations between treatments or over the diurnal period could not be accurately gauged from DGGE analysis. A better insight into methanogen dynamics could thus be obtained upon estimation of their population numbers and activity through a real time PCR.

The cDNA and DNA bands matching with *Methanobrevibacter* species decreased in intensity 16h and 20h after morning feeding but no such change was detected in twice fed animals. A possible explanation for this could be establishment of more stable ruminal conditions upon feeding twice daily (Robles *et al.* 2007). This may suggest that when fed twice a day there is a continuous supply of nutrients to the microbes, rumen fermentation is continuous ensuring regular availability of substrates to methanogens which does not lead to any major shift in methanogen numbers or activity whereas, when the same animals were fed only once a day, there would be dramatic shifts in rumen environment and thus the methanogen community. The bands showing lower intensity at 16h and 20h after morning feeding in once a day fed animals belonged to *Methanobrevibacter* sp., the predominant species of rumen which synthesizes methane through hydrogenotrophic pathway i.e. by reducing $CO_2$ with molecular $H_2$ (Miller *et al.* 1986; Mohammed *et al.* 2011). Thus, a decrease in rumen fermentation end products ($CO_2$ and $H_2$), could be a possible reason for the decreased *mcr*A gene expression after a prolonged period of starvation. But, a complete inhibition of methanogenesis did not occur, because many other *Methanobrevibacter* strains/ species as well as other species could still be active.

For the qPCR assay a reference gene could not be used because a suitable gene which would have a consistent level of transcription across all the microbial communities has not been identified till yet. No indication of a suitable reference gene in earlier studies on methanogen community could be found (Denman *et al.* 2007; Guo *et al.* 2008). These studies also relied on the efficiency of standard curve to generate quantification data. Since the purpose of this

study was to measure changes between different times or treatments, extrapolation from a standard curve prepared by serial dilutions of a methanogen plasmid would serve the purpose. Though it was desirable to have a reference gene, it was believed that any technical variations during assay would be negated by the statistical significance of results from biological replicates. Another possible option could have been spiking the samples with a known amount of RNA before conversion to cDNA. It would have contributed to the synthesis efficiency but was less likely to contribute in overall estimations. Therefore, in order to account for any difference in RNA conversion to cDNA, estimation was done against a fixed amount of DNA or RNA which would negate the effects of any technical errors during nucleic acid processing. For the assay itself, it was highly desirable to use TaqMan assay against SYBR® Green assay because of high specificity of the products obtained. But at this stage of experimentation, it was decided to use SYBR® Green assay because a TaqMan probe may not be able to capture all the species/ strains of the methanogen community which was the main focus of experiment. The assay was further controlled by using highly specific primers, performing dissociation (melting) curve analysis after every reaction to rule out any non specific amplification, controlling the efficiency of reaction to a minimum of 95% and the technical variability with a minimum $R^2$ of 0.900. The SYBR® Green assay performed could be further developed for a TaqMan assay if the sample number is very large where it would be more time and cost effective.

The transcription level of a gene has been associated with its expression in a physiological function. The expression level of *mcr*A gene has been reported to be proportional to the methane production (Denman *et al.* 2007) and estimation of *mcr*A specific mRNA out of the total pool of RNA can give an idea of the methanogen activity within a given sample. The RT-qPCR analysis revealed that a change in feeding frequency from once a day feeding to twice a day feeding caused a decrease in *mcr*A gene expression indicating a decreased methanogen activity (table 5.4, figure 5.10). Though there was no significant change in methanogen copy numbers, maximum difference in *mcr*A gene quantity between two feeding frequencies was observed at 8h after morning feed (table 5.1, figure 5.9). Also, the greatest difference in *mcr*A gene expression between two feeding managements was detected at 8h after morning feeding and the gene expression was generally lowest for both 8h and 4h before the morning feeding. For animals fed twice a day, gene expression differed only at 8h (4.30pm) after morning feed and ≈2h after evening feed, the methanogen numbers did not vary significantly between times (table 5.3, figure 5.10). In contrast, for once a day fed animals, the gene expression started increasing immediately after feeding and was on a relative high till about 12h after feeding from where it decreased gradually though

maintaining a numerical but not significant high over twice fed animals, to its lowest at 8h and 4h before morning feeding (table 5.1, figure 5.10). The methanogen numbers as revealed from *mcr*A DNA quantity though were not significantly different in once a day from twice fed animals but within the diurnal period they varied considerably in once a day fed animals being highest at 8h (4.30am) after feeding and lowest just before feeding (table 5.2, figure 5.9).

Though these results could be compared with the DGGE analysis where few bands showed decreased intensity at 16h and 20h after once a day feeding and a constant intensity in twice a day feeding, but the subtle shifts in the gene expression or total population could not be detected from corresponding DGGE analysis. Further, it is evident that analysis of spot samples i.e. at a particular time within 24h would not have given an accurate representation of the methanogen community dynamics and a larger number of samples spread over the diurnal period provided better information about the shifts in methanogen population and their gene expression.

The peak in gene expression observed after 8h of feeding could be explained by a small increase in methanogen population combined with an increase in substrate availability leading to an increased fermentation by rumen microbes. The increase in fermentation may lead to enhanced release of fermentation end products which can cause a spurt in methanogen population and activity.

No significant changes were detected in numbers and gene expression over the diurnal period when animals were fed twice a day except a peak 8h after feeding (table 5.3 and table 5.6). The possible explanation for this could be that when animals were fed on kale twice a day there was a constant supply of readily fermentable carbohydrates which may have increased the $H_2$ partial pressure or a decrease in the rumen pH. It has been reported earlier that a constant supply of water soluble carbohydrates can decrease the pH *in vitro* though no effect on VFA production was noted (Lee *et al.* 2003). A pH below 6 has been reported to reduce methane production *in vitro* and as the pH rose above 6, the methane production returned suggesting that methanogens are not automatically killed by low pH, although their activity is influenced (Kessel and Russell 1997a).

In a parallel study with this experiment (Ruguho, *unpublished data*), the ruminal pH of the steers was measured over the 24h diurnal period and it was found that steers fed twice a day tended to have a comparatively lower pH than steers fed once day. Though the average pH was above 6 for most of the diurnal period, the period when rumen pH was 5.8-6.0, though a low proportion in the overall 24h period was comparatively greater for twice fed animals than

once a day fed animals. This may explain the difference in activity of methanogens. Since the rumen provides a more stable environment than an *in vitro* medium; the activity of methanogens can be expected to decrease, rather than ceasing completely. Robles *et al*., (2007) had reported that an increased feeding frequency provides more stable rumen conditions without any changes in the average rumen pH. But in a slight contrast to our study, they found that pH tended to be higher 12h after feeding when animals were fed twice daily. Since the animals were fed on high concentrate diets and the feeding interval was 12h as against 7h interval followed by 18h of no feeding in this study and a high forage diet, a different rumen pattern could be expected. The overall decrease in gene expression 8h or 4h before morning feeding could be due to the starvation effect which might occur on rumen microbes upon prolonged gaps in feeding. Kessel and Russell (1997b) had proposed that methanogens are more prone to starvation effect and a prolonged starvation for more than 12h could lead to almost complete inhibition of methanogenesis. Similarly, Khafipour *et al*., (2009) had also reported that the number of methanogens was lowest 15min prior to feeding. As discussed earlier, under the starvation conditions in rumen, methanogen activity may be reduced but there may not be any effect on the microorganisms because of rumen being a very dynamic environment.

Further, when the rumen microbes, especially reductive acetogens, are subjected to extreme changes in rumen environment, they may not be able to cope with starvation effects which include very low partial pressure of $H_2$ and may be dominated by the methanogens which are capable of surviving under extremely low $H_2$ partial pressure (Fievez *et al.* 2001; Greening and Leedle 1989; Joblin 1999). In the scenario where animals are fed twice daily, there is more stable rumen environment and relatively continuous fermentation which raises the partial pressure of $H_2$ (low pH). As a result of this increased $H_2$ partial pressure, the reductive acetogens may become more active and compete with methanogens for $H_2$ (Greening and Leedle 1989; Joblin 1999). The $H_2$ may thus be channelled towards more VFA production. Methanogen activity would then be decreased for a similar DMI. Certain species such as *Methanosphaera stadtmanae* which have high requirements for pH and growth optima might be more effected than other species such as *Methanobrevibacter ruminantium*, which has a wider range of pH (5.5-7.0) for growth and activity.

One limitation of this study was establishing the relationship between actual methane production and *mcr*A gene expression. Calculating methane production through respiratory chambers was outside of the ambit of this work, and the limitations of the $SF_6$ technique mean

it is not sensitive enough to detect subtle changes within the diurnal period. Since no reliable and feasible alternative method for estimation of rumen methanogenesis within the diurnal 24h cycle for forage fed cattle is available, a molecular analysis of the rumen milieu was the only reliable technique to estimate the effect of a feed management on rumen methanogenesis within the diurnal cycle. The *mcr*A gene expression and *mcr*A gene quantity have earlier been correlated with the actual methane production in some of the earlier studies (Denman *et al.* 2007; Guo *et al.* 2008; Hook *et al.* 2011).

## 5.4 Conclusion

The development of modified method to extract DNA and RNA from the rumens of cows fed high quality forage has made possible the sensitive detection of changes in methanogen quantities and gene expression, and facilitated high sample number throughput. This enabled the use of serial rumen sampling across the diurnal period, and improved the analysis of the methanogen population dynamics compared to spot sampling protocols. While DGGE analysis provided an initial screening of the methanogen community and some important information regarding the effect of a long feeding gap on some methanogen community members, a comprehensive understanding of methanogen community dynamics could however be obtained only through quantitative real time and reverse transcriptase PCR.

It was possible to conclude from this experiment that a diurnal pattern of methanogen activity is present in cattle fed high quality forages, and appears to be determined by their feeding pattern. This is the first report of such a finding, and is a significant contribution to the study of rumen methanogen ecology.

The applicability of this technique needs to be validated further in the pasture based systems through experimentation on animals under actual grazing conditions The ability to detect changes in methanogen population and gene expression over a diurnal period under grazing conditions would further validate the application of this technique to pasture based ruminants, which is the subject of the following chapter.

# Chapter 6

# Quantification of Rumen *mcr*A mRNA and Methanogen DNA within the Diurnal Cycle in Cows Grazing High Quality Pastures.

In the previous chapters, a reliable method of extracting and quantifying rumen methanogen mRNA/ DNA from cattle pen fed forages was developed and used to describe the diurnal variation in rumen methanogen numbers and their activity. However, to be of use in investigating the dynamics of methanogenesis in high intensity pasture based grazing systems, this method was required to be validated in cows grazing very high quality pasture under a typical South Island pasture management and grazing protocol.

This experiment was planned to assess the use of the method developed in Chapter 4 in determining diurnal patterns of rumen methanogenesis in grazing cows on high quality pasture, using the effect of a proven methanogenic inhibitor (fish oil), as a treatment, and unsupplemented cows as controls. Since, it has been reported that fish oil can have a direct toxic effect on methanogens (Dong *et al.* 1997; Fievez *et al.* 2003), DGGE analysis was also performed on both DNA and RNA/ cDNA samples to investigate any changes in methanogen community profile.

The supplementation of ruminant diets with fats has been proposed as a promising strategy to reduce rumen methane emissions (Grainger and Beauchemin 2011). Fats are generally added in the finishing diets in many farm systems internationally in order to increase the energy density of diets. Considering the fact that addition of ionophores and synthetic analogues has been banned by many governments, fats offer a natural source of methane mitigation. Earlier reviews have concluded that the effect of fat supplementation on milk production is complex and depends upon the type of diet: pasture or TMR based, type of forage offered, total fat content of diet, physiological state and genetic merit of the animal (Garnsworthy 1997). Grainger and Beauchemin (2011) reported that in diets containing fat content less than 80g/kg DMI (8%), a 10g/kg increase in dietary fat decreased methane yield by 1g/kg DM intake in cattle. Based upon a statistical analysis, they also concluded that the effect of fat on methane production was more consistent with total dietary fat rather than amount of added fat.

Dietary lipids have been proposed to exert their effect on methane inhibition by (i) decreasing ruminal organic fermentation, (ii) through diverting $H_2$ towards biohydrogenation of the lipids

containing unsaturated fatty acids (Czerkawski *et al.* 1966; Johnson and Johnson 1995), (iii) indirectly through decreasing the ruminal bacteria and protozoa or (iv) a direct toxic effect on the methanogens, or a combination of these modes (Soliva *et al.* 2003).

Grainger and Beauchemin (2011) concluded that the type of fatty acid or the formulation in which it is fed does not have any effect on methane production, though some oilseeds need to be processed before feeding to increase digestibility. But, Galbraith *et al*., (1971) had reported that for efficient adsorption, the fatty acids must be in solution and remain sufficiently lipophilic. To support this observation, the fats which have a lower melting temperature and are able to melt more efficiently at the rumen temperature e.g. coconut oil and palm kernel oil, which are particularly rich in lauric acid (C12:0), have been found to be more effective in methane suppression (Dohme *et al.* 2000).

Osborne *et al*., (2007) have shown that it is possible to supplement fish oil to pasture fed dairy cows without decreasing feed or water intake relative to cows fed fish oil in the diet, and the low volume of oil required, enabled the cows to be fed at milking.

Fish oil supplementation has also been reported to shift rumen fermentation towards propionate production at the expense of acetate and butyrate (Doreau and Chilliard 1997; Shingfield *et al.* 2010). It has already been discussed earlier (chapter 2) that a higher propionate production at the cost of acetate implies a decrease in $H_2$ available for methane production. Further, the fish oil also contains substantial quantity of polyunsaturated fatty acids (PUFA) such as linoleic acid (C18:2n-6, LA), linolenic acid (C18:3n-3, LNA), eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA), which have potential health benefits and its supplementation may alter the composition and percentage of unsaturated fat in milk, which is desirable to consumers (Kim *et al.* 2008).

A DGGE analysis and qPCR of rumen fluid from steers fed fish oil at different levels of DMI has been shown to change bacterial diversity as well as total DNA concentration (Huws *et al.* 2010). Its supplementation has been shown to cause a direct toxic effect on certain Gram +ve rumen bacteria which produce more $H_2$ (Kim *et al.* 2008). Further, Fievez *et al*. (2003) observed up to 80% reduction in methane production of batch cultures *in vitro,* 48h after addition of fish oil and Petrie *et al*., (2009) reported a decrease in both daily and intake corrected methane emission of steers fed 50: 50:: hay: concentrate diet upon fish oil supplementation at 2% of DMI.

For these reasons, fish oil was expected to greatly reduce rumen methanogenesis in pasture based cows grazed under the typical production system of the South Island. It was therefore

used as a treatment along with unsupplemented controls in this experiment to determine the efficacy of the method developed in Chapter 4, in quantifying the rumen *mcr*A mRNA and methanogen DNA within the diurnal cycle of this grazing system.

## 6.1 Materials and methods

### *6.1.1 Animals, diets and experimental design*

Six ruminally fistulated, non-lactating Holstein Friesian cows with an average liveweight of 525kg were used in the experiment. They were strip grazed on a ryegrass (*Lolium perennes*) and white clover (*Trifolium repens*) pasture. The cows were allocated 10kg DM/animal of pasture once daily, at 5pm. Plate meter reading of the pasture was done pre and post grazing to monitor the supply of pasture. In addition, 3kg DM/animal of a similar ryegrass and clover silage was allocated each day at 5pm.

Experiment was conducted in a 3x3 crossover design. The six cows were randomly divided into two groups of three cows each. The treatment group cows were given 350ml of fish oil (United Fisheries, Christchurch, NZ) (2.7% of total DMI or 3.5% of pasture DMI) through the rumen canula via a tube guided into the reticulum to simulate oesophageal entry. The control group cows were administered an equal amount of water to maintain rumen volume and compensate for the time the fistula was opened. Cows had access to fresh water throughout the day. The fish oil or water supplementation was done at 5pm daily before giving the fresh pasture break to cows. First sample collection was done 72h after supplementation and the second sample collection was done on 15d of supplementation. The cows were then given a washout period of 21d grazing the same pasture and the treatments were then switched between groups.

Samples of WRC were collected from the rumen of all six cows every 4h over a 24h period. Sample collection was done before the respective supplementation at 5pm, then at 9pm, 1am, 5am, 9am and 1pm (0, 4, 8, 12, 16 and 20h after feeding). 400g of WRC were collected from the ventral sac of the rumen via the canula, placed in an ice bath for 5min for transportation and then aliquoted to microtubes. The samples were then processed as described earlier in section 5.1.1 and stored at -80$^{\circ}$C until further analysis.

Spot samples of rumen fluid were also collected at 0h (5pm), 8h (1am) and 16h (9am) after the morning feeding on all four days of sample collection. The WRC samples were taken through rumen canula by hand from the ventral sac of rumen and squeezed through two layers of cheesecloth to collect rumen fluid in two vials for VFA and $NH_3$ samples. Samples for

VFA were immediately placed on ice and 1ml of 6M sulphuric acid was added to samples for $NH_3$ analysis (to prevent volatilisation by ensuring pH remained under 4). The samples were then immediately stored under -20°C until further analysis.

## *6.1.2 Sample analysis*

Samples were taken out from -80°C and RNA & DNA were extracted using the procedure described in section 4.1.2 (appendix A.7) and quantified with fluorometry (Qubit™ , Invitrogen, Christchurch, New Zealand). RNA was also treated with Turbo DNA-free™ DNase (Ambion, Applied Biosystems, Auckland, New Zealand) to remove any contaminating DNA. RNA samples were checked for DNA contamination by performing a polymerase chain reaction (PCR) using archaeal 344f-GC and 522r primers targeting archaeal V2V3 region as described in section 3.1.1.1.3. Complementary DNA (cDNA) was synthesized from the RNA using TaKaRa Blue Print ™ reverse transcriptase kit according to the manufacturers' instructions (Norrie Biotech, Auckland, New Zealand).

### 6.1.2.1 PCR amplification for DGGE

The cDNA & DNA were PCR amplified for DGGE using Archaeal V2V3 primers 344f-GC and 522r (Akarsubasi *et al.* 2005) according to the procedure described earlier in section 3.1.1.3.3. and appendix A.3.

### 6.1.2.2 Denaturing gradient gel electrophoresis (DGGE) analysis

In Chapter 5 it was reported that the most significant differences in methanogen numbers and activity through DNA and cDNA estimation could be found at 8h after feeding. Therefore for initial screening, DNA and cDNA extracted from samples collected at 1am (8h after feeding) from both treatment and control cows were used for DGGE analysis. For detecting any changes in composition of methanogen community, PCR-DNA samples from all six animals were subjected to DGGE and two gels were constructed. One gel contained both control and treatment samples after 72h and 15d of fish oil supplementation for three animals. For detecting the relative activity after 72h or 15d of fish oil supplementation, cDNA-PCR products from control and treatment samples of all six animals were run on one gel. One gel was for samples collected after 72h of supplementation and second gel was for samples collected after 15d of supplementation.

DGGE was performed for PCR product analysis according to the procedure described earlier in section 3.1.1.3.5. and appendix A.4.

#### 6.1.2.3 qPCR/ qRT-PCR design and analysis

Quantitative PCR (qPCR) or reverse transcriptase quantitative PCR (qRT-PCR) were performed on DNA/ cDNA obtained from rumen contents of cows to enumerate the number of methanogens across different time periods (pre-prandial/ post-prandial) in cows supplemented with fish oil against control group cows. The remaining procedure was described earlier in section 5.1.2.3.

#### 6.1.2.4 VFA and ammonia analysis

The concentration of VFAs in the sampled rumen fluid was measured by thawing the frozen vials then vortexing, inverting and removing 2ml for sub-sample analysis. These were then centrifuged at 13000rpm for 30min at 4$^{o}$C. From each of these sub-samples, 500µl of the supernatant was placed into a 1.5ml centrifuge tube and 100µl of internal standard, 200µl of metaphosphoric acid and 200µl of deionised water ($dH_2O$) were added. Samples were then vortexed and placed at 4$^{o}$C for 30min. They were then centrifuged at 13,000rpm for 15min and filtered through a 0.45µm nylon syringe filter and placed into tubes ready for injection into the high performance liquid chromatography (HPLC) machine (Hewlett Packard 1100 Series, HPLC system) (Chen and Lifschlth 1989).

The frozen acidified samples for ammonia analysis were thawed out over night at 4$^{o}$C and maintained at this temperature or below throughout the extraction protocol. Samples were vortexed and inverted to ensure homogeneity and subsamples were removed into 2ml centrifuge tubes. They were centrifuged at 13000rpm in a refrigerated bench-top centrifuge set at 4$^{o}$C for 30min. After centrifugation, samples were filtered through a 0.45µl syringe top filter placed into a 2ml syringe. A 1000µl (1ml) aliquot was then added to 9ml of deionised sterilised water ($sdH_2O$). All samples were then analysed for ammonia concentration via Flow Injection Analyser (FIA) analysis immediately following the dilution process as described by Blakemore *et al*., (1987). Samples of fish oil used for supplementation in both parts of experiment were also analysed for their fatty acid composition (appendix B, table B.5).

### *6.1.3 Statistical analysis*

For each animal and variable, the data values were averaged for control and treated (fish oil supplemented) separately, and the difference between control and treated was calculated. These differences were then statistically analysed using a two tailed paired samples t test using SPSS software (version 16.0, IBM SPSS statistics). The variables analysed were: *mcr*A gene expression, *mcr*A gene quantity, VFA and ammonia concentration (mmol/L) over days after treatment and within the diurnal period after treatment.

The animal no.5 was inappetant at the third day (72h) of fish oil supplementation, and the yields of nucleic acid from these samples were precipitously reduced, and were omitted for 72h treatment and the corresponding 72h control period from the statistical analysis of qPCR and qRT-PCR.

## 6.2 Results

### *6.2.1 PCR-DGGE*

Approximately 21 to 23 bands were obtained upon DGGE analysis of DNA (DNA-PCR) and RNA (cDNA-PCR) samples obtained from whole rumen contents of control and fish oil supplemented (treatment) animals. Except for minor changes in several bands in animals 1, 2 and 3, no major changes were observed between control and fish oil treatments. These bands represent species closest to *Methanosphaera stadtmanae* and family *Methanobacteriacae* in all three animals (figure 6.1, box 'B-1'). Two bands having closest match to *Methanobrevibacter* sp. (figure 6.1, arrow 'A' & 'B') were also absent in animals 2 and 3 with fish oil supplementation. No similar changes were detected in animals 4, 5 and 6 (figure 6.2).

Similarly, the cDNA DGGE demonstrated a variable response to fish oil supplementation. In the samples collected after 72hr of fish oil supplementation, only animals 1 and 2 showed some changes (figure 6.3). The band relating to species matching closest with *Methanobrevibacter* sp. (arrow A) had a decreased intensity in animal 1 while bands matching closest to *Methanosphaera stadtmanae* were decreasing in intensity in animal 2 (arrow B). After 15d of fish oil supplementation (figure 6.4), no such effect was visible in animal 1 but animal 2 did have decreased intensities of bands relating to species matching closest to *Methanosphaera stadtmanae* (group 'A') and of two bands relating to *Methanobrevibacter* sp. (group B). The cluster of bands relating to *Methanosphaera stadtmanae* (group 'A') showed some peculiarity in that while one band was decreasing in intensity, other bands which were absent in control appeared in the treatment samples. Two bands relating to *Methanobrevibacter* sp. were also absent in animal 4 (arrows 'C' and 'D').

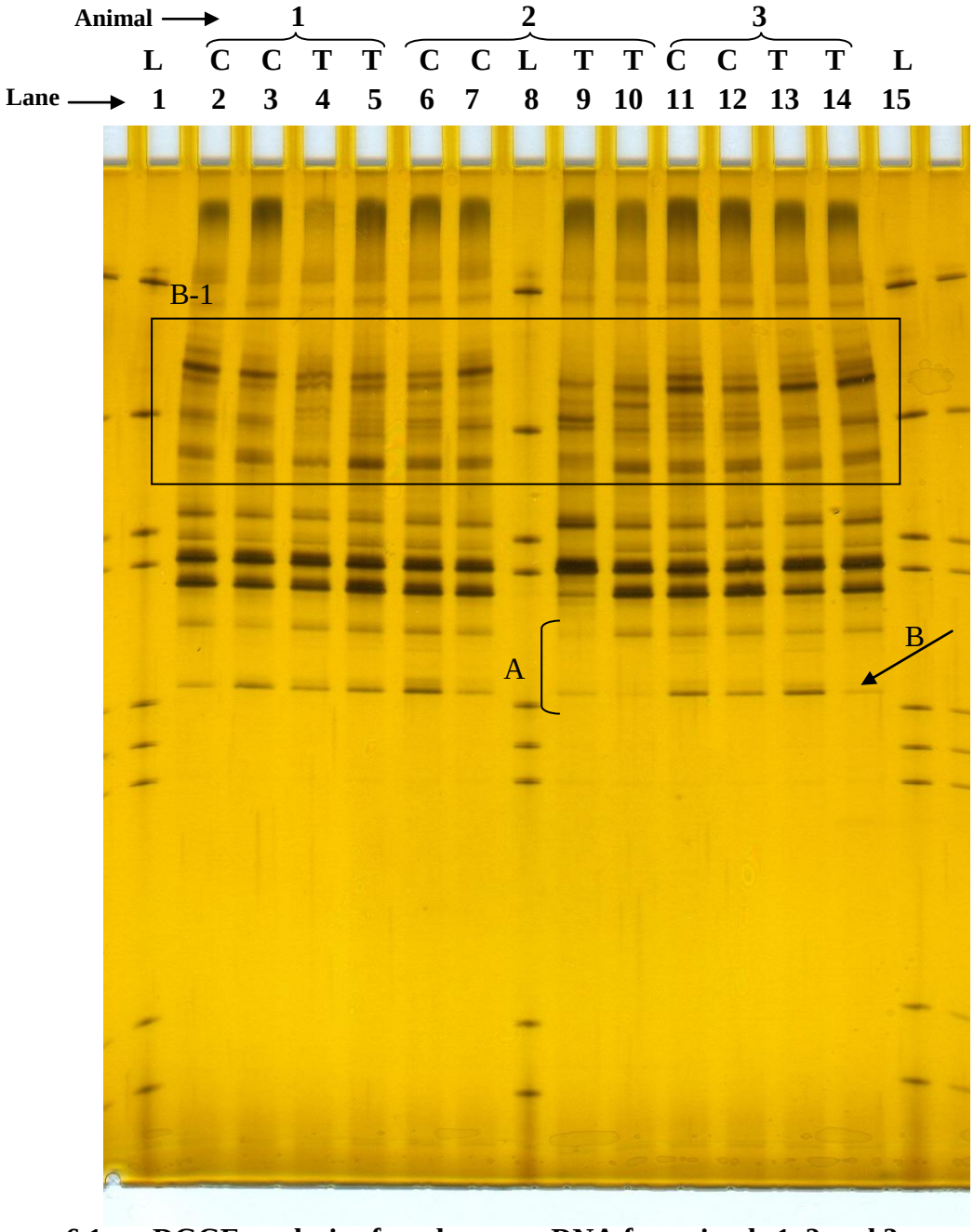



**Figure 6.1 DGGE analysis of methanogen DNA for animals 1, 2 and 3.**

*** Arrows in the figure indicate the site of changes detected in gels. L- ladder, C- control, T-treatment. Samples collected 8h (1am) after fresh pasture break at 72h and 15d of control/ fish oil supplementation (treatment).**
**Lane 1- ladder, Lane 2- 72h, Lane 3- 15d, Lane 4- 72h, Lane 5- 15d, Lane 6- 72h, Lane 7- 15d , Lane 8- ladder, Lane 9- 72h, Lane 10- 15d, Lane 11- 72h, Lane 12- 15d, Lane 13- 72h, Lane 14- 15d, Lane 15- ladder.**

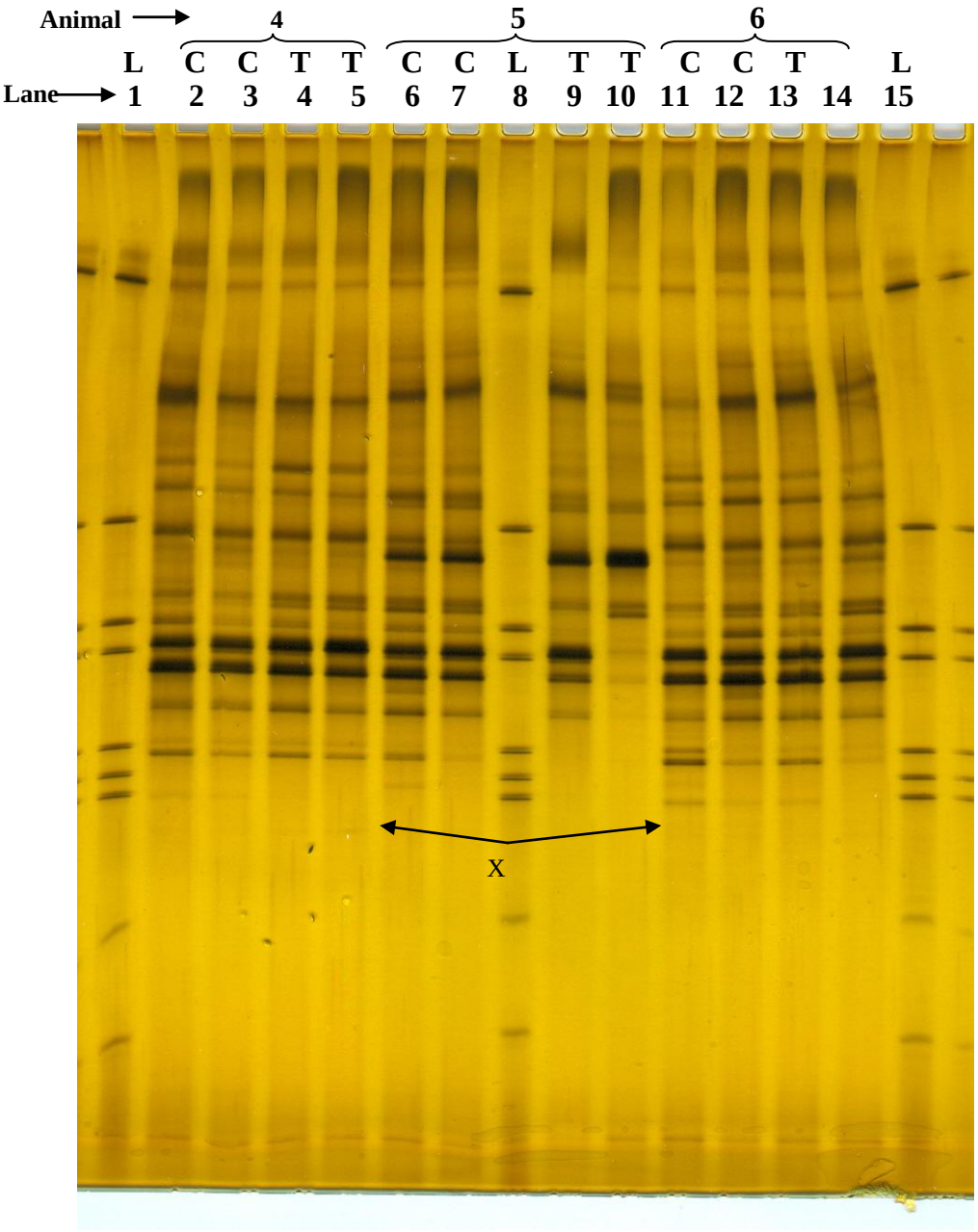



**Figure 6.2 DGGE analysis of methanogen DNA for animals 4, 5 and 6.**

*** Arrows in the figure indicate the site of changes detected in gels. L- ladder, C- control, T-treatment. Samples collected 8h (1am) after fresh pasture break at 72h and 15d of control/ fish oil supplementation (treatment).**

**Lane 1- ladder, Lane 2- 72h, Lane 3- 15d, Lane 4- 72h, Lane 5- 15d, Lane 6- 72h, Lane 7- 15d , Lane 8- ladder, Lane 9- 72h, Lane 10- 15d, Lane 11- 72h, Lane 12- 15d, Lane 13- 72h, Lane 14- 15d, Lane 15- ladder.**

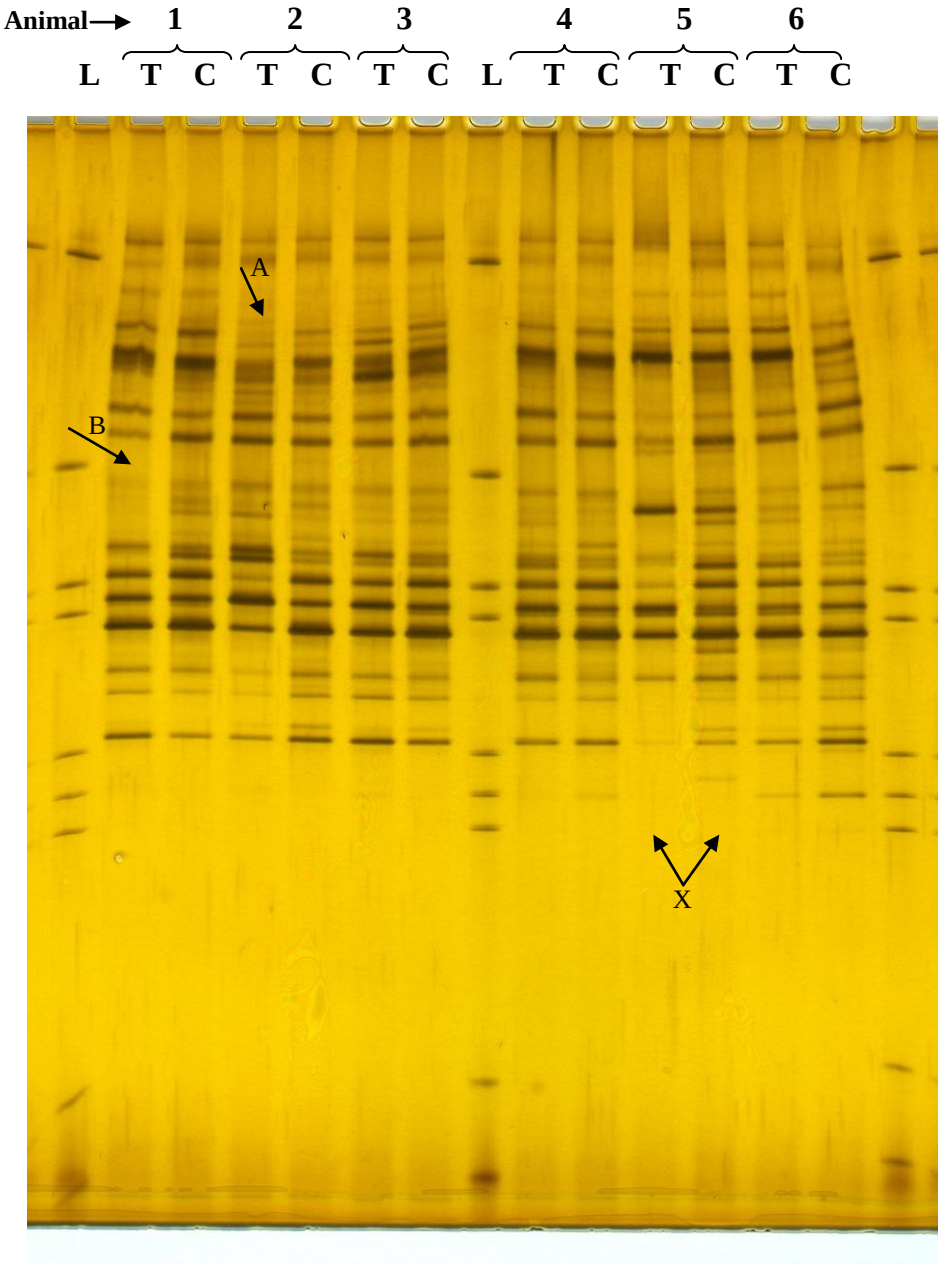



**Figure 6.3 DGGE analysis of methanogen cDNA for animals 1 to 6 at 72h after control or treatment.**

*** Arrows in the figure indicate the site of changes detected in gels. L- Ladder, C- Control, T- Treatment. Samples collected 8h (1am) after fresh pasture break at 72h of control/ fish oil supplementation (treatment).**

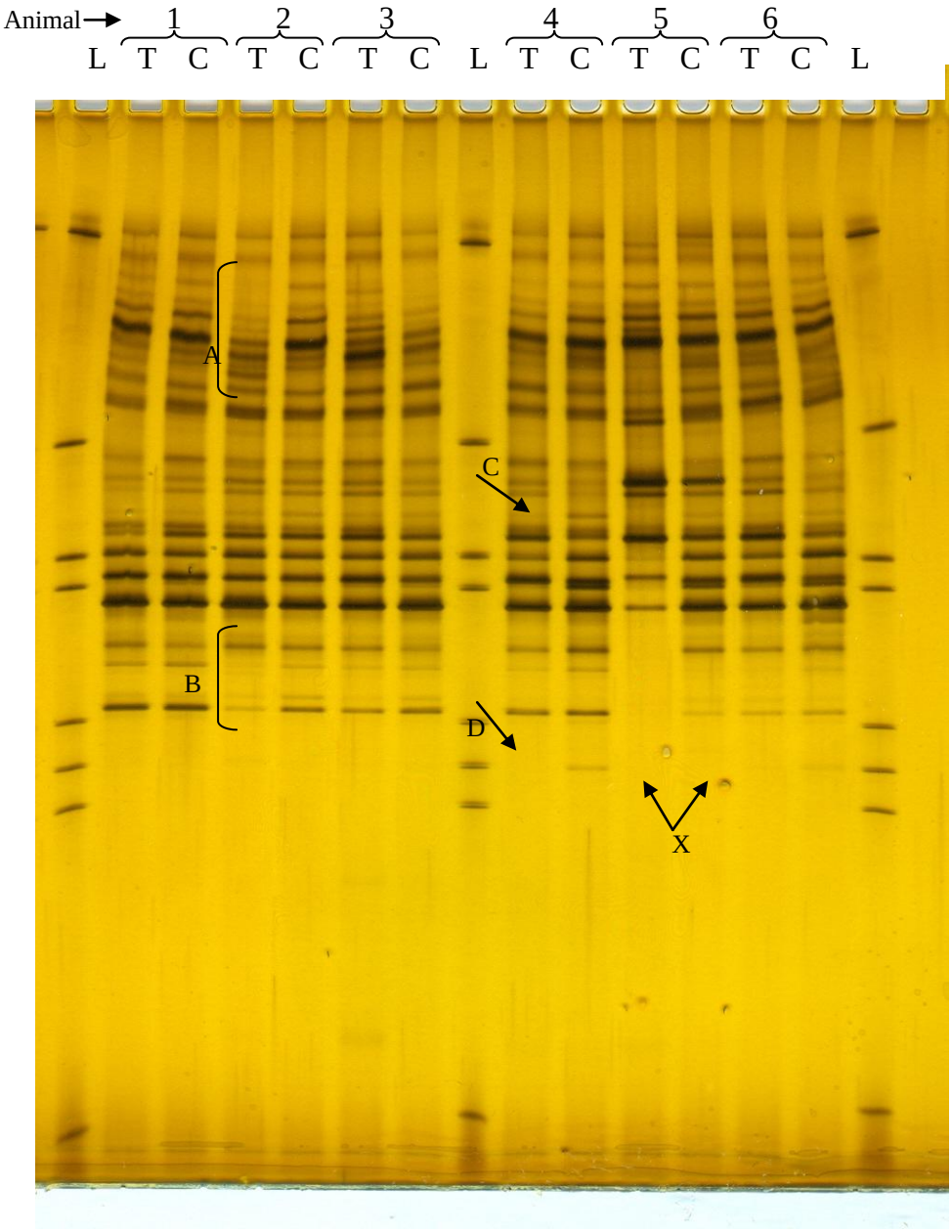



**Figure 6.4 DGGE analysis of methanogen cDNA for animals 1 to 6 at 15d after treatment or control.**

*** Arrows in the figure indicate the site of changes detected in gels. L- Ladder, C- Control, T- Treatment. Samples collected 8h (1am) after fresh pasture break at 15d of control/ fish oil supplementation (treatment).**

### *6.2.2 qPCR/qRT-PCR analysis*

The *mcr*A gene quantity (methanogen numbers) and *mcr*A gene expression values of all animals were analysed, except for animal 5, who was excluded from the statistical analysis of the results for the reasons detailed above.

The *mcr*A gene quantity as measured by the amount of *mcr*A DNA present per 100ng of total DNA decreased numerically upon the initial 72h of fish oil supplementation (treatment) but a

significant decrease in methanogen quantities was detected only after 15d of fish oil supplementation (table 6.1, figure 6.5) and on this day amongst all the times analysed, a significant difference in *mcr*A gene quantity between control and fish oil supplemented occurred only 8h (1pm) after the fresh pasture break was given to animals ($p \leq 0.05$, $t=5.90$). When the control and treatment values of *mcr*A gene quantity were averaged for 72h and 15d over all animals (table 6.2), a significant ($p \leq 0.05$, $t=6.10$) decrease was again observed in the treatment animals only 8h (1am) after break.

The average *mcr*A gene expression values were numerically less than corresponding control values upon initial 72h of fish oil supplementation but a significant decrease was found only after 15d of fish oil supplementation at 4h (9pm) and 8h (1am) ($p \leq 0.05$, $t=2.86$, $t=7.90$) after fresh pasture break was given (table 6.4, figure 6.6). When all the values were averaged for 72h and 15d of control and treatment for all animals (table 6.3), the comparative decrease in *mcr*A gene expression was again significant at 4h (9pm) and 8h (1am) after fish oil supplementation ($p \leq 0.05$, $t=4.81$ and $t=4.30$).

Within the diurnal period, significant changes ($p \leq 0.05$) were observed between average *mcr*A gene expressions of control animals, 4h (9pm) and 8h (1am) after fresh pasture break was given to the animals (appendix B, table B.1). Generally, lowest gene expression was observed at 1pm and 5pm, i.e. 4h before and just before the next fresh pasture break and thereafter the gene expression peaked till 8h after which it started decreasing. A small but not significant peak in gene expression was again observed in the morning at 9am from whereon the gene expression again decreased. In the fish oil supplemented animals, no such significant changes were detected within the diurnal period (appendix B, table B.2). Though some small peaks (not significant) were observed, the time and the relative increase in gene expressions varied between animals. Generally, these peaks occurred at 1am (8h after fresh break) and 9am, but the *mcr*A gene expression was relatively stable from 12h (5am) after pasture break till the next pasture break (5pm).

Similarly, the *mcr*A gene quantity was significantly high 8h (1am) after pasture break in control animals (appendix B, table B.3) but showed insignificant changes at other times. But, in the treatment animals, no significant changes were observed for *mcr*A gene quantity which generally remained stable throughout the diurnal period (appendix B, table B.4).

### 6.2.3 VFA and ammonia analysis

Statistical analysis of VFA concentrations in rumen fluid of control and fish oil supplemented animals, showed a significant decrease in average (of 72h and 15d) acetic acid concentration (table 6.5, figure 6.7) upon fish oil supplementation ($p \leq 0.05$, $t=2.63$). Also, there was no significant change in acetic acid concentrations after 72h of fish oil supplementation, but the concentrations decreased significantly after 15d of fish oil supplementation. When average values of treatment and control periods were analysed, significant difference ($p \leq 0.05$, $t=5.17$) was observed at 16h after pasture break (9am). Also, significant changes in acetic acid concentration were observed between times (table 6.5, figure 6.7) within both the control and fish oil supplemented animals. Highest concentrations were observed 8h after fresh pasture break (1am) followed by a decrease at 16h (9am) to the lowest at 5pm (0h) or just before next pasture break was to be given.

In spite of a numerical increase in propionic acid concentrations from control to fish oil supplemented group, the concentrations did not vary significantly between control and fish oil supplemented animals (figure 6.8, table B.6, appendix B). Also the highest (not significant at $p \leq 0.05$, but widest confidence interval) difference in propionic acid concentrations were observed for the 15d fish oil supplementation period at 8h (1am) after fresh pasture break. Within the diurnal period, propionic acid concentrations changed significantly for both control and fish oil supplemented animals, with highest concentrations at 8h after fresh pasture break (1am) followed by a decrease at 16h (9am) to the lowest at 5pm (0h) or just before next pasture break was to be given. There was no significant change in butyric acid concentration between control and treatment animals and the highest butyric acid concentrations were detected at 8h after fresh pasture break (1am) followed by a decrease at 16h (9am) to the lowest at 5pm (0h) or just before next pasture break was to be given. The ammonia concentration also did not vary significantly between control and treatment animals but the highest ammonia concentration was observed at 8h after pastures break (1am) and low but almost similar mean ammonia concentrations at other two times.

**Table 6.1 *mcr*A gene quantity for control and fish oil supplemented (treatment) animals at 72h and 15d.**

| Time | *mcr*A gene quantity for control at 72h (pg/100ng of total DNA) | | | | | | | *mcr*A gene quantity for treatment at 72h (pg/100ng of total DNA) | | | | | | | Paired t test | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5[×] | 6 | Mean | 1 | 2 | 3 | 4 | 5[×] | 6 | Mean | LSD 5% | t value |
| 5pm | 0.28 | 0.36 | 0.32 | 0.07 | 0.07[×] | 0.21 | 0.25 | 0.11 | 0.11 | 0.20 | 0.17 | 0.62[×] | 0.16 | 0.15 | 0.16 | 1.70 |
| 9pm | 0.35 | 0.32 | 0.56 | 0.13 | 0.06[×] | 0.20 | 0.31 | 0.09 | 0.06 | 0.18 | 0.22 | 2.16 | 0.14 | 0.14 | 0.23 | 2.08 |
| 1am | 0.36 | 0.37 | 0.11 | 0.32 | 0.31[×] | 0.20 | 0.27 | 0.24 | 0.04 | 0.06 | 0.27 | 6.86[×] | 0.09 | 0.14 | 0.15 | 2.55 |
| 5am | 0.28 | 0.17 | 0.11 | 0.03 | 0.17[×] | 0.16 | 0.15 | 0.17 | 0.21 | 0.08 | 0.09 | 2.67[×] | 0.28 | 0.17 | 0.11 | 0.39 |
| 9am | 0.22 | 0.13 | 0.42 | 0.17 | 0.02[×] | 0.09 | 0.21 | 0.14 | 0.03 | 0.20 | 0.14 | 0.81[×] | 0.16 | 0.13 | 0.13 | 1.59 |
| 1pm | 0.30 | 0.14 | 0.06 | 0.03 | 0.13[×] | 0.16 | 0.14 | 0.22 | 0.12 | 0.08 | 0.09 | 2.23[×] | 0.11 | 0.12 | 0.07 | 0.64 |
| MAUC | 0.30 | 0.25 | 0.28 | 0.14 | 0.13[×] | 0.17 | **0.23** | 0.16 | 0.09 | 0.13 | 0.17 | 2.78[×] | 0.16 | **0.14** | 0.11 | 2.15 |
| Time | *mcr*A gene quantity for control at 15d (pg/100ng of total DNA) | | | | | | | *mcr*A gene quantity for treatment at 15d (pg/100ng of total DNA) | | | | | | | Paired t test | |
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean | 1 | 2 | 3 | 4 | 5 | 6 | Mean | LSD 5% | t value |
| 5pm | 0.11 | 0.34 | 0.36 | 0.21 | 0.15 | 0.12 | 0.22 | 0.16 | 0.09 | 0.30 | 0.13 | 0.23 | 0.14 | 0.17 | 0.13 | 0.85 |
| 9pm | 0.16 | 0.37 | 0.37 | 0.19 | 0.08 | 0.10 | 0.21 | 0.11 | 0.14 | 0.20 | 0.19 | 0.74 | 0.24 | 0.27 | 0.34 | 0.44 |
| 1am | 0.33 | 0.51 | 0.32 | 0.49 | 0.48 | 0.62 | 0.46 | 0.15 | 0.11 | 0.06 | 0.21 | 0.26 | 0.09 | 0.15 | 0.14 | 5.90* |
| 5am | 0.12 | 0.14 | 0.07 | 0.03 | 0.57 | 0.41 | 0.22 | 0.26 | 0.38 | 0.15 | 0.08 | 0.31 | 0.07 | 0.21 | 0.25 | 0.15 |
| 9am | 0.05 | 0.18 | 0.32 | 0.21 | 0.06 | 0.04 | 0.14 | 0.10 | 0.09 | 0.31 | 0.07 | 0.32 | 0.33 | 0.20 | 0.19 | 0.82 |
| 1pm | 0.06 | 0.11 | 0.17 | 0.15 | 0.44 | 0.30 | 0.21 | 0.24 | 0.21 | 0.13 | 0.14 | 0.67 | 0.10 | 0.25 | 0.17 | 0.61 |
| MAUC | 0.15 | 0.28 | 0.27 | 0.22 | 0.30 | 0.28 | **0.25** | 0.17 | 0.17 | 0.19 | 0.14 | 0.42 | 0.17 | **0.21** | 0.21 | 0.98 |

***values are significant at p≤0.05, MAUC- Mean area under curve for 20h, [×]values for 72h control and treatment of animal 5 excluded from statistical analysis.**

**Table 6.2 Paired t test between averages (72h and 15d) of *mcr*A gene quantity for control and fish oil supplemented (treatment) animals.**

| Time | Average *mcr*A gene quantity for control animals (pg/100ng of total DNA) | | | | | | | Average *mcr*A gene quantity for treatment animals (pg/100ng of total DNA) | | | | | | | Paired t test | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5× | 6 | Mean | 1 | 2 | 3 | 4 | 5× | 6 | Mean | LSD 5% | t value |
| **5pm** | 0.20 | 0.35 | 0.34 | 0.14 | 0.15 | 0.16 | 0.22 | 0.13 | 0.10 | 0.25 | 0.15 | 0.23 | 0.15 | 0.17 | 0.12 | 1.21 |
| **9pm** | 0.25 | 0.34 | 0.47 | 0.16 | 0.08 | 0.15 | 0.24 | 0.10 | 0.10 | 0.19 | 0.20 | 0.74 | 0.19 | 0.25 | 0.36 | 0.09 |
| **1am** | 0.35 | 0.44 | 0.22 | 0.41 | 0.48 | 0.41 | 0.38 | 0.20 | 0.07 | 0.06 | 0.24 | 0.26 | 0.09 | 0.15 | 0.10 | 6.10* |
| **5am** | 0.20 | 0.15 | 0.09 | 0.03 | 0.57 | 0.29 | 0.22 | 0.21 | 0.29 | 0.11 | 0.09 | 0.31 | 0.18 | 0.20 | 0.15 | 0.39 |
| **9am** | 0.14 | 0.16 | 0.37 | 0.19 | 0.06 | 0.07 | 0.16 | 0.12 | 0.06 | 0.26 | 0.10 | 0.32 | 0.24 | 0.18 | 0.17 | 0.32 |
| **1pm** | 0.18 | 0.13 | 0.12 | 0.09 | 0.44 | 0.23 | 0.20 | 0.23 | 0.17 | 0.10 | 0.12 | 0.67 | 0.10 | 0.23 | 0.12 | 0.67 |
| **MAUC** | 0.23 | 0.27 | 0.27 | 0.18 | 0.30 | 0.22 | **0.24** | 0.16 | 0.13 | 0.16 | 0.15 | 0.42 | 0.16 | **0.20** | 0.18 | 1.27 |

***values are significant at p≤0.05, MAUC- Mean area under curve for 20h, ×values for 72h control and treatment of animal 5 excluded from statistical analysis.**

**Table 6.3 Paired t test between averages (72h and 15d) of *mcr*A gene expression for control and fish oil supplemented (treatment) animals.**

| Time | Average *mcr*A gene expression for control animals (pg/100ng of total RNA) | | | | | | | Average *mcr*A gene expression for treatment animals (pg/100ng of total RNA) | | | | | | | Paired t test | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5× | 6 | Mean | 1 | 2 | 3 | 4 | 5× | 6 | Mean | LSD 5% | t value |
| **5pm** | 0.36 | 0.53 | 0.70 | 0.13 | 0.59 | 0.46 | 0.46 | 0.30 | 0.32 | 0.53 | 0.36 | 3.49 | 0.58 | 0.93 | 1.26 | 0.96 |
| **9pm** | 0.37 | 0.75 | 0.97 | 0.32 | 0.78 | 0.56 | 0.63 | 0.18 | 0.46 | 0.59 | 0.20 | 0.28 | 0.39 | 0.35 | 0.15 | 4.81* |
| **1am** | 2.49 | 1.35 | 1.63 | 1.92 | 4.31 | 1.45 | 2.19 | 0.47 | 0.38 | 0.33 | 1.58 | 2.26 | 0.75 | 0.96 | 0.74 | 4.30* |
| **5am** | 0.60 | 0.47 | 0.42 | 0.40 | 1.46 | 0.43 | 0.63 | 0.64 | 0.28 | 0.41 | 0.63 | 2.19 | 0.35 | 0.75 | 0.35 | 0.89 |
| **9am** | 0.59 | 0.87 | 0.69 | 0.28 | 0.81 | 0.54 | 0.63 | 0.44 | 0.46 | 0.68 | 0.19 | 1.70 | 1.11 | 0.76 | 0.52 | 0.66 |
| **1pm** | 0.36 | 0.24 | 0.45 | 0.32 | 1.16 | 0.25 | 0.46 | 0.36 | 0.16 | 0.25 | 0.87 | 3.90 | 0.38 | 0.99 | 1.17 | 1.15 |
| **MAUC** | 0.88 | 0.76 | 0.86 | 0.63 | 1.65 | 0.67 | 0.91 | 0.41 | 0.36 | 0.48 | 0.64 | 2.02 | 0.61 | 0.76 | 0.59 | 1.30 |

***values are significant at p≤0.05, MAUC- Mean area under curve for 20h, ×values for 72h control and treatment of animal 5 excluded from statistical analysis.**

**Table 6.4 *mcr*A gene expression for control and fish oil supplemented (treatment) animals at 72h and 15d.**

| Time | ***mcr*A gene expression for control at 72h (pg/100ng of total RNA)** | | | | | | | ***mcr*A gene expression for treatment at 72h (pg/100ng of total RNA)** | | | | | | | **Paired t test** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5×** | **6** | **Mean** | **1** | **2** | **3** | **4** | **5×** | **6** | **Mean** | **LSD 5%** | **t value** |
| **5pm** | 0.01 | 0.05 | 0.06 | 0.05 | 0.08× | 0.37 | 0.11 | 0.18 | 0.43 | 0.47 | 0.02 | 0.16× | 0.05 | 0.23 | 0.38 | 0.92 |
| **9pm** | 0.53 | 0.58 | 0.42 | 0.32 | 0.27× | 0.45 | 0.46 | 0.19 | 0.30 | 0.58 | 0.12 | xxx | 0.67 | 0.37 | 0.33 | 0.76 |
| **1am** | 2.66 | 1.33 | 1.61 | 1.56 | 2.48× | 0.74 | 1.58 | 0.44 | 0.34 | 0.28 | 1.88 | xxx | 0.93 | 0.77 | 1.33 | 1.68 |
| **5am** | 0.72 | 0.33 | 0.47 | 0.23 | 0.82× | 0.28 | 0.41 | 0.65 | 0.16 | 0.32 | 0.59 | 2.20× | 0.39 | 0.42 | 0.27 | 0.16 |
| **9am** | 0.72 | 0.92 | 0.82 | 0.13 | 0.36× | 0.32 | 0.58 | 0.49 | 0.34 | 0.58 | 0.13 | 1.58× | 1.11 | 0.53 | 0.64 | 0.23 |
| **1pm** | 0.69 | 0.20 | 0.37 | 0.36 | 0.35× | 0.24 | 0.37 | 0.31 | 0.07 | 0.24 | 0.90 | 1.79× | 0.43 | 0.39 | 0.44 | 0.15 |
| **MAUC** | 1.00 | 0.66 | 0.71 | 0.49 | 0.83× | 0.42 | **0.65** | 0.40 | 0.28 | 0.42 | 0.64 | 0.95× | 0.67 | **0.48** | 0.44 | 1.07 |
| **Time** | ***mcr*A gene expression for control at 15d (pg/100ng of total RNA)** | | | | | | | ***mcr*A gene expression for treatment at 15d (pg/100ng of total RNA)** | | | | | | | **Paired T test** | |
| | **1** | **2** | **3** | **4** | **5** | **6** | **Mean** | **1** | **2** | **3** | **4** | **5** | **6** | **Mean** | **LSD 5%** | **t value** |
| **5pm** | 0.72 | 1.01 | 1.34 | 0.20 | 0.59 | 0.55 | 0.74 | 0.42 | 0.21 | 0.59 | 0.69 | 3.49 | 1.10 | 1.09 | 1.45 | 0.62 |
| **9pm** | 0.21 | 0.91 | 1.53 | 0.32 | 0.78 | 0.67 | 0.74 | 0.17 | 0.63 | 0.61 | 0.27 | 0.28 | 0.10 | 0.34 | 0.35 | 2.86* |
| **1am** | 2.32 | 1.37 | 1.65 | 2.28 | 4.31 | 2.16 | 2.35 | 0.50 | 0.42 | 0.38 | 1.27 | 2.26 | 0.56 | 0.90 | 0.47 | 7.90* |
| **5am** | 0.49 | 0.61 | 0.37 | 0.57 | 1.46 | 0.59 | 0.68 | 0.64 | 0.40 | 0.51 | 0.67 | 2.19 | 0.31 | 0.78 | 0.38 | 0.71 |
| **9am** | 0.45 | 0.82 | 0.56 | 0.43 | 0.81 | 0.75 | 0.64 | 0.39 | 0.57 | 0.77 | 0.24 | 1.70 | 1.12 | 0.80 | 0.45 | 0.93 |
| **1pm** | 0.03 | 0.29 | 0.52 | 0.29 | 1.16 | 0.26 | 0.42 | 0.41 | 0.24 | 0.26 | 0.84 | 3.90 | 0.32 | 1.00 | 1.16 | 1.27 |
| **MAUC** | 0.77 | 0.87 | 1.01 | 0.77 | 1.65 | 0.91 | **1.00** | 0.42 | 0.45 | 0.54 | 0.64 | 2.02 | 0.56 | **0.77** | 0.59 | **1.63** |

***values are significant at p≤0.05, MAUC- Mean area under curve for 20h, ×values for 72h control and treatment of animal 5 excluded from statistical analysis.**

**Table 6.5 Acetic acid concentration (mmol/L) in control and fish oil supplemented (treatment) animals.**

| ime | Acetic acid concentration for control at 72h | | | | | | | Acetic acid concentration for treatment at 72h | | | | | | | Paired t test | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean | 1 | 2 | 3 | 4 | 5 | 6 | Mean | LSD 5% | t value |
| 5pm | 74.03 | 84.95 | 46.24 | 55.58 | 99.53 | 77.35 | 72.95 | 53.22 | 81.16 | 52.78 | 53.71 | 28.03 | 72.56 | 56.91 | 30.00 | 1.37 |
| 1am | 138.60 | 124.43 | 107.92 | 81.79 | 121.42 | 98.76 | 112.15 | 89.62 | 107.53 | 86.72 | 90.08 | 42.10 | 111.70 | 87.96 | 36.80 | 1.69 |
| 9am | 112.96 | 113.03 | 103.92 | 72.74 | 103.63 | 96.90 | 100.53 | 80.55 | 72.13 | 55.32 | 102.20 | 33.89 | 85.27 | 71.56 | 36.10 | 2.06 |
| MAUC | 116.05 | 111.71 | 91.49 | 72.98 | 111.49 | 92.94 | 99.45 | 78.26 | 92.08 | 70.38 | 84.02 | 36.53 | 95.31 | 76.10 | 32.30 | 1.86 |
| Time | Acetic acid concentration for control at 15d | | | | | | | Acetic acid concentration for treatment at 15d | | | | | | | Paired t test | |
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean | 1 | 2 | 3 | 4 | 5 | 6 | Mean | LSD 5% | t value |
| 5pm | 66.31 | 67.70 | 50.04 | 49.76 | 68.84 | 77.93 | 63.43 | 60.27 | 69.93 | 42.78 | 45.51 | 54.96 | 61.66 | 55.85 | 7.04 | 2.77* |
| 1am | 94.30 | 91.23 | 84.58 | 104.99 | 85.28 | 121.74 | 97.02 | 109.62 | 111.40 | 118.76 | 62.24 | 75.86 | 86.45 | 94.06 | 32.91 | 0.23 |
| 9am | 97.00 | 115.14 | 57.83 | 102.07 | 80.53 | 112.98 | 94.26 | 92.73 | 88.40 | 78.34 | 46.58 | 70.30 | 82.83 | 76.53 | 27.21 | 1.67 |
| MAUC | 87.98 | 91.33 | 69.26 | 90.45 | 79.98 | 108.59 | 87.93 | 93.06 | 95.28 | 89.66 | 54.14 | 69.24 | 79.35 | 80.12 | 22.90 | 0.88 |
| Time | Acetic acid concentration for control average | | | | | | | Acetic acid concentration for treatment average | | | | | | | Paired t test | |
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean | 1 | 2 | 3 | 4 | 5 | 6 | Mean | LSD 5% | t value |
| 5pm | 70.17 | 76.32 | 48.14 | 52.67 | 84.18 | 77.64 | 68.19 | 56.75 | 75.54 | 47.78 | 49.61 | 41.50 | 67.11 | 56.38 | 16.84 | 1.80 |
| 1am | 116.45 | 107.83 | 96.25 | 93.39 | 103.35 | 110.25 | 104.59 | 99.62 | 109.46 | 102.74 | 76.16 | 58.98 | 99.07 | 91.01 | 18.84 | 1.85 |
| 9am | 104.98 | 114.08 | 80.88 | 87.40 | 92.08 | 104.94 | 97.39 | 86.64 | 80.27 | 66.83 | 74.39 | 52.09 | 84.05 | 74.05 | 11.60 | 5.17* |
| MAUC | 102.01 | 101.52 | 80.38 | 81.72 | 95.74 | 100.77 | 93.69 | 85.66 | 93.68 | 80.02 | 69.08 | 52.89 | 87.33 | 78.11 | 15.20 | 2.63* |

***values are significant at p≤0.05, MAUC- Mean area under curve for 20h.**

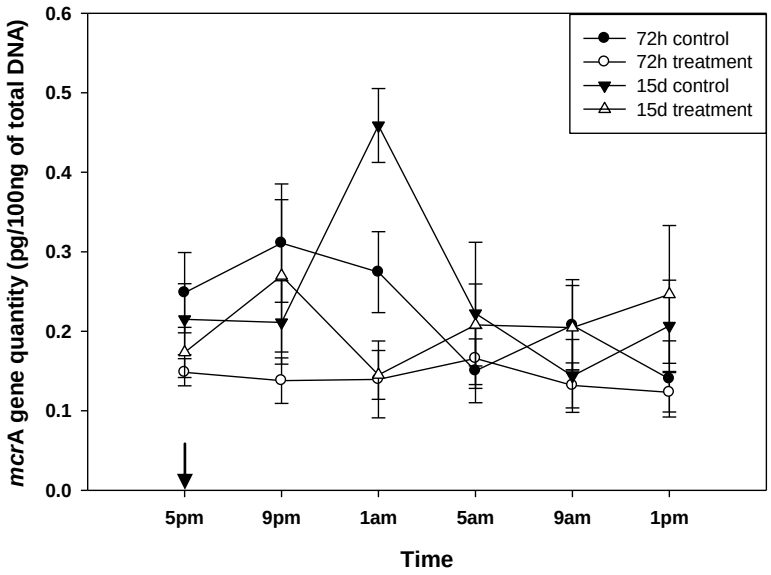



**Figure 6.5 *mcr*A gene quantity in control and fish oil supplemented (treatment) animals after 72h and 15d.**

***Values plotted are average of data in table 6.1, over 20h diurnal period, error bars represent SE, n=6, ˣvalues for 72h control and treatment of animal 5 excluded from statistical analysis. Arrows on the time axis indicate time of pasture break and fish oil supplementation.**

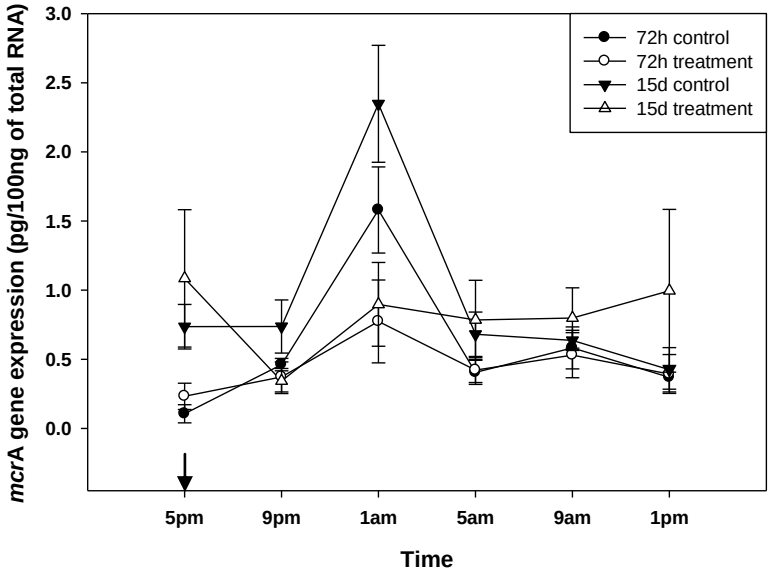



**Figure 6.6 *mcr*A gene expression in control and fish oil supplemented (treatment) animals after 72h and 15d.**

***Values plotted are average of data in table 6.4, over 20h diurnal period, error bars represent SE, n=6, ˣvalues for 72h control and treatment of animal 5 excluded from statistical analysis. Arrows on the time axis indicate time of pasture break and fish oil supplementation.**

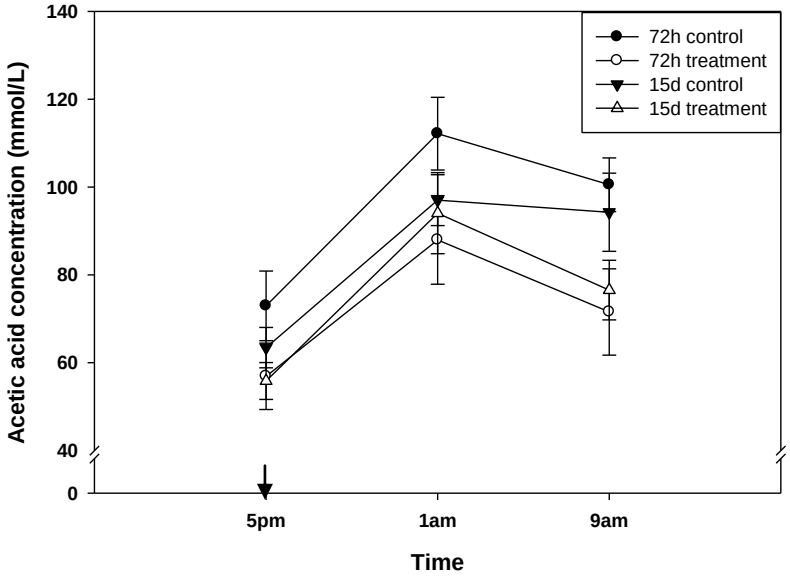



**Figure 6.7 Acetic acid concentration (mmol/L) in control and fish oil supplemented animals.**

***Values plotted are average of data in table 6.5, over 20h diurnal period, error bars represent SE, n=6. Arrows on the time axis indicate time of pasture break and fish oil supplementation.**

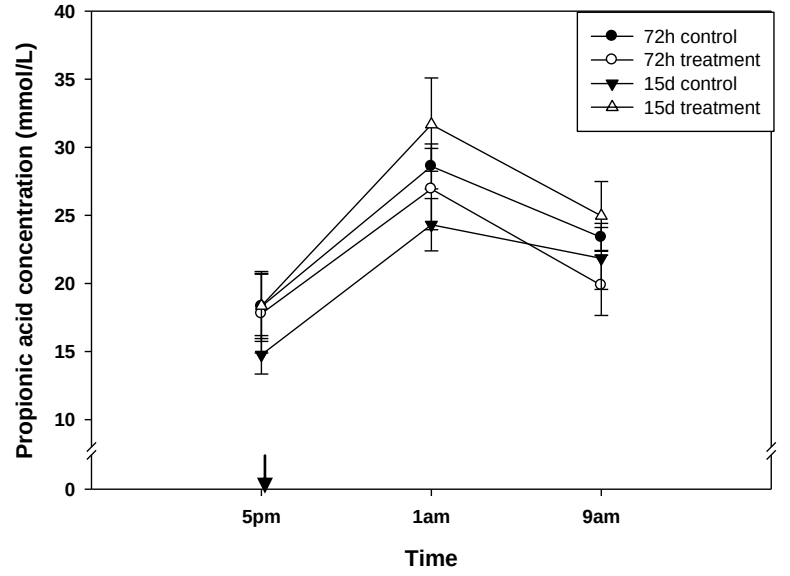



**Figure 6.8 Propionic acid concentration (mmol/L) in control and fish oil supplemented animals.**

***Values plotted are average of data in table B.6 in appendix B, over 20h diurnal period, error bars represent SE, n=6. Arrows on the time axis indicate time of pasture break and fish oil supplementation.**

## 6.3 Discussion

DGGE analysis of both DNA and cDNA samples of control and fish oil supplemented animals did not show any distinct band changes across the treatments (figures 6.1 to 6.4). In general, there were few consistent changes, and considerable inter-animal variation. The observed band changes were matched most closely to *Methanosphaera stadtmanae* and *Methanobrevibacter* sp. Changes in the DNA bands were visible in animal 1 and 2 only, while changes in cDNA bands were visible in animals 1, 2 and 4. However, it was interesting to note that decreased feed intake in animal 5 reduced the number of both DNA and cDNA bands in this animal. This suggests some methanogen species in the rumen may survive under starvation conditions while other fragile or less predominant species may be eliminated or their activity decreased.

But, DGGE was again demonstrated insufficient to deliver conclusive results as to the effect of dietary change (fish oil supplementation) on methanogen community. Given that, long chain polyunsaturated fatty acids which are an essential component of fish oil (appendix B, table B.1.5) have been found to have a direct toxic effect on methanogens (Dong *et al.* 1997; Fievez *et al.* 2003; Prins *et al.* 1972; Zhang *et al.* 2008) and that DGGE has proved successful in an earlier experiment of fish oil supplementation where changes in bacterial community were detected (Huws *et al.* 2010), it was expected that the same technique would be able to show distinct changes in the methanogen community, perhaps even within the diurnal period. But only animals 1, 2 and 4 showed some changes in the methanogen profile and animal 3 and 6 did not show any changes. This also suggests the role of a possible animal to animal variation in methanogen community response to fish oil supplementation. While few species in animal 1, 2 and 4 were affected as to minimise their quantity, the effect in animals 3 and 6 could be more generalised, e.g. fish oil supplementation may not be causing disappearance of specific methanogen species but may have caused an overall decrease in methanogen numbers. However, these shifts in methanogen numbers or their activity could not be analysed from DGGE analysis and therefore qPCR and RT-qPCR were required for this quantification.

The observed qPCR results revealed that fish oil supplementation decreased methanogen populations numerically but not significantly at 72h after fish oil supplementation (table 6.1, figure 6.5). The significant decrease in methanogen quantity (per 100ng of total DNA) occurred only after fish oil supplementation for 15d and maximum difference was observed at 8h (1am) after fresh pasture break was given to animals. An earlier *in vitro* experiment by Fievez *et al*., (2003) had reported a decrease in methanogen quantities after 48h of fish oil

addition, but since under the actual grazing conditions, rumen environment is much more complex and stable (Hino and Russell, 1985), it needed a prolonged supplementation of fish oil for 15d to affect the methanogens in present experiment. Similarly, the significant decrease in *mcr*A gene expression also indicated that fish oil supplementation caused a decrease in methanogen activity (table 6.4, figure 6.6). Though the gene expression started decreasing after 72h of fish oil supplementation, a significant decrease was observed only after 15d of supplementation. This decrease in gene expression was significant at 4h (9pm) and at 8h (1am) after fresh pasture break. This indicates that in the present experiment, fish oil supplementation directly reduced the number of methanogens as well as their activity which is in accordance with earlier studies (Dong *et al.* 1997; Fievez *et al.* 2003; Zhang *et al.* 2008).

The presence of high amount of unsaturated fatty acids in the fish oil (appendix B.5), may also divert the available $H_2$ towards biohydrogenation of the fatty acids decreasing the availability of $H_2$ to methanogens, thereby, decreasing their activity. The findings of Fievez *et al.*, (2003) that an increase in the amount of PUFA increased the suppression of methanogenesis also support this observation.

Within the diurnal period significant changes were observed in both *mcr*A gene quantity and expression in the control animals (figure 6.5 and 6.6). The peak in *mcr*A gene quantity and *mcr*A gene expression occurred at 8h (1am) after animals were given fresh break. This coincided with the peak in VFA and $NH_3$ concentrations in the rumen fluid. A higher concentration of VFA in the rumen fluid indicates a higher rate of fermentation at that time and more availability of $H_2$ (end product of fermentation) to the methanogens thus causing an increase in their quantity and activity. After this peak both the *mcr*A expression and quantity decreased till morning at 9am when a smaller but insignificant increase was again observed in *mcr*A gene expression. The possible reason for this could be that animals spent less time grazing during the night and they would again graze the residual pasture mass in early morning. A parallel experiment on the grazing behaviour of these cows fed once daily had shown that they consumed most of the forage offered within first few hours of feeding and spent the rest of time ruminating and idling (Rugoho, unpublished data). Most of the time during night was spent in idling. In the present experiment, since they were offered fresh break at 5 in the evening, they would graze off fresh grass within the first few hours and hence a peak occurred in the night; spent night time ruminating or idling and graze again in the morning which would explain another smaller peak in *mcr*A gene expression and the VFA concentration in the morning. Minimum *mcr*A gene quantity, *mcr*A gene expression and VFA concentration was found at 5pm i.e. just before feeding or a fresh pasture break. This is

supported by earlier studies where the methane emission pattern matched with the grazing behaviour of sheep (Lockyer 1997). In cattle, Gao *et al*., (2011) measured methane emissions from a herd of feedlot cattle using an inverse dispersion technique in conjunction with open path lasers and reported that methane emissions corresponded with the feeding pattern of cattle.

In the fish oil supplemented animals, though there was an increase in *mcr*A gene quantity and gene expression 4h (9pm) and 8h (1am) after fresh pasture break was given, it was not significant within the diurnal period. Interestingly, the *mcr*A gene expression did not decrease dramatically after 8h (1am) of fresh pasture break as in control animals but it decreased gradually till 16h (9am) when another slight increase was observed and then remained relatively stable till the next break was given. The *mcr*A gene quantity also, did not vary significantly within the diurnal period. Initially, addition of fish oil might have affected the rumen digestibility of organic matter (Wachira *et al.* 2000) by covering the feed particles and thus instead of a sharp increase in methanogen quantity and expression after pasture intake, as was observed in control animals, a relatively low increase in methanogen quantity and expression was observed in fish oil supplemented animals.

It has been reported that fish oil supplementation causes a shift in VFA proportion with an increase in propionic acid at the expense of acetic acid. Though no significant increase in propionic acid was observed in the present experiment, acetic acid concentrations decreased significantly upon fish oil supplementation, indirectly decreasing the acetic acid to propionic acid ratio which has been correlated many times with a decrease in methane production (Johnson and Johnson 1995; Russell 1998). It may be possible that fish oil has selective inhibitory effect on bacteria which produce acetic acid in rumen (Belenguer *et al.* 2010; Kim *et al.* 2008). But, there was no significant change in total VFA production upon fish oil supplementation. These findings are in contrast to a report where fish oil supplementation had been found to decrease the net VFA concentration (Shingfield *et al.* 2010). Yet, they are consistent with earlier studies where acetic acid concentrations were decreased upon fish oil supplementation but no significant effect of fish oil supplementation on total VFA concentration was observed (Fievez *et al.* 2003; Lee *et al.* 2005; Zhang *et al.* 2008).

Thus, it can be summarized that fish oil supplementation was able to decrease methanogen numbers as well as methanogen activity. The inhibition may have been through one or a combination of different mechanisms i.e. decreasing ruminal digestibility through covering feed particles, diversion of $H_2$ for biohydrogenation of the PUFA present in fish oil or, a direct effect on the methanogens (Czerkawski *et al.* 1966; Johnson and Johnson 1995; Soliva

*et al.* 2003). Since, the decrease in *mcr*A gene expression was relatively more than decrease in *mcr*A gene quantity, the effect of fish oil is likely to be more on methanogen activity than the population numbers.

The estimation of *mcr*A gene quantity and *mcr*A gene expression through qPCR and qRT-PCR provided a useful tool for studying the effect of fish oil as a possible methane inhibitor in pasture systems, though further studies to correlate the methane emissions with methanogen numbers and their activity and to prove this effect over a longer period need to be carried out. More importantly, it could be concluded that a diurnal pattern of methanogen activity exists in pasture grazing animals and it is related to the grazing pattern of animals. This is a significant advancement in the methanogenesis studies in pasture systems. A step forward from this would be to develop species specific primers which would help in analysing the effect of fish oil or any other mitigation agent on individual species and their activities, and help in exploration of other possible mitigation options.

# Chapter 7
# General discussion

Dairy cows in the South Island of New Zealand are commonly grazed on high quality pastures each day with minimum or no concentrate. In pasture based systems production is typically limited by the DMI, but high quality pastures encourage high DMI and high milk production. The South Island system of once daily allocation also encourages a grazing pattern where most of the daily DMI is consumed within a few hours and this produces a clear diurnal pattern of rumen fermentation (Gibbs and Laporte 2009). It has been reported that the amount of methane produced per unit of product decreases, with an increase in DMI (Johnson and Johnson 1995). Enteric methane produced from ruminants is of topical interest due to the energy loss it represents to livestock production and the widely held association with climate change.

The rapid growth of the high intensity pasture based dairying has focused interest on the methane production of this system, and possible mitigation strategies given the practical difficulties of diet manipulation with a pasture base. Improved methods that could help achieve these aims by quantification of actual methanogenesis within specific diurnal windows of rumen activity were the subject of this series of experiments.

The techniques currently available to measure methane production are not suitable for determining diurnal fluctuations in rumen methane production from grazing animals (Gao *et al.* 2011; Lockyer 1997; Ulyatt *et al.* 1999; Williams *et al.* 2011; Wright *et al.* 2004). Molecular techniques are attractive as they offer an alternative approach (Firkins 2010; Firkins *et al.* 2008; Zhou *et al.* 2011).

The molecular fingerprinting technique, DGGE, had been reported to be successful in earlier studies (Knight *et al.* 2011; Mohammed *et al.* 2011; Ouwerkerk *et al.* 2008; Zhou *et al.* 2010) to identify changes in the rumen methanogen community under the influence of different diets or supplements. A decrease in the number of bands in DGGE profiles had been detected with chloroform administration, which is a strong chemical inhibitor of methanogenesis (Knight *et al.* 2011), and is known to cause a toxic effect on all microbes. In Chapter 3, DGGE was used to characterise the rumen methanogen community in dairy cows in a typical South Island system, by comparison of unsupplemented controls against diet treatments of known methanogen effect – cereal grain, palm oil, and monensin – in a series of cross-over design

experiments. In Chapter 5, it was also used to study diurnal changes in methanogen community under the influence of different feeding frequencies. Approximately, 22-25 bands were routinely detected, and the DGGE profile was found to be closely similar over all the experiments, suggesting the presence of a similar community in all animals even under different feeding conditions.

Phylogenetic analysis of the DGGE bands revealed that the majority of the bands had closest matching (100%) with *Methanobrevibacter* sp. indicating that this is the most predominant species in rumen which is similar to many studies in cattle and sheep all over world (Ouwerkerk *et al.* 2008; Wright *et al.* 2007; Wright *et al.* 2008; Wright *et al.* 2006; Zhou *et al.* 2010), although bands closely matching *Methanosphaera stadtmanae* were also found. Thus, it is likely that irrespective of production system and geographical location, *Methanobrevibacter* sp. is dominant methanogen species in ruminants all over the world, despite some strains/ species being associated with specific diets in earlier studies (Wright and Klieve 2011). Due to shorter product size in this experiment, some of the bands could not be identified to species level which may have revealed any novel strains/ species in this system. But based on a combination of phylogenetic analysis and reference ladders, as had been done in previous studies (Mohammed *et al.* 2011; Zhou *et al.* 2010), 12 bands were identified and used for analysis of the methanogen community under different times and feeding conditions. Notably, using the same primers and protocol more bands were observed in the rumen samples of this study than of Ouwerkerk *et al*. (2008), which may suggest a more diverse methanogen community in this higher quality and intake forage system.

Between animal variations in response to different supplements was detected. *Methanosphaera stadtmanae* was generally most affected by grain supplementation and different feeding management, along with *Methanobrevibacter*. Since these changes were not consistent in all animals across different experiments, it suggests that animal to animal variation has a significant contribution in response of methanogen community to any modification (Grainger *et al.* 2007).

Overall, relatively minor changes with few clear differences in the DGGE profiles were observed between treatments in this experimental series, which suggests the rumen methanogen community is highly stable. However, the lack of observed differences compared with published reports of such distinct differences could be due to markedly different diets used in the earlier studies, or the sharp variation in the levels of supplementation (low to high concentrate), or the use of strong chemicals to eliminate methanogens (e.g. chloroform) (Knight *et al.* 2011; Mohammed *et al.* 2011; Ouwerkerk *et al.* 2008; Zhou *et al.* 2010).

Alternatively, it is also possible that supplementation given in present study would have caused changes in methanogen community but DGGE may not have been sensitive enough to detect them. DGGE detects major community changes, such as presence or absence of a species, which may not have happened with these supplements in the present pasture system due to very robust rumen environment. It is possible that the effect of these supplements could have been more on methanogen activity rather than the methanogen numbers which could not be detected by DGGE.

Further, when DGGE analysis was conducted for both DNA and RNA/ cDNA samples over diurnal period, it showed decreased intensity in few bands of RNA/ cDNA during periods of very low intake (figures 5.1 to 5.8). It did show that while some species may not constitute a significant proportion of the population, they may still contribute significantly to methane production. But it does not provide highly sensitive and suitably robust qualitative or quantitative insight into comparative methanogen concentrations or activities between two times or between two treatments at a given time or periods of high intake. It was thus concluded that DGGE was not suitable to be used as a technique for determining diurnal changes in rumen methanogenesis in high intensity pasture systems.

The qPCR and qRT-PCR techniques offered a viable alternative for measuring the methanogen community dynamics over the diurnal period. But the applicability of these techniques was initially limited due to the difficulty and cost involved in extracting high quality RNA and DNA from rumen samples (Yu and Morrison 2004). In Chapter 4, a method was developed for this study by adaptation and modification of two earlier methods of nucleic acid extraction (Gambino *et al.* 2008; Whitford *et al.* 1998) which made the analysis of a large number of rumen samples possible in routine experimental practice. This study demonstrated it is possible to extract DNA and RNA simultaneously from a rumen sample, which is both efficient and economical. The quality of RNA obtained after extraction is also higher than other published techniques (Kang *et al.* 2009), and it was shown to be efficient over different diets. It also proved more sensitive in determining *mcr*A gene expression than other methods (Kang *et al.* 2009) which is a crucial requirement for any study on methanogens.

The mcrA gene and its expression has been reported to be suitable (Denman *et al.* 2007; Guo *et al.* 2008; Springer *et al.* 1995; Steinberg and Regan 2009) for quantifying methanogen population and its activity (which can be an indicator of methane production). The use of mcrA targeted primers in qPCR and qRT-PCR for measuring methanogen population numbers and activity *in vitro* and *in vivo* with different diets has been documented earlier

(Denman *et al.* 2007; Guo *et al.* 2008). Therefore, in Chapters 5 and 6, the *mcr*A gene sequence was targeted for use in investigating methanogen quantity and activity in cattle grazing high quality forages, to determine any changes within the diurnal period. This is the first report of such work, and included free grazing cows under typical South Island pasture management.

As discussed earlier, these cattle are routinely given once daily allocations of pasture. This encourages high consumption within the first few hours, and then inactivity until a fresh break is given (Gibbs and Laporte 2009). The rumen microbes are then routinely exposed to a high load of substrates in the first few hours ('feast') followed by a lean period ('famine'). This is also reflected in the diurnal pattern of rumen parameters such as pH, VFA and $NH_3$ production (Wales *et al.* 2004). If the animals are given the same amount of feed spread over a longer period, it is assumed that the dramatic shifts in rumen environment would be decreased (Robles *et al.* 2007) and would have a stabilizing effect on rumen microbial population. Therefore, the qPCR and qRT-PCR quantifications for *mcr*A gene were done over the diurnal period to detect any diurnal pattern in methanogen numbers or activity, and any change in methanogen population or activity over the diurnal period under the influence of a different rumen environment.

A diurnal pattern of methanogen activity was detected in these animals. A peak in *mcr*A gene expression occurred after 8h of morning feeding in both once a day and twice a day fed animals (figure 5.10, table 5.4). Also within the diurnal period, the methanogen number and activity changed significantly in animals fed once a day (tables 5.2 and 5.5) but it did not change significantly in animals fed twice a day (tables 5.3 and 5.6). It was concluded that animals fed once a day were exposed to more dramatic shifts in rumen environment which caused marked changes in their activity and numbers. However, feeding the animals twice a day did not significantly decrease the methanogen population numbers but had a significant effect on their activity. It may be because when animals were fed twice a day, the rumen pH went below 6 for a comparatively longer period (parallel unpublished data) causing a decrease in methanogen activity (Kessel and Russell 1997a). Another possible explanation for this could be that availability of substrates for a longer period results in prolonged production of $H_2$. This can cause a comparative increase in the partial pressure of $H_2$ (Lee *et al.* 2003) at which other bacteria such as reductive acetogens can also proliferate and compete with methanogens for available $H_2$ (Cottle *et al.* 2011). Thus, methanogen activity can be affected for the same amount of DMI.

Further, the gap between morning and evening feedings was 7h instead of a 12h difference and thus when all animals were exposed to prolonged periods of no feeding in early morning, methanogen activity was lowest in the samples collected 4h and just before morning feeding. This supports earlier reports that methanogens' activity is very sensitive to starvation (Kessel and Russell 1997a; b; Khafipour *et al.* 2009). Under unfavourable conditions, methanogen number is not affected, only their activity is decreased which again resumes upon favourable conditions (Kessel and Russell 1997a; b). It could then be inferred that the effect of different feeding frequencies or the presence of diurnal pattern in methanogens is more a function of their activity than their numbers.

This technique thus proved competent in establishing a diurnal pattern of methanogen activity. In Chapter 6 the efficacy of this method was then tested in actual grazing conditions over the diurnal period, an area in which there are no existing studies. Since a diurnal pattern in ruminal parameters existed under grazing conditions in this typical production system (Gibbs and Laporte 2009), it was hypothesized that a similar pattern would exist in methanogen community also. To make a comparison, fish oil was used a treatment because the fish oil had been repeatedly demonstrated to be effective in decreasing methane production, methanogen numbers and their activity in previous *in vitro* and *in vivo* experiments (Fievez *et al.* 2003; Huws *et al.* 2010; Prins *et al.* 1972; Zhang *et al.* 2008), though typically using concentrate based diets.

A diurnal pattern was detected in grazing animals also (figures 6.5 and 6.6, tables 6.1 to 6.4). The highest methanogen numbers and activity was detected 8h after fresh break was given (5pm) to animals, and in fish oil supplemented animals where rumen methanogenesis was likely to be dramatically reduced, this increase was correspondingly not significant. Another small insignificant peak was observed at 9am which was after the morning grazing by animals. The methanogen activity corresponded with the grazing pattern (Rugoho, unpublished data) of animals. This matched with earlier studies where a relation between methane production from a herd and its feeding pattern had been reported using inverse dispersion technique and a tunnel method (Gao *et al.* 2011; Lockyer 1997).

The methanogen numbers didn't show any significant change over the diurnal period in fish oil supplemented animals and their activity was also low but stable after 9am. This upheld the use of this treatment as an effective comparison to unsupplemented controls for determining diurnal rumen methanogen patterns. It is also possible fish oil supplementation decreased or delayed the rumen digestion of feed particles or caused a shift in the site of digestion towards intestine. But this is not supported by the limited effects of fish oil supplementation on

production parameters such as VFA concentrations that were observed. Acetic acid concentration did decrease in fish oil supplemented animals (figure 6.7, table 6.5) along with a numerical increase in propionic acid (figure 6.8, appendix B table B.6). The production of acetic acid has been reported to cause loss of more $H_2$ to methane and formation of propionic acid to be more beneficial in terms of mitigating methane (Lana *et al.* 1998; Russell 1998). Though the ratio of acetic acid to propionic acid decreased with fish oil, no significant effect on total VFA production of animal could be found in animals with or without fish oil supplementation. It is thus possible that the fish oil may have affected methanogen numbers and activity through a multipronged effect; decreasing the digestibility of feed particles by engulfing them, diverting the $H_2$ away from methanogens by biohydrogenation of PUFA present in fish oil and a direct toxic effect on the methanogens (Czerkawski *et al.* 1966; Johnson and Johnson 1995; Soliva *et al.* 2003). The possible use of fish oil as a mitigating agent in pasture systems thus needs to be explored further in conjunction with further digestibility and production studies.

To conclude, the estimation of *mcr*A quantity and expression through qPCR and qRT-PCR is a reliable and feasible indicator of methanogen community behaviour, and was able to detect the influence of an external ruminal modification. It would appear this technique can be successfully applied in the pastoral systems to gain significant new understandings of the methanogen community. However, there is a need to correlate the changes in *mcr*A gene quantity and expression with the actual methane production to validate the use of this technique in measuring methanogenesis. Though some studies to this nature have been done earlier, but the methods used for RNA extraction were different and it has been shown earlier (Chapter 4) that as compared to other methods, the modified method developed for RNA extraction in this study is more sensitive to detect changes in *mcr*A gene expression over different samples and diets. Therefore, using this RNA extraction method, a higher degree of sensitivity would be achieved in establishing correlation between *mcr*A gene expression and actual methane production and thus this method may serve as a metabolic proxy for methane production at any given time.

The clear diurnal pattern of rumen methanogen activity demonstrated in this work is the first report of its kind in grazing studies. This opens up new avenues for methane mitigation strategies involving some focal managemental changes which may include the use of specific diet supplements at particular times of day to alter total daily methane production, rather than the use of strategies that seek to universally reduce either methanogens or methanogenesis.

A next step ahead would be development of species- specific primers for performing inter-species analysis over the diurnal period or under the influence of any rumen modification. This will help in analysing inter-species interactions for possible development of more effective mitigation options. Pyrosequencing, the modern approach to large scale sequencing of multiple DNA strands (Ahmadian *et al.* 2000; Huse *et al.* 2007) also offers a novel approach to study the whole microbial community within a given sample and using specific markers, multiple samples can be studied at the same time. It is currently still an expensive technique but with advances in technology, its usage is becoming more cost effective and error proof. In a complex rumen environment, it has the promise of combining diversity analysis with quantitative estimates (Brulc *et al.* 2010; Callaway *et al.* 2010; de Menezes *et al.* 2011). It may even be possible to apply this technique for mRNA analysis and gain information about specific microbial activity or the relative activities of different species under specific rumen conditions. It is hoped that this technique will provide important insights into inter-species relationships (Wright and Klieve 2011) within the complex rumen environment in the near future.

# Appendix A
# Protocols

## A.1 DNA extraction protocol

***Protocol***

- To 0.5g of sample add 700µl of TE buffer and vortex them to mix the contents.
- Transfer to a bead beating tube containing 0.5g of 0.1mm sterile zirconia/silica beads (catalog no. 11079101z, Daintree Scientific, 20 Kiama Pde., St. Helens, Tasmania 7216) and add 20µl 20% SDS and 700µl of buffered phenol (pH of phenol phase 6.7±0.2).
- Bead beat the samples in a Mini Beadbeater$^{TM}$ (Biospec products) at maximum speed for 3 cycles of 2min bead beating and 2min on ice.
- Centrifuge for 5min at 6000rpm. Transfer the supernatant to a fresh, sterile microtube (MCT-175-C, 1.7ml ultra clear tubes, Axygen, INC. California 94587, USA) and add 700µl of chloroform (Merck, KGaA, 64271, Darmstadt, Germany). Mix the samples by inversion.
- Centrifuge for 10min at 10,000rpm. Transfer the supernatant into fresh microtube, taking care not to disrupt the interphasic protein layer, and add 20µl of 10mg/ml RNase enzyme (REF 10109134001, Roche Diagnostics GmbH, Mannheim, Germany).
- Incubate the tubes in a 39°C waterbath for 1 hour and then add an equal volume of phenol (700µl). Mix the samples by inversion and centrifuge at 10,000rpm for 10min.
- To the supernatant add an equal volume of phenol: chloroform (350: 350µl), mix by inversion for 5min and centrifuge at 13,500 rpm for 10min.
- Add 700µl of chloroform to the supernatant transferred in a fresh tube and centrifuge at 13,200rpm for 10min.
- In order to precipitate the DNA, add 0.25X volume of 3M sodium acetate and 2.5X volume of absolute ethanol, where X is the volume of supernatant containing DNA. Keep under -20◦C for a minimum of 1 hour.
- Centrifuge at 13,200rpm for 10min to pellet the DNA. Remove the supernatant.
- Wash with 1ml of 70% alcohol and centrifuge at 13,200rpm for 10min. Remove the supernatant.
- The precipitated DNA is then dried in a vacuum centrifuge at 60°C and is resuspended in sterile distilled water and stored at -20°C till further analysis.

## A.2 Gel electrophoresis

***Reagents required***

- Agarose (DNase/RNase free)
- 10X TBE buffer distilled water
- 1 Kb/ 100bp ladder
- Ethidium Bromide

**Table A.1 Composition of 10X TBE buffer.**

| Component | Mass/ Volume per 1 Litre |
|---|---|
| Tris Base | 108g |
| Boric Acid | 55g |
| 0.5 M EDTA (pH 8.0) | 20ml |
| Distilled water | Make up volume to 1 Litre |

Autoclave at 121◦C.

***Protocol***

- Make solution in a 500ml Schott bottle depending upon the concentration of agar required e.g. to make 100ml of 1% agarose gel, take 1g of agarose, 10ml of 10X TBE buffer and 80ml of distilled water and for 100ml of 2% agarose gel, take 2g of Agarose, 10ml of 10X TBE buffer and 80ml of distilled water. Label the bottle with date and concentration of the gel.
- Melt the gel by microwaving for short intervals with the cap loose until the agarose is fully dissolved and the gel has attained a clear consistency. Add 2µl/ 100ml of ethidium bromide to the prepared gel.
- Pour the gel into a container first for cooling the gel.
- Meanwhile seal the sides of the gel setting tray with a masking tape.
- Pour the gel from the container into the gel tray and insert combs inside the tray. Let the gel set for about 20min.
- After setting, take out the combs from inside the gel carefully without damaging the wells. Remove sealing tape from the sides of the gel and put the tray into the electrophoresis tank containing 1X TBE running buffer.
- Load 5µl of the standard, 1Kb ladder for genomic DNA or 100bp ladder for PCR products.

- On a parafilm take out 2µl of gel loading buffer. Pipette out 5µl of sample and mix with the loading buffer. Load this sample into the well in the gel. Repeat this for all the samples.
- After loading all the samples run the gel on 95V for 30min or 45min depending upon the size of sample.
- Examine the gel under UV light in Gel Doc XR$^+$ System (catalogue no. 170-8195, Bio-Rad).

## A.3 PCR protocol

***Reaction setup for archaeal 16S rDNA specific PCR***

| Component | Volume in 50µl | Final concentration |
|---|---|---|
| sd$H_2O$ | 31.8µl | |
| 10x buffer | 5.0µl | |
| dNTP mix | 8.0µl | 0.2mM of each dNTP |
| $MgCl_2$ | 2.0µl | 2.5mM including buffer concentration |
| 46F (10 pmol/µL) | 1.0µl | 0.2µM |
| 1017R (10 pmol/µL) | 1.0µl | 0.2µM |
| Taq polymerase | 0.2µl | 1U |
| Template gDNA/cDNA | 1.0µl | 16-20ng/µl |
| Total volume | 50.0µl | |

***Reaction setup for archaeal V2V3 PCR***

| Component | Volume in 50µl | Final concentration |
|---|---|---|
| sd$H_2O$ | 31.8µl | |
| 10x buffer | 5.0µl | |
| dNTP mix | 8.0µl | 0.2mM of each dNTP |
| $MgCl_2$ | 2.0µl | 2.5mM including buffer concentration |
| 344F-GC (10 pmol/µL) | 1.0µl | 0.2µM |
| 522R (10 pmol/µL) | 1.0µl | 0.2µM |
| Taq polymerase | 0.2µl | 1U |
| Product of first PCR | 1.0µl | |
| Total volume | 50.0µl | |

- 10pmol/µl concentration of primers was prepared by doing 1:10 dilution of 100pmol/µl stock solution of primers.
- dNTP mix was prepared by taking 5µl of each dNTP and adding it to 380µl of sterile distilled water.

## A.4 DGGE protocol

**Table A.2 Composition of denaturing solutions.**

| Component (volume/mass per 120ml) | 30% Denaturing Solution | 60% Denaturing Solution |
|---|---|---|
| 40% Acrylamide | 24ml | 24ml |
| 20X TAE Buffer | 3ml | 3ml |
| Formamide | 14.4ml | 28.8ml |
| Urea | 15.12g | 30.24g |

**Table A. 3 Compositon of 8% Acrylamide seal.**

| Composition | Volume in mixture |
|---|---|
| 40% Acrylamide/Bis | 5ml |
| 20X TAE | 625µl |
| Distilled Water | 19.375ml |

**Table A. 4 Composition of 20X TAE Buffer.**

| Component | Mass/volume per litre | Final concentration |
|---|---|---|
| Tris base | 96.8g | 800mM |
| Glacial Acetic Acid | 22.84ml | |
| 0.5M EDTA pH 8.0 | 40ml | 20mM |
| Sterile Distilled Water | Make up to 1 Litre | |

### A.4.1 Protocol for silver staining

Solutions required:

- Silver stain buffer A (10% Ethanol; 0.5% Acetic Acid)
- Silver stain buffer B (0.1% $AgNO_3$)
- Silver stain buffer C (1.5% NaOH; 0.01% $NaBH_3$; 0.015% formaldehyde). Has to be prepared fresh.
- Silver stain buffer D (0.75% $Na_2CO_3$)

**Table A.5 Composition of silver staining buffer A.**

| Component | Volume in 1 Litre |
|---|---|
| Absolute ethanol | 100ml |
| Acetic Acid (>80%) | 5ml |
| Sterile distilled water | 895ml |

**Table A.6 Composition of silver staining buffer B.**

| Component | Volume/mass in 1 Litre |
|---|---|
| Silver nitrate | 1.0g |
| Sterile distilled water | 1000 ml |

**Table A.7 Composition of silver staining buffer C.**

| Component | Volume/mass in 1 Litre |
|---|---|
| Sodium hydroxide | 15.0g |
| Formaldehyde (40% in water) | 3.7ml |
| Sodium borohydrate | 0.1g |
| Sterile distilled water | make up volume to 1 Litre |

**Table A. 8 Composition of silver staining buffer D.**

| Component | Volume/mass in 1 Litre |
|---|---|
| Sodium carbonate | 7.5g |
| Sterile distilled water | 1000ml |

***Staining method:***

This protocol is optimised for sliver staining of DNA and RNA in polyacrylamide DGGE gels. For all incubations, gently agitate the gel while submerged in approximately 300ml of the appropriate solution. Handle only the gel support film. Trim the PAG film, place the gel into the glass tray with the gel support film oriented against the bottom of the tray.

- Cover the gel with silver stain buffer A and incubate for 5min. Discard the buffer.
- Repeat steps 2 and 3 twice.
- Incubate in silver stain buffer B for 10min (Keep buffer B as it may be used for staining 5 – 10 gels).
- Wash the gel twice with distilled water for 10sec.
- Incubate the gel in silver stain buffer C for 10-20min or until it stains and the bands are visible but with no background. Discard the buffer.
- Incubate the gel in silver stain buffer D for 5-10min. Discard the buffer.

## A.5 Gel electrophoresis for isolating PCR product

- Load 45µl of PCR product in a 1% gel and run it at 100V for 45min. Examine the gel under UV light and carefully excised the band ensuring minimum amount of extra gel.
- Then followed with DNA extraction from the gel using gel extraction kit from Axygen (Catalogue no. AP-GX-250, Axygen, Raylab, Auckland, New Zealand). 5µl of the extracted product was again run on 1% agarose to check the yield.

## A.6 Cloning reaction and transformation

Cloning reaction and transformation were carried out using TOPO® TA Cloning® kit by Invitrogen (Catalogue no.K4510-20, Auckland, New Zealand ) through chemical transformation using pCR® 2.1-TOPO® vectors (Invitrogen, Auckland, New Zealand).

### A.6.1 Materials required

- Water bath or heating block set at 42◦C.
- SOC medium warmed at room temperature (available with the cloning kit).
- 40 mg/ml X-gal in dimethylformamide (DMF).

- 37°C shaking and non-shaking incubator.
- LB + ampicillin plates prewarmed at 37◦C.

**Table A.9 Composition of LB broth and agar.**

| **Component** | **Mass/volume per 1 Litre** |
|---|---|
| Tryptone | 10.0g |
| Yeast extract | 5.0g |
| Sodium chloride | 10.0g |
| Agar ( if required) | 15.0g |
| **After Autoclaving, add,** | |
| Ampicillin solution (100 µg/ml) | 1ml (@1µl/1ml) |

### A.6.2 Protocol

The cloning reaction was set up as:

| | |
|---|---|
| Fresh PCR product | 4µl |
| Salt solution | 1µl |
| TOPO vector® | 1µl |

i. Briefly centrifuge to combine all the components and incubate at room temperature for 15min.
ii. Remove one tube of One Shot TOP$^{TM}$ 10 *E. Coli* competent cells (Invitrogen) per sample from -80°C freezer and thaw on ice.
iii. Add 2µl of the above cloning reaction to the tube of competent cells and gently tap the sides of tube to mix the contents.
iv. Incubate on ice for 20min.
v. Heat shock at 42°C for 30sec and then place on ice.
vi. Add 250µl of room temperature SOC medium to the tube and shake horizontally at 37°C, 200rpm for 1 hour.
vii. Prewarm two LB + ampicillin agar plates and spread 40µl of 40mg/ml X-gal on the plates. Let them dry.

viii. Make dilutions of the ligation mix e.g. in one microtube add 50µl of ligation mix and 200µl of room temperature SOC medium and in the other tube add a different volume of ligation mix and make the volume to 250µl using SOC medium.

ix. In the laminar flow spread the ligation mix on prewarmed LB + ampicillin plates and incubate at 37°C overnight.

x. After the colonies have grown pick up white colonies using sterilized tooth picks or pipette tips and transfer to culture tubes containing 3ml of LB media and 3µl of ampicillin (100mg/ml).

xi. Shake at 37°C, 250rpm for 16h.

xii. Then follow with plasmid extraction.

### A.6.3 E. coli Plasmid Mini-preps

***Reagents required***

- Alkaline lysis solution I (250ml)

| | |
|---|---|
| 50mM | Glucose (2.25g) |
| 25mM | Tris-Cl pH 8.0 (6.25ml from 1M) |
| 10mM | EDTA pH 8.0 (5ml from 0.5M) |

Add sterilised distilled water to make up the volume to 250ml and autoclave.

- Alkaline lysis solution II (5ml). Prepared fresh.

| | |
|---|---|
| 0.2N | NaOH (0.1ml from 10N) |
| 1% | SDS (0.5ml from 10%) |
| 4.4ml | Sterile water |

- Alkaline lysis solution III (100ml)

| | |
|---|---|
| 60ml | 5M Potassium Acetate |
| 11.5ml | Glacial acetic acid |
| 28.5ml | Sterile water |

Store at 4°C.

Keep solutions I and III on ice while using.

***Protocol***

Place alkaline lysis solutions I and III on ice and set the microcentrifuge at 4°C.

i. Transfer all culture to a 1.5ml microtube and spin for 1min at maximum speed. Discard supernatant.

ii. Add 250µl of solution I and vortex to resuspend cells.

iii. Add 250µl of solution II and invert gently to mix, solution should clear slightly.

iv. Add 350µl of solution III and invert gently to mix completely.

v. Spin in centrifuge at maximum speed for 10min and then transfer supernatant to fresh tube without transferring the white precipitate.

vi. Add 750µl (equal volume) of isopropanol and mix well. Spin at maximum speed for 10min at 4°C. Remove supernatant.

vii. Wash pellet with 500µl of 75% ethanol and centrifuge at maximum speed for 2min.

viii. Remove supernatant and let air dry for 15min.

ix. Resuspend pellet in 50µl of TE buffer containing RNase A (5µl per ml).

### A.6.4 Restriction enzyme digest

Restriction enzyme digest was done to separate the inserts from rest of plasmid. Enzyme Eco R1 (BioLabs, R0101S, 20,000 U/ml) was used for performing the reaction.

***Reaction set up***

| | |
|---|---|
| Eco R1 enzyme | 1µl |
| Buffer 10X | 2µl |
| Mini prep (plasmid) | 5µl |
| Sterile distilled water | 12µl |
| | 20µl |

i. Incubate at 37°C for 2-3h.

ii. Run on 2% agarose gel at 100V for 40min along with the ladder and the original PCR product. In the gel there will be two bands, one corresponding to the PCR product and the other for the plasmid residue.

### A.6.5 Sequencing

The samples (plasmid mini-preps) having the right inserts as checked on 2% agarose gel were subjected to purification using AXYPREP™ PCR cleanup kit (AXYGEN, catalogue no. AP-PCR-50). Quantify the DNA with Nanodrop™ 1000 spectrophotometer .The samples were then submitted for sequencing where they were prepared for sequencing using ABI Big Dye Terminator v3.1 cycle sequencing reactions with M13 forward primer.

## A.7 Parallel extraction of RNA and DNA

### A.7.1 Reagents/Materials required

- Sterile bead beating tubes (2ml screw cap tubes with o-ring), each containing 0.5g of 0.1mm zirconia/silica beads.
- 10% SDS (sodium dodecyl sulphate) solution
- TE buffer pH 8.0 constituted in DPEC treated water (10mM Tris-HCl, pH 8.0; 1mM EDTA)

**Table A.10 Composition of TE buffer.**

| Component | Mass/volume per 1 Litre |
|---|---|
| 10mM Tris-HCl (pH 8.0) | 1.21g |
| 1mM EDTA | 0.37g |
| Distilled water | Make up volume to 1Litre |

Autoclave at 121◦C.

- Phenol (pH 6.7±0.2)
- Chloroform
- 3M sodium acetate, pH5.2
- 5M lithium chloride (final concentration 3M in solution)
- 70% ethanol solution
- Absolute ethanol
- 1.7ml microtubes

### A.7.2 Protocol

i. Thaw 0.5g of Whole Rumen Contents (WRC) or 200µl of rumen fluid (RF) sample (contained in microtube) over ice.
ii. Add 500µl of TE buffer and mix by vortexing. Add to the corresponding labelled bead beating tube.
iii. Add 10µl of 20% SDS to each of bead beating tube.
iv. In the fume hood add 500µl of Phenol.
v. Place tubes in the bead beater and beat for 2min.
vi. Remove tubes from bead beater and place on ice for 2min.
vii. Repeat steps ‘v’ and ‘vi’ twice (total of three repetitions).

viii. Centrifuge tubes in a refrigerated microcentrifuge (4°C) at 6000rpm for 5min. Remove upper aqueous phase into a fresh tube and discard the interphase and lower phase.

ix. Add 200µl of TE buffer and 700µl of chloroform and mix well by inversion. Centrifuge at 10,000rpm, 4°C for 10min. Remove upper aqueous phase into a fresh tube and discard the interphase and lower phase.

x. Add 700µl of phenol and mix well by inversion. Centrifuge at 10,000rpm, 4°C for 10min. Remove upper aqueous phase into a fresh tube and discard the interphase and lower phase.

xi. Add an equal volume of phenol: chloroform (350µl: 350µl) solution to the tube and mix by inversion. Centrifuge at 13,200rpm, 4°C for 10min. Remove upper aqueous phase into a fresh tube and discard the interphase and lower phase.

xii. Add 700µl of chloroform and mix by inversion. Centrifuge at 13,200rpm, 4°C for 10min. Remove upper aqueous phase into a fresh tube and discard the interphase and lower phase.

xiii. Measure the volume of aqueous phase (supernatant) and divide into two tubes. Add 5M lithium chloride 2.5 times the volume in tube and mix by inversion.

xiv. Keep on ice for 30min.

xv. Centrifuge at 13,200rpm, 4°C for 30min.

xvi. Remove the supernatant into 15ml tube for DNA precipitation and add equal volume of isopropanol. Keep in -20°C freezer for at least 1h.

xvii. Resuspend the pellet from step 'xv' in 100µl of TE buffer. Pool the volumes from both tubes (total of 200µl).

xviii. Mix by vortexing. Add 20µl of 3M sodium acetate (0.1 times the volume) and 500µl of absolute ethanol (2.5 times the volume) which has been stored at -20°C. Mix well and keep in -20°C freezer for at least 1h.

xix. Centrifuge at 13,200rpm for 25min. Drain supernatant.

xx. Wash the pellet in 1ml of 70% ethanol. Centrifuge at 13,200rpm for 15min.

xxi. Drain supernatant and air dry tubes for 15min. Resuspend the pellet in 100µl of TE buffer (pH 7.2).

xxii. Check the quality of the RNA samples in denaturing gel electrophoresis and quantify by Qubit Fluorometer or Nanodrop.

**Parallel DNA extraction**

i. After the supernatant has been precipitated with equal volume of Isopropanol for 1 hour at -20°C (carried on from step xvi) centrifuge at 3000g for 30min.

ii. Remove the supernatant and resuspend the pellet in 500µl of TE buffer. Do not vortex but resuspend gently by inversion.

iii. Centrifuge the 1.5ml Phase lock gel (Light)$^{TM}$ suspension tubes (Eppendorf, Auckland, New Zealand) at 14000g (rcf) for 30sec.

iv. Transfer the suspended DNA to 1.5ml Phase lock gel suspension tube and add Phenol: Chloroform:: 250 µl : 250µl to the tube. Mix thoroughly by inversion.

v. Centrifuge at 14000g for 5min.

vi. Transfer the supernatant to a fresh Phase lock gel suspension tube and add 500µl (equal volume) of chloroform. Centrifuge at maximum speed (14000g) for 5min.

vii. Transfer the supernatant to a fresh 2ml tube and add 1/10 volume of sodium acetate (50µl) and 2.5 volume (1.5ml) of absolute ethanol. Incubate at -20°C for at least 1h.

viii. Centrifuge at 13200rpm for 20min.

ix. Wash the pellet with 1 ml of 70% ethanol. Centrifuge at 13200 rpm for 10 minutes. Remove the supernatant.

x. Resuspend the pellet in 500 µl of TE buffer and store at -20°C till further analysis.

xi. Check the quality of DNA on 1% agarose gel.

## A.8 Denaturing gel electrophoresis for RNA

### A.8.1 Reagents required

- **10X MOPS buffer** (500ml)

| | |
|---|---|
| MOPS (pH7.0) | 0.2M (20.9g) |

Dissolve MOPS first and adjust pH to 7.0.

| | |
|---|---|
| 3M Sodium acetate | 20mM (3.33ml) |
| 0.5M EDTA (pH 8.0) | 10mM (10ml) |

Make volume to 500ml. Autoclave at 121°C.

- **Formamide Loading Dye**

| | |
|---|---|
| Formamide | 8ml |
| Sterile distilled water | 1.8ml |
| 0.5M EDTA (pH 8.0) | 0.2ml |
| Xylene cyanol FF | 10mg |
| Bromophenol blue | 10mg |

- **Denaturing loading buffer**

| | |
|---|---|
| Formaldehyde | 150µl |
| Formamide | 500µl |
| 10X MOPS | 500µl |
| Ethidium bromide | 2µl |

- **Agarose gel**

| | |
|---|---|
| DNA/ RNA grade agar | 0.75g |
| 10X MOPS | 5ml |
| Sterile distilled water | 37.5ml |

Melt Agarose and then add,

| | |
|---|---|
| Formaldehyde | 7.5ml |

- **Running buffer**

1X MOPS buffer (dilute from 10X MOPS)

### A.8.2 Protocol

i. Spray the electrophoresis apparatus with RNase Zap and set the heating block to 65°C.
ii. Make up the Agarose gel and pour in a fume hood. Leave it to set for about 20min.
iii. Make up the Denaturing Loading buffer.
iv. Aliquot 15µl of denaturing loading buffer into the required number of microtubes (1 per sample).
v. Add 5µl of sample to the tubes containing 15µl of denaturing loading buffer and heat the samples at 65°C for 10min.
vi. Immediately place on ice to snap chill for at least 1min. Then while keeping on ice add 1µl of Formamide loading dye to each sample.
vii. Place the gel in the gel tank and completely cover with 1X MOPS loading buffer.
viii. Load the samples into the gel and run at 85V for 1 hour.
ix. Examine under UV light in the Gel Doc System.

### A.8.3 DNA Digestion

DNA digestion was done to remove any residual DNA with Turbo DNA-free™ ( Ambion).

#### *Protocol*

The standard protocol as described in the kit was followed and the reagents supplied with the kit were used.

i. Add 45µl of RNA sample, 1µl of Turbo DNA-free and 4.5µl of 10X Turbo DNase Buffer in a microtube and mix gently.
ii. Incubate at 37°C for 30min.
iii. Add 5µl of resuspended DNase Inactivation reagent (supplied with kit) and mix well.
iv. Incubate at room temperature for 2min while mixing occasionally.
v. Centrifuge at 10,000g for 1.5min and transfer RNA to a fresh tube without touching the white precipitate at the bottom.
vi. Resuspend the RNA in 450µl of TE buffer to make up the volume to 500µl.
vii. Centrifuge the 1.5ml Phase lock gel (Light)™ suspension tubes (Eppendorf, Auckland, New Zealand) at 14000g for 30sec.
viii. Transfer the RNA to 1.5ml Phase lock gel suspension tube and add Phenol: Chloroform :: 250µl : 250µl to the tube. Mix thoroughly by inversion.
ix. Centrifuge at 14000g for 5min.
x. Transfer the supernatant to a fresh Phase lock gel suspension tube and add 500µl (equal volume) of Chloroform. Centrifuge at maximum speed (14000g) for 5min.
xi. Transfer the supernatant to a fresh 2ml tube and add 1/10 volume of Sodium Acetate (50µl) and 2.5 volume (1.5ml) of absolute ethanol. Incubate at -20°C for at least 1 hour.
xii. Centrifuge at 13200rpm for 20min.
xiii. Wash the pellet with 1ml of 70% ethanol. Centrifuge at 13200rpm for 10min. Remove the supernatant.
xiv. Resuspend the pellet in 500µl of TE buffer and store at -80°C till further analysis.
xv. The integrity and quantity of RNA was again checked using Denaturing RNA Gel Electrophoresis and Qubit Fluorometer (Invitrogen, Auckland, New Zealand) or Nanodrop (NanoDrop Technologies, Thermo Fisher Scientific, Auckland, New Zealand).

## A.9 PCR amplification of cDNA/ DNA samples using Methyl Coenzyme Reductase (*mcr*A) gene specific primers

### A.9.1 Reaction setup

| Component | Volume in 50µl | Final concentration |
|---|---|---|
| Sterile distilled water | 31.8µl | |
| 10X Buffer | 5µl | |
| dNTP mix | 8µl | 0.2mM |
| $MgCl_2$ | 2µl | 2.5mM including buffer concentration |
| *mcr*-F | 1µl | 0.2µM |
| *mcr*-R | 1µl | 0.2µM |
| Taq Polymearse | 0.2µl | 1U |
| cDNA/DNA template | 1µl | |
| | 50µl | |

### A.9.2 Program Parameters

| | | |
|---|---|---|
| Initial denaturation at 95°C | 30sec | x 1 cycle |
| Denaturation at 95°C | 5sec | 40 cycles |
| Annealing at 60°C | 10sec | |
| Extension at 72°C | 30sec | |
| Final extension at 72°C | 3min | x 1 cycle |
| 10°C | Hold | |

Run the samples on 2% agarose gel with a 100bp standard to check the size of amplicon.

# Appendix B

# Tables

## B.1 Tables for fish oil supplementation experiment

**Table B. 1 Paired t-test on raw differences between times for average (of 72h and 15d) *mcr*A gene expression (cDNA) of control animals.**

| Time (Time1-time 2) | 1 | 2 | 3 | 4 | 5× | 6 | Mean | LSD 5% | t value |
|---|---|---|---|---|---|---|---|---|---|
| 9pm-5pm | 0.01 | 0.22 | 0.27 | 0.19 | 0.18 | 0.11 | 0.16 | 0.10 | 4.33* |
| 1am-5pm | 2.13 | 0.82 | 0.93 | 1.79 | 3.72 | 1.00 | 1.73 | 1.16 | 3.83* |
| 5am-5pm | 0.24 | -0.06 | -0.28 | 0.27 | 0.87 | -0.02 | 0.17 | 0.42 | 1.04 |
| 9am-5pm | 0.22 | 0.34 | -0.01 | 0.15 | 0.22 | 0.08 | 0.17 | 0.13 | 3.33* |
| 1pm-5pm | -0.01 | -0.29 | -0.25 | 0.19 | 0.57 | -0.21 | 0.00 | 0.35 | 0.01 |
| 1am-9pm | 2.12 | 0.60 | 0.66 | 1.60 | 3.54 | 0.89 | 1.57 | 1.19 | 3.39* |
| 5am-9pm | 0.23 | -0.28 | -0.55 | 0.08 | 0.68 | -0.13 | 0.01 | 0.45 | 0.03 |
| 9am-9pm | 0.21 | 0.12 | -0.29 | -0.04 | 0.04 | -0.03 | 0.00 | 0.18 | 0.04 |
| 1pm-9pm | -0.02 | -0.50 | -0.53 | 0.00 | 0.38 | -0.31 | -0.16 | 0.37 | 1.13 |
| 5am-1am | -1.89 | -0.88 | -1.21 | -1.52 | -2.85 | -1.02 | -1.56 | 0.77 | 5.24* |
| 9am-1am | -1.91 | -0.47 | -0.94 | -1.64 | -3.50 | -0.92 | -1.56 | 1.14 | 3.53* |
| 1pm-1am | -2.13 | -1.10 | -1.18 | -1.60 | -3.15 | -1.20 | -1.73 | 0.84 | 5.31* |
| 9am-5am | -0.02 | 0.40 | 0.27 | -0.12 | -0.65 | 0.10 | 0.00 | 0.39 | 0.02 |
| 1pm-5am | -0.24 | -0.23 | 0.03 | -0.08 | -0.30 | -0.18 | -0.17 | 0.13 | 3.42* |
| 1pm-9am | -0.23 | -0.63 | -0.24 | 0.04 | 0.35 | -0.29 | -0.17 | 0.35 | 1.23 |

*** values are significant at p≤ 0.05, individual values are calculated by subtracting the value of time 2 from time 1, -ve value represents a decrease in expression, ×values for 72h control and treatment of animal 5 excluded from statistical analysis.**

**Table B.2 Paired t-test on raw differences between times for average (of 72h and 15d) *mcr*A gene expression (cDNA) of fish oil supplemented animals.**

| Time (Time 1-Time 2) | 1 | 2 | 3 | 4 | 5× | 6 | Mean | LSD 5% | t value |
|---|---|---|---|---|---|---|---|---|---|
| 9pm-5pm | -0.12 | 0.14 | 0.06 | -0.16 | -3.21 | -0.19 | -0.58 | 1.36 | 1.10 |
| 1am-5pm | 0.16 | 0.06 | -0.21 | 1.22 | -1.24 | 0.17 | 0.03 | 0.83 | 0.09 |
| 5am-5pm | 0.34 | -0.04 | -0.12 | 0.27 | -1.30 | -0.23 | -0.18 | 0.62 | 0.74 |
| 9am-5pm | 0.14 | 0.14 | 0.14 | -0.17 | -1.80 | 0.54 | -0.17 | 0.87 | 0.50 |
| 1pm-5pm | 0.06 | -0.17 | -0.28 | 0.52 | 0.41 | -0.20 | 0.06 | 0.35 | 0.41 |
| 1am-9pm | 0.28 | -0.08 | -0.26 | 1.38 | 1.97 | 0.36 | 0.61 | 0.92 | 1.70 |
| 5am-9pm | 0.46 | -0.18 | -0.18 | 0.43 | 1.91 | -0.04 | 0.40 | 0.83 | 1.24 |
| 9am-9pm | 0.26 | 0.00 | 0.08 | -0.01 | 1.41 | 0.72 | 0.41 | 0.59 | 1.79 |
| 1pm-9pm | 0.18 | -0.30 | -0.34 | 0.68 | 3.62 | -0.01 | 0.64 | 1.58 | 1.03 |
| 5am-1am | 0.18 | -0.10 | 0.09 | -0.95 | -0.06 | -0.40 | -0.21 | 0.43 | 1.24 |
| 9am-1am | -0.03 | 0.08 | 0.35 | -1.39 | -0.56 | 0.37 | -0.20 | 0.71 | 0.72 |
| 1pm-1am | -0.10 | -0.22 | -0.07 | -0.71 | 1.65 | -0.37 | 0.03 | 0.87 | 0.08 |
| 9am-5am | -0.21 | 0.18 | 0.26 | -0.44 | -0.49 | 0.76 | 0.01 | 0.51 | 0.06 |
| 1pm-5am | -0.28 | -0.12 | -0.16 | 0.24 | 1.71 | 0.03 | 0.24 | 0.78 | 0.78 |
| 1pm-9am | -0.08 | -0.30 | -0.42 | 0.68 | 2.21 | -0.74 | 0.23 | 1.14 | 0.51 |

*** values are significant at p≤ 0.05, individual values are calculated by subtracting the value of time 2 from time 1, -ve value represents a decrease in expression, ×values for 72h control and treatment of animal 5 excluded from statistical analysis.**

**Table B. 3 Paired t-test on raw differences between times for average (of 72h and 15d) *mcr*A gene quantity (DNA) of control animals.**

| Time (Time 1-time 2) | 1 | 2 | 3 | 4 | 5× | 6 | Mean | LSD 5% | t value |
|---|---|---|---|---|---|---|---|---|---|
| **9pm-5pm** | 0.06 | 0.00 | 0.12 | 0.02 | -0.07 | -0.01 | 0.02 | 0.07 | 0.68 |
| **1am-5pm** | 0.15 | 0.10 | -0.13 | 0.27 | 0.33 | 0.24 | 0.16 | 0.17 | 2.40 |
| **5am-5pm** | 0.00 | -0.20 | -0.25 | -0.11 | 0.42 | 0.12 | 0.00 | 0.26 | 0.02 |
| **9am-5pm** | -0.06 | -0.19 | 0.03 | 0.05 | -0.09 | -0.10 | -0.06 | 0.09 | 1.68 |
| **1pm-5pm** | -0.01 | -0.22 | -0.23 | -0.05 | 0.29 | 0.07 | -0.02 | 0.20 | 0.31 |
| **1am-9pm** | 0.10 | 0.10 | -0.25 | 0.25 | 0.40 | 0.25 | 0.14 | 0.23 | 1.56 |
| **5am-9pm** | -0.05 | -0.19 | -0.38 | -0.13 | 0.50 | 0.13 | -0.02 | 0.32 | 0.16 |
| **9am-9pm** | -0.11 | -0.19 | -0.09 | 0.03 | -0.02 | -0.09 | -0.08 | 0.08 | 2.51 |
| **1pm-9pm** | -0.07 | -0.22 | -0.35 | -0.07 | 0.37 | 0.08 | -0.04 | 0.26 | 0.42 |
| **5am-1am** | -0.15 | -0.29 | -0.13 | -0.38 | 0.10 | -0.12 | -0.16 | 0.17 | 2.43 |
| **9am-1am** | -0.21 | -0.29 | 0.15 | -0.22 | -0.42 | -0.34 | -0.22 | 0.21 | 2.70* |
| **1pm-1am** | -0.16 | -0.31 | -0.10 | -0.32 | -0.03 | -0.18 | -0.18 | 0.12 | 3.98* |
| **9am-5am** | -0.06 | 0.01 | 0.28 | 0.16 | -0.52 | -0.22 | -0.06 | 0.30 | 0.50 |
| **1pm-5am** | -0.01 | -0.02 | 0.03 | 0.06 | -0.13 | -0.06 | -0.02 | 0.07 | 0.84 |
| **1pm-9am** | 0.04 | -0.03 | -0.25 | -0.10 | 0.38 | 0.17 | 0.04 | 0.23 | 0.39 |

*** values are significant at p≤ 0.05, individual values are calculated by subtracting the value of time 2 from time 1, -ve value represents a decrease in expression, ×values for 72h control and treatment of animal 5 excluded from statistical analysis.**

**Table B. 4 Paired t-test on raw differences between times for average (of 72h and 15d) *mcr*A gene quantity (DNA) of fish oil supplemented animals.**

| Time (Time 1-time 2) | 1 | 2 | 3 | 4 | 5× | 6 | Mean | LSD 5% | t value |
|---|---|---|---|---|---|---|---|---|---|
| **9pm-5pm** | -0.03 | 0.00 | -0.06 | 0.05 | 0.51 | 0.04 | 0.09 | 0.22 | 1.00 |
| **1am-5pm** | 0.06 | -0.03 | -0.19 | 0.09 | 0.02 | -0.06 | -0.02 | 0.10 | 0.39 |
| **5am-5pm** | 0.08 | 0.20 | -0.13 | -0.06 | 0.08 | 0.03 | 0.03 | 0.12 | 0.65 |
| **9am-5pm** | -0.01 | -0.04 | 0.01 | -0.05 | 0.09 | 0.10 | 0.02 | 0.07 | 0.63 |
| **1pm-5pm** | 0.09 | 0.07 | -0.15 | -0.03 | 0.43 | -0.04 | 0.06 | 0.21 | 0.76 |
| **1am-9pm** | 0.10 | -0.03 | -0.13 | 0.03 | -0.48 | -0.10 | -0.10 | 0.22 | 1.22 |
| **5am-9pm** | 0.11 | 0.19 | -0.08 | -0.12 | -0.43 | -0.01 | -0.06 | 0.23 | 0.62 |
| **9am-9pm** | 0.02 | -0.04 | 0.07 | -0.10 | -0.42 | 0.05 | -0.07 | 0.19 | 0.94 |
| **1pm-9pm** | 0.13 | 0.06 | -0.09 | -0.08 | -0.07 | -0.09 | -0.02 | 0.10 | 0.62 |
| **5am-1am** | 0.02 | 0.22 | 0.05 | -0.15 | 0.05 | 0.09 | 0.05 | 0.13 | 0.95 |
| **9am-1am** | -0.08 | -0.01 | 0.20 | -0.13 | 0.07 | 0.15 | 0.03 | 0.14 | 0.61 |
| **1pm-1am** | 0.03 | 0.09 | 0.04 | -0.12 | 0.41 | 0.01 | 0.08 | 0.19 | 1.08 |
| **9am-5am** | -0.09 | -0.24 | 0.14 | 0.02 | 0.02 | 0.07 | -0.01 | 0.14 | 0.27 |
| **1pm-5am** | 0.01 | -0.13 | -0.01 | 0.03 | 0.36 | -0.07 | 0.03 | 0.18 | 0.45 |
| **1pm-9am** | 0.10 | 0.11 | -0.15 | 0.02 | 0.34 | -0.14 | 0.05 | 0.19 | 0.61 |

*** values are significant at p≤ 0.05, individual values are calculated by subtracting the value of time 2 from time 1, -ve value represents a decrease in expression, ×values for 72h control and treatment of animal 5 excluded from statistical analysis.**

**Table B.5 Fatty acid composition (g/100g of total fatty acid) of fish oil used for supplementation in treatment animals.**

| Fatty acid | Fish oil 1* | Fish oil 2** |
|---|---|---|
| C6:0 | 0.010 | 0.009 |
| C8:0 | 0.019 | 0.012 |
| C10:0 | 0.016 | 0.008 |
| C12:0 | 0.109 | 0.109 |
| C14:0 | 4.863 | 4.784 |
| C14:1 t9 | 0.018 | 0.016 |
| C14:1 c9 | 0.143 | 0.131 |
| C16:0 | 13.837 | 13.580 |
| C16:1 t9 | 0.020 | 0.020 |
| C16:1 c9 | 7.185 | 7.139 |
| 7,10,13-hexadecatrienoatecosatetraenoate | 0.313 | 0.318 |
| C18:0 anteiso | 0.030 | 0.027 |
| C18:0 | 3.230 | 3.170 |
| C18:1 t9 | 0.654 | 0.659 |
| C18:1 c9 | 29.003 | 28.899 |
| C18:1 c11 | 3.349 | 3.282 |
| C18:2 c9,12 (LA) | 4.415 | 4.428 |
| C20:1 c11 | 4.809 | 4.788 |
| C20:4 | 0.634 | 0.653 |
| C20:5 (EPA) | 4.861 | 5.065 |
| C22:0 | 0.058 | 0.061 |
| C22:1 t13 | 0.875 | 0.903 |
| C22:1 | 1.959 | 1.965 |
| C22:1 c13 | 1.172 | 1.177 |
| C22:5 | 1.545 | 1.600 |
| C22:6 (DHA) | 5.442 | 5.737 |
| C24:0 | 0.028 | 0.026 |
| C24:1 | 0.403 | 0.399 |
| C26:0 | 0.011 | 0.012 |

***Fish oil 1 was used in first part of experiment,**
****Fish oil 2 used in second part of experiment.**

**Table B.6 Propionic acid concentration (mmol/L) in control and fish oil supplemented (treatment) animals.**

| Time | Propionic acid concentration for control at 72h | | | | | | | Propionic acid concentration for treatment at 72h | | | | | | | Paired t test | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean | 1 | 2 | 3 | 4 | 5 | 6 | Mean | LSD 5% | t value |
| 5pm | 14.99 | 17.93 | 9.51 | 16.96 | 27.65 | 22.84 | 18.31 | 18.76 | 30.25 | 19.19 | 13.74 | 9.44 | 15.34 | 17.79 | 12.02 | 0.11 |
| 1am | 26.19 | 32.39 | 23.87 | 25.45 | 33.90 | 29.79 | 28.60 | 31.13 | 36.58 | 29.80 | 23.75 | 15.40 | 24.94 | 26.93 | 9.72 | 0.44 |
| 9am | 21.30 | 23.40 | 21.93 | 20.87 | 25.98 | 26.85 | 23.39 | 27.08 | 23.93 | 18.79 | 21.29 | 12.16 | 15.93 | 19.86 | 7.85 | 1.15 |
| MAUC | 22.17 | 26.53 | 19.79 | 22.18 | 30.36 | 27.32 | 24.73 | 27.02 | 31.84 | 24.40 | 20.63 | 13.10 | 20.29 | 22.88 | 9.41 | 0.50 |
| Time | Propionic acid concentration for control at 15d | | | | | | | Propionic acid concentration for treatment at 15d | | | | | | | Paired T test | |
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean | 1 | 2 | 3 | 4 | 5 | 6 | Mean | LSD 5% | t value |
| 5pm | 14.60 | 16.93 | 11.05 | 12.53 | 12.96 | 20.52 | 14.77 | 22.13 | 26.81 | 13.16 | 11.28 | 20.48 | 16.24 | 18.35 | 5.90 | 1.56 |
| 1am | 23.26 | 24.81 | 20.55 | 28.02 | 18.24 | 31.02 | 24.32 | 35.84 | 38.23 | 40.06 | 17.28 | 30.40 | 28.20 | 31.67 | 12.11 | 1.56 |
| 9am | 22.38 | 26.69 | 13.56 | 25.24 | 16.53 | 26.65 | 21.84 | 27.35 | 31.09 | 24.99 | 13.05 | 27.53 | 25.76 | 24.96 | 9.23 | 0.87 |
| MAUC | 20.88 | 23.31 | 16.41 | 23.45 | 16.49 | 27.30 | 21.31 | 30.29 | 33.59 | 29.57 | 14.72 | 27.20 | 24.60 | 26.66 | 9.31 | 1.48 |
| Time | Propionic acid concentration for control average | | | | | | | Propionic acid concentration for treatment average | | | | | | | Paired T test | |
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean | 1 | 2 | 3 | 4 | 5 | 6 | Mean | LSD 5% | t value |
| 5pm | 14.80 | 17.43 | 10.28 | 14.75 | 20.31 | 21.68 | 16.54 | 20.44 | 28.53 | 16.17 | 12.51 | 14.96 | 15.79 | 18.07 | 7.33 | 0.54 |
| 1am | 24.72 | 28.60 | 22.21 | 26.73 | 26.07 | 30.40 | 26.46 | 33.49 | 37.41 | 34.93 | 20.51 | 22.90 | 26.57 | 29.30 | 8.54 | 0.86 |
| 9am | 21.84 | 25.04 | 17.74 | 23.05 | 21.26 | 26.75 | 22.61 | 27.22 | 27.51 | 21.89 | 17.17 | 19.84 | 20.84 | 22.41 | 5.21 | 0.10 |
| MAUC | 21.52 | 24.92 | 18.11 | 22.82 | 23.43 | 27.31 | 23.02 | 28.66 | 32.72 | 26.98 | 17.68 | 20.15 | 22.44 | 24.77 | 7.16 | 0.63 |

***values are significant at p≤0.05, ˣ values not considered for statistical analysis, MAUC- Mean area under curve for 20h.**

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