Enumeration, Identification and Characterisation of Methanogens Colonising Pre-Ruminant Calves

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Abstract

Methane-producing archaea, methanogens, in ruminant animals are a major source of anthropogenic methane. With a global warming potential 23 times greater than carbon dioxide, methane has been targeted for reduction under the Kyoto protocol. In New Zealand methane emissions from ruminant animals are major contributor to the national greenhouse gas inventory. For this reason agricultural industries are challenged with reducing methane emissions from ruminants. This investigation on methanogens in young dairy calves was carried out to obtain information on methanogen colonisation and establishment in the rumen because little is known about this process.

In this study, methanogen colonisation occurred within two days after birth in four calves that were raised in the absence of cows. Anaerobic culture techniques were used to enumerate methanogens in gut samples and showed that methanogen numbers increased over time, but dropped below detection limits in two of four calves between six and 11 days after birth. Methanogens in these two calves then reappeared at day 13. By three weeks of age methanogen densities in all four calves were approximately 10^8 cells ml^{-1}. These densities are similar to those found by other workers for 3-week old and mature ruminants. Colonies picked from anaerobic agar roll-tubes prepared from enumeration cultures yielded 31 methanogenic isolates and 28 isolates that utilised hydrogen but did not produce methane. Eleven of the 31 methanogenic isolates were selected for purification. Despite extensive efforts only four methanogens were able to be purified from the eleven isolates because of persistent non-methanogenic eubacteria also present in cultures.

A phylogenetic analysis of 16S rRNA gene sequences from purified and partially-purified methanogen isolates was carried out and dendograms constructed to identify methanogens. Some phenotypic characteristics of purified methanogens were determined. This revealed a number of methanogen species previously not found in the rumen. The results showed *Methanofollis liminatans* (three isolates), *Methanoculleus palmolei* (three isolates) or *Methanosarcina barkeri* (one isolate) were the predominant culturable methanogens colonising the rumen two days after birth. The three isolates identified as

M. liminatans were only 96.0% identical at the 16S rRNA gene level to the *M. liminatans* type strain, DSM 4140, and appear to be new a methanogen species. In gut samples collected 3-5 days after birth, *Methanobacterium bryantii* (three isolates) was found to be a predominant methanogen in some calves apparently replacing the first methanogens colonising the developing rumen. Twenty two days after birth *Methanobrevibacter thaueri* (one isolate) was identified as a predominant methanogen in one calf. These results are the first to suggest that there is a successional change in the methanogen populations as the rumen develops in young ruminants.

Consideration of the colonising species showed that *Mcl. palmolei* were obtained from only two calves (calves 10 and 12) and that *Mfl. liminatans*-like isolates were obtained only from a different cohort of calves penned separately to calves 10 and 12. These methanogens, previously found only in terrestrial or aquatic environments, are probably the primary colonising methanogens because there were no mature ruminants to provide alternative inocula. It appears that the developing rumen of young calves provides a niche suitable for opportunistic hydrogenotrophic methanogens.

A PCR investigation using targeted primers specific for seven groupings of methanogens was carried out on all rumen samples to obtain information not dependant on culturing. This analysis on DNA extracts showed methanogens belonging to the *Methanobacteriales* were present in almost every sample. Methanogens belonging to the *Methanosarcinales* and *Methanomicrobiales* were not detected in any sample. At the end of the trial (22 days), PCR analysis showed the presence of *Methanobacterium* spp. and *Methanobrevibacter* spp. in all four calves. Although there were some disagreements with results for isolates cultured, overall, PCR results confirmed the concept of successional changes in methanogen populations in pre-ruminant calves.

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Abbreviations

A.M.	*ante meridiem*	UV	ultraviolet
BLAST	Basic Local Alignment Search Tool	V	Volt
°C	degrees Celsius	v/v	volume per volume
cells ml^{-1}	cells per millilitre	w/v	weight per volume
DMSO	dimethylsulphoxide		
DNA	deoxyribonucleic acid		
g	gram		
kb	kilobase		
kPa	kilopascal		
L	litre		
$litre^{-1}$	per litre		
M	molar		
mg	milligram		
mg ml^{-1}	milligrams per millilitre		
ml	millilitre		
mm	millimetre		
mV	millivolt		
nm	nanometre		
pmol	picomoles		
PCR	polymerase chain reaction		
P.M.	*post meridiem*		
RNA	ribonucleic acid		
rDNA	ribosomal deoxyribonucleic acid		
rRNA	ribosomal ribonucleic acid		
rpm	revolutions per minute		
s	second		
SEM	Scanning Electron Microscope		
µg	microgram		
µg ml^{-1}	micrograms per millilitre		
µl	microlitre		

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1 Rumen Colonisation by Methanogens

1.1 Ruminants as Production Animals

Ruminants play an important role in the provision of meat, milk, wool and skin as sources of raw materials for food, clothing and textiles. In New Zealand, the major farmed ruminants are cattle and sheep, with fewer numbers of other ruminants, like deer, goats, alpacas and llamas also present. The numbers of farmed ruminants reached a peak in the mid 1980s (Statistics New Zealand, 2004a) with a sheep population of 70.3 million in June 1982. Sheep numbers steadily declined to 44 million by June 2001. In June 2001, dairy cattle numbered 4.73 million, up from 1.72 million animals in June 1982. Meanwhile, total cattle numbers (beef and dairy) have remained static for the period 1996 to 2001. Exports of dairy and meat products, mostly from ruminants, are important to New Zealand's economy, with 10 billion of the 31 billion dollars earned from exported goods in 2000/2001 being derived from agriculture of which ruminants predominate (Statistics New Zealand, 2004b).

Ruminants are herbivorous mammals that attain their energy from a symbiosis with gut microbes. Forage consumed by ruminants serves as substrates for microbial fermentation in the gut. In return, microbes provide the ruminant animal with energy and protein from volatile fatty acids and microbial cells, respectively. The ruminant/microbe symbiosis has developed because without forage breakdown by microbes, ruminant digestive processes are unable to fully degrade plant materials (Hobson, 1997). However, not all microbial products are metabolised by ruminants. Methane, a gaseous by-product formed within the rumen is one end product not utilised by ruminants. Methane is a potent greenhouse gas, identified as causing 23 times the Global Warming Potential (GWP) compared to an equivalent mass of carbon dioxide (Ramaswamy *et al.*, 2001). Among the developed world New Zealand has an unusual greenhouse gas profile with a high proportion of total emissions, 49%, from agriculture and 36.2% of the total gas emissions are methane (Ministry for the Environment, 2006). The majority of methane emissions in New Zealand are from farmed ruminants (Brown and Petrie, 2003). Under the Kyoto protocol there is a directive to reduce methane emissions from farmed ruminants both in New Zealand and globally.

1.2 The Rumen

The rumen is a multi-chambered stomach containing microbes that ferment feed consumed by the host ruminant. The reticulo-rumen is comprised of two compartments, the reticulum and the rumen. The reticulum, where a minor part of microbial fermentation takes place, completes a passage from the oesophagus to the omasum (Hobson, 1997). The rumen is a blind sac adjoining the reticulum where the majority of microbial fermentation occurs (Figure 1A). Together, the reticulo-rumen occupies large volumes of the animal's digestive tract; about 100 L in cattle and 10 L in sheep (Annison and Lewis, 1959; Hobson, 1997).

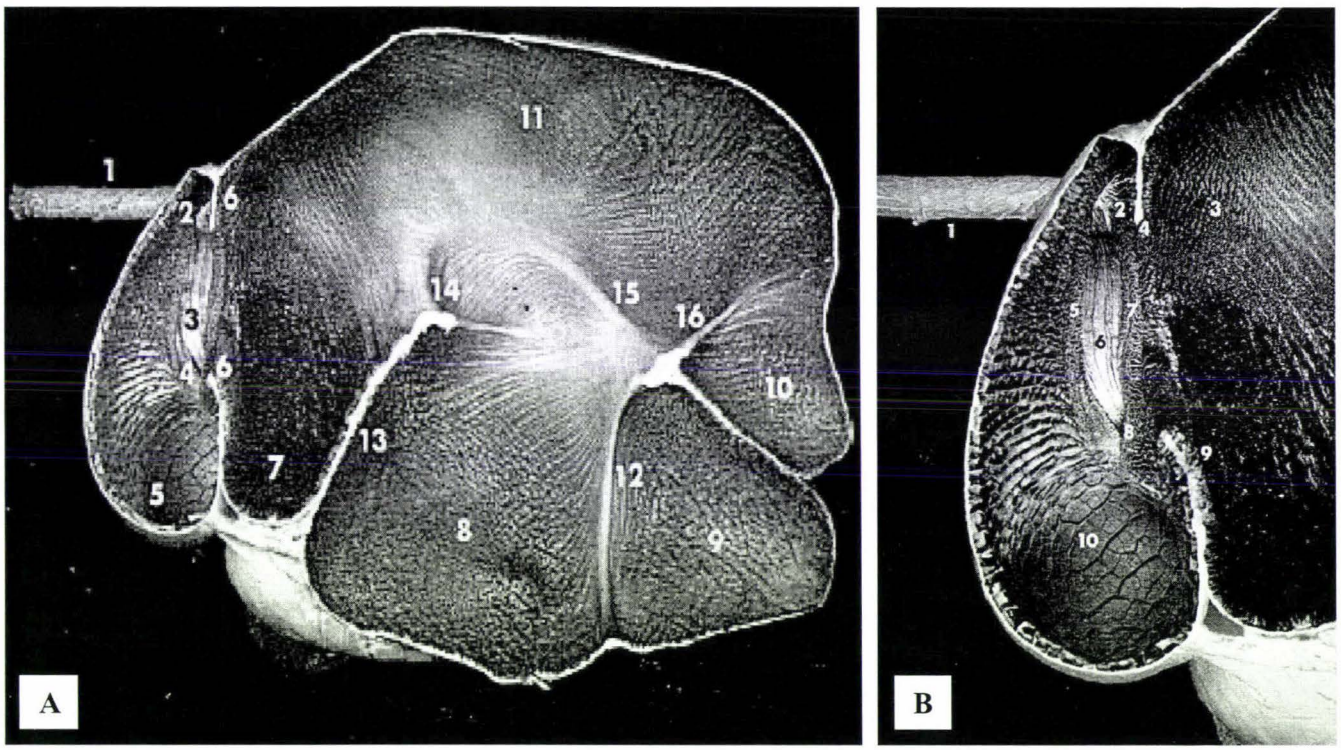


Figure 1: Cross Section of the Major Rumen Components. (A) Cross section of reticulo-rumen: (1) oesophagus; (2) cardia; (3) esophageal groove; (4) reticulo-omasal opening; (5) reticulum; (6) rumino-reticular fold; (7-11) rumen compartments; (12) ventral pillar; (13) anterior fold; (14) anterior pillar; (15) longitudinal pillar; (16) dorsal pillar. **(B)** cross section of reticulum: (1) oesophagus; (2) cardia; (3) wall of anterior rumen; (4) rumino-reticular fold; (5) lip of oesophageal grove; (6) oesophageal groove; (7) lip of esophageal groove; (8) omasal-reticulum orifice (9) rumino-reticular fold; (10) reticulum. Adapted from Hungate (1966).

In young ruminants the microbial fermentation of plant materials present in the adult, is lacking and they are unable to digest plant material due to the rumen being underdeveloped. Young ruminants consume milk that bypasses the underdeveloped rumen via the oesophageal groove (Figure 1B) and enters the abomasum directly. In the abomasum the milk is curdled by stomach acids and absorbed across the gut wall in the lower digestive tract. As the young ruminant matures, less time is spent suckling milk and more time spent consuming plant materials. Greater levels of plant fibre consumption in the young ruminant allow earlier development of a mature rumen microbial population (Fonty *et al.*, 1987). The first microbial colonisation of the young ruminant happens during birth when microbes from the maternal reproductive tract attach to the newborn. Further contact between mother and offspring is an important source of microbes for gut colonisation during the first days after birth (Fonty *et al.*, 1986). In commercial farming systems young ruminants are sometimes removed from the mother soon after birth and other sources of microbial inoculation become important. After removal from the dam sources of inoculation can now include human handlers, and other young animals, also removed from their dams. Removal from the dam can retard the development of gut microbe populations (Lysons *et al.*, 1976). As an extreme example, lambs reared in sterile conditions consuming sterile feed have slower colonisation rates compared to those reared under natural conditions (Fonty *et al.*, 1991). Conversely, inoculation of young calves with natural adult rumen flora causes an earlier development of microbial populations than in uninoculated calves (Pounden and Hibbs, 1948). Once in the rumen, microbial species may or may not become permanent. Succession and competition for substrates by microbes causes some species to flourish, and others to disappear from the rumen (Hobson *et al.*, 1958).

1.3 Rumen Microbial Activity

Rumen bacterial populations have evolved to breakdown most plant materials to provide the host ruminant the majority of its nutritional requirements. Plant materials are comprised mainly of structural carbohydrate polymers including celluloses, hemicelluloses, lignins and starches. Lesser components are soluble sugars, pectins, fructosans, proteins and small amounts of lipid (Chesson and Forsberg, 1997). Microbial fermentation of feeds in the rumen can be divided into major two steps: in the first,

complex plant components like starches and plant cell wall polymers are broken down into volatile fatty acids (acetate, propionate and butyrate) and by-product gases (H_2 and CO_2). In addition, the conversion of proteins into amino acids and then ammonia also takes place. In the second step, the conversion of ammonia into microbial protein and the conversion of H_2 and CO_2 into methane occur. Nutrition for the ruminant is obtained by the absorption of volatile fatty acids, microbial protein and unaltered protein across the gut wall. The major nutrient flows are illustrated in Figure 2, including examples of representative microbes able to perform each conversion. Figure 2 is not totally inclusive of all the metabolic transformations that occur; a more detailed flowchart is given in Hobson (1997).

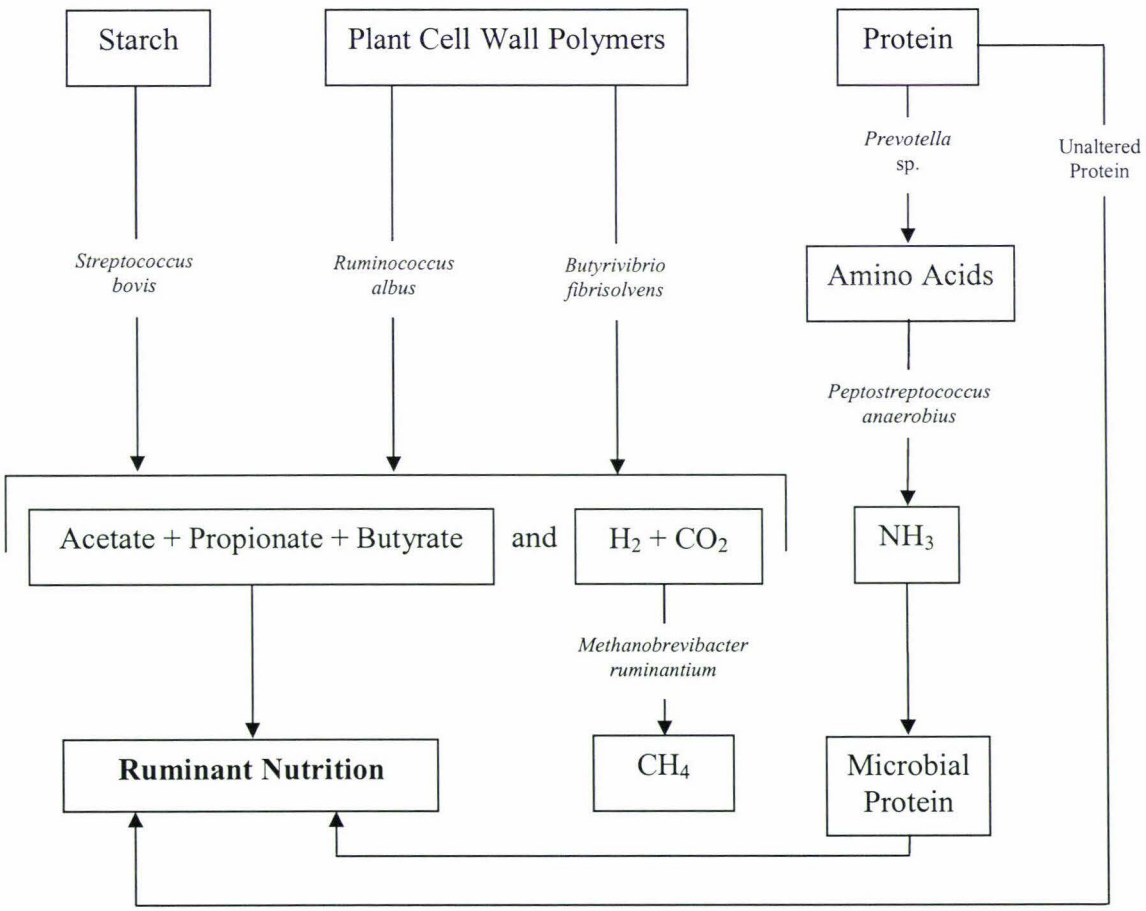


Figure 2: Starch, Plant Cell Wall Polymers and Protein Metabolism in the Rumen. Adapted from Hobson (1997).

1.3.1 Carbohydrate Breakdown

Polysaccharides entering the rumen are considered to belong to one of two general types. These include plant storage polysaccharides, such as starch, and the structural polysaccharides, such as celluloses, hemicelluloses and lignin, which represent the greater part of plant cell walls and forms the fibrous components of animal feedstuffs (Chesson and Forsberg, 1997). Many rumen microbes can breakdown both structural and storage polysaccharides. Laboratory based simulations of fibre degradation in the rumen suggest that bacteria and fungi together degrade approximately 80% of fibre, with protozoa degrading the remaining 20% (Dijkstra and Tamminga, 1995). *Ruminococcus albus* and *Ruminococcus flavefaciens* are bacterial species considered to be the predominant fibre degrading bacteria in the rumen (Chesson and Forsberg, 1997). *Streptococcus bovis* is a bacterium that ferments the storage carbohydrate starch to volatile fatty acids (Figure 2). In addition, species exemplified by *Butyrivibrio fibrisolvens* are able to produce volatile fatty acids from plant cell wall polymers (Figure 2). Hydrogen and carbon dioxide are products of carbohydrate degradation, but are not utilised by the ruminant.

1.3.2 Protein Breakdown

Upon entry to the rumen most plant proteins are broken down into constituent amino acids by bacteria like *Prevotella* sp. (Wallace *et al.*, 1997) (Figure 2). However, not all proteins are broken down by bacteria. Some protein can pass through the rumen to the lower digestive tract unaltered by microbes (Figure 2). The majority of amino acids are deaminated by bacteria such as *Peptostreptococcus anaerobius* into branched chain fatty acids and ammonia (Russell *et al.*, 1988) (Figure 2). Ammonia is then synthesised back into amino acids for protein synthesis in microbial cells. Microbial proteins and unaltered protein are available for digestion by the ruminant further down the intestinal tract.

1.3.3 Lipid Breakdown

Lipids represent only a small proportion of the ruminant diet. Consequently, few lipolytic bacteria inhabit the rumen, and few have been isolated from the rumen to date (Cirne *et al.*, 2006). Lipolytic microbes function to hydrolyse lipids into their component parts, glycerol backbone and free fatty acids. Subsequently, some of the hydrolysed unsaturated

fatty acids are saturated to varying degrees by combinations of biohydrogenating bacteria. Many microbial species are able to perform biohydrogenation steps, including those isolated by Verhulst *et al.* (1985). The resulting saturated fats, in addition to the remaining unsaturated free fatty acids, are absorbed across the jejunum, where they are synthesised into triacylglycerides and deposited in animal tissue.

1.3.4 Hydrogen and Carbon Dioxide Production

Microbial fermentation in the rumen produces large quantities of hydrogen and carbon dioxide, but these products are not utilised by the ruminant. Few microbes in the rumen are able to use hydrogen and carbon dioxide as a substrate. The majority of hydrogen, and some carbon dioxide, produced by rumen microbes are consumed by methanogenic microbes forming methane (Hungate, 1966). Acetogenic bacteria in the rumen also use hydrogen and carbon dioxide for growth, but produce acetate instead of methane. Methanogens typically out-compete acetogens for hydrogen as they are able to utilise hydrogen at a lower concentration in the rumen (Cord-Ruwisch *et al.*, 1988). The high affinity of methanogens for hydrogen means that little hydrogen accumulates in the rumen, even though large quantities are produced each day (Hungate *et al.*, 1970). Methane and carbon dioxide are eructated from the host representing a loss of energy to the animal. If rumen methane production were to be inhibited, the six to 13% of dietary energy lost as methane could be recovered by the animal (Miller and Wolin, 2001). Inhibition of methanogenesis would also result in a reduction in emissions of greenhouse gases (Ramaswamy *et al.*, 2001).

1.4 Methanogens

Methane is one end product of the anaerobic breakdown of organic matter and is emitted from a variety of environments where anaerobic microbes exist. Hungate (1966), noted that in 1863 Jules Reiset was the first to demonstrate that methane is produced in the rumen. In 1947, Schellen was the first to isolate methane-producing microbes, from an anaerobic digester. These species were later identified *Methanosarcina barkeri* and *Methanobacterium bryantii* (Wolfe, 1993). In 1952 the first ruminal methanogen, *Methanosarcina barkeri*, was isolated from cattle (Beijer, 1952).

Methane-producing microbes belong to the domain Archaea, a different domain from the Bacteria, they are however prokaryotic like Bacteria (Woese *et al.*, 1990). Along with the domains Eukarya and Bacteria, the Archaea are believed to share a common ancestor with division occurring approximately 3.5 billion years ago (Maugh, 1977). The domain Archaea is separated into 4 kingdoms, the Crenarchaeota, Euryarchaeota (Woese *et al.*, 1990), Korarchaeota (Barns *et al.*, 1996) and Nanoarchaeota (Huber *et al.*, 2002). The Nanoarchaeaota are recently discovered obligate symbionts of other archaea. The Korarchaeotal kingdom consists only of 16S rRNA gene sequences from hot water springs. The culturable microbes in the Crenarchaeotal kingdom have a relatively small phenotypic diversity, with all cultivable organisms being hyperthermophiles (Woese *et al.*, 1990). The Euryarchaeotal kingdom is phenotypically more diverse than the Crenarchaeota. Known cultivable microbes within the Euryarchaeotal kingdom include halophiles, acidophiles, hyperthermophiles and methanogens (Woese *et al.*, 1990).

Methanogens are obligate anaerobes and grow in environments with redox potentials of less than -300 mV (Stewart and Bryant, 1988). They occupy the terminal position in anaerobic organic matter breakdown. Methanogen species have been isolated from a range of environments and include psychrophilic (*Methanogenium fridigum),* mesophilic (*Methanobrevibacter ruminantium*), thermophilic (*Methanothermobacter thermoautotrophicus*) and extremely thermophilic (*Methanothermobacter jannaschii*) species (Garcia *et al.*, 2000). There are said to be five orders of methanogens within the domain Archaea, *Methanosarcinales*, *Methanomicrobiales*, *Methanococcales*,

Methanobacteriales and *Methanopyrales* (Garcia *et al.*, 2000) (Figure 3), although some controversy exists as to the assignments of methanogen orders due in some cases to sequence quality (Ludwig and Klenk, 2001).

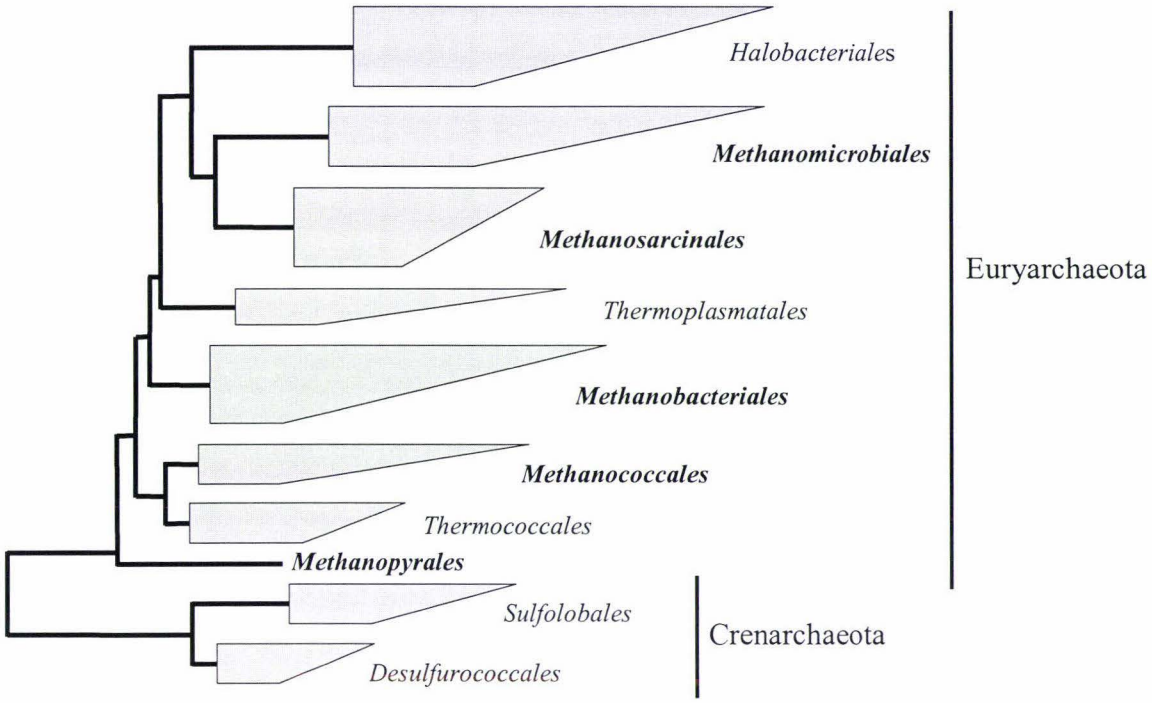


Figure 3: Phylogeny of the Five Methanogen Orders. Phylogeny of the major orders within the *Crenarchaeota* and *Euryarchaeota* are shown. Methanogen orders are in bold type. Adapted from Garcia *et al.* (2000).

Key differences exist between archaea and bacteria. Methanogens, as archaea, posses both structural and genetic differences when compared to bacteria. For example methanogen cell walls do not contain bacterial peptidoglycan, instead, they contain either pseudomurein, protein, heteropolysaccarides or glycoprotein complexes dependent on their phylogenetic position (Kandler and Hippe, 1977). Bacterial cell membranes contain ester-linked alkane-lipids whereas methanogen membranes posses ether-linked isoprenoid glycerol lipids (Grant *et al.*, 1985). Methanogen gene arrangements are similar to bacterial operons, and in some methanogens tRNA and rRNA gene introns have been discovered (Brown *et al.*, 1989). RNA polymerases of methanogens are more complex than bacterial polymerases, and have some characteristics of both bacterial and eukaryotic polymerases (Woese, 1977).

1.5 Ruminal Methanogens

Ruminal methanogens that have been isolated to date include *Methanosarcina barkeri* (Beijer, 1952), *Methanobacterium formicicum* (Oppermann *et al.*, 1957), *Methanobrevibacter ruminantium* (Smith and Hungate, 1958), *Methanomicrobium mobile* (Paynter and Hungate, 1968), *Methanosarcina* sp. (Patterson and Hespell, 1979), *Methanoculleus olentangyi* (Skillman *et al.*, 2001) and *Methanobrevibacter* spp. (Lovley *et al.*, 1984; Miller *et al.*, 1986). Currently, it is believed that *Methanobrevibacter ruminantium* is the predominant ruminal methanogen species (Wolin and Miller, 1997). In contrast to Wolin and Miller (1997), other studies using 16S rRNA gene cloning have concluded that *Methanomicrobium mobile* is the predominant ruminal methanogen (Yanagita *et al.*, 2000; Tajima *et al.*, 2001). 16S rRNA gene cloning studies have also shown that novel methanogens, and methanogens novel to the rumen, such as *Methanosphaera stadtmanae*, are present in the rumen (Whitford *et al.*, 2001; Tajima *et al.*, 2001, Nicholson *et al.*, 2007). Most previous studies that have isolated methanogens have done so from animals fed a diet high in grain. The first characterised methanogens from pasture-fed ruminants were identified as *Methanobacterium formicicum*, *Methanomicrobium mobile* and *Methanosarcina barkeri* (Jarvis *et al.*, 2000).

Relatively few studies detailing methanogen densities in the rumen exist. Published studies have typically found methanogens populations between 10^8 and 10^9 cells ml^{-1} (Smith and Hungate, 1958; Fonty *et al.*, 1986; Miller *et al.*, 1986; Morvan *et al.*, 1994; Skillman *et al.*, 2004). Substrates for methanogenesis in the rumen are either hydrogen/carbon dioxide, or formate. Hungate *et al.* (1970) found that 82% of methane formed in the rumen is from hydrogen/carbon dioxide, and the remainder from formate. However, methanogens that are known to utilise acetate, like *Methanosarcina barkeri* (Beijer, 1952) have also been isolated from the rumen. *Methanosphaera stadtmanae*, an obligate methanol-utilising methanogen, has also been detected in the rumen from clone libraries (Whitford *et al.*, 2001; Skillman *et al.*, 2006; Nicholson *et al.*, 2007). These studies suggest that substrates other than hydrogen/carbon dioxide and formate may be used for ruminal methanogenesis.

The metabolic association of methanogens with other ruminal microbes has an important role in rumen function. Under anaerobic conditions continued oxidation of NADH to NAD^+ is necessary to provide reduced cofactors for pyruvate formation during glycolysis. Accumulation of hydrogen, produced during rumen fermentation, reduces the process of NADH oxidation and lowers the efficiency of the rumen fermentation (Wolin and Miller, 1997). In the presence of methanogens, under normal rumen conditions, hydrogen does not accumulate because of interspecies hydrogen transfer (Hungate *et al.*, 1970) and as a result end products of fermentation are carbon dioxide, methane, acetate, propionate and butyrate (Wolin and Weinberg, 1960).

Interspecies hydrogen transfer in methanogenic co-cultures is exemplified by the cellulolytic and hydrogen-producing rumen bacterium *Ruminococcus albus*. In pure culture *R. albus* ferments cellulose to acetate, ethanol, hydrogen and carbon dioxide (Stewart and Bryant, 1988), but when grown in the presence of methanogens only carbon dioxide, methane and acetate are produced from cellulose. Therefore, acetate, for ruminant nutrition, is produced more efficiently by *R. albus* when methanogens are present than when absent.

The effects of interspecies hydrogen transfer to methanogens are also evident with other rumen microbes. Marked increases in specific activities of fungal xylanolytic enzymes were found when rumen fungi were co-cultured with *Methanobrevibacter smithii* (Joblin *et al.*, 1990) and xylan degradation increased markedly. Methanogens are also associated with rumen protozoa through the attachment to protozoal cells (Finlay *et al.*, 1994) and are involved in interspecies hydrogen transfer (Ushida *et al.*, 1997). Hillman et al., 1982 Because of the methanogen-protozoa association, methane emissions were reduced when protozoa were removed from the rumens of grain fed cattle (Whitelaw *et al.*, 1984).

1.6 Methanogen Anaerobic Culturing and Molecular Techniques

Prior to 1950 studies of rumen microbiology were limited in scope because of the difficulty in culturing anaerobic microbes. Development of the anaerobic roll tube technique by Hungate (1950) has allowed greater ease and speed of culturing intestinal and sewage microbes (Holdeman and Moore, 1972). A few methanogen species, such as *Methanobacillus omelianskii* (Barker, 1940), were cultured before the development of anaerobic culture techniques by Hungate. Since 1950 many methanogen species have been isolated from a variety of environments using variations of the Hungate technique.

Rumen methanogens are cultured in anaerobic media consisting of salt solutions with rumen fluid added to the basal methanogen media to provide undefined growth factors (Smith and Hungate, 1958). Media for methanogen cultivation is prepared using CO_2 exposed to heated copper filings to remove residual oxygen (Jarvis *et al.*, 2000), reproducing anaerobic rumen conditions. Methanogens are cultured by the Hungate technique in anaerobic media pressurised with a H_2/CO_2 (80%:20%) atmosphere to serve as the substrate (Smith and Hungate, 1958). Additional substrates for methanogen growth, like methanol, can be added to anaerobic media for those species with special growth requirements (Biavati *et al.*, 1988). Growth of methanogens in media can be slow compared to other rumen microbes, with visible colony formation taking up to 16 days on agar roll tubes (Jarvis *et al.*, 2000). Methanogen isolates often lose viability during purification, or upon subculturing (Jarvis *et al.*, 2000). The low tolerance of methanogens to oxygen creates difficulties when culturing and enumerating these microbes (Smith and Hungate, 1958; Jarvis *et al.*, 2000; Skillman *et al.*, 2004). The anaerobic roll tube technique and serial decimal dilution (Smith and Hungate, 1958) are commonly used in methanogen quantification.

Use of non-culture-based techniques, like clone libraries, have allowed both the abundance and diversity of methanogens to be examined directly from the rumen ecosystem. The limitations due to the fastidious growth requirements of methanogens can be often overcome using molecular techniques. For instance, strains of *Methanosphaera stadtmanae* have been detected by analysis of 16S rRNA genes in clone

libraries from rumen contents, but have yet to be isolated in pure culture from the rumen (Whitford *et al.*, 2001; Wright *et al.*, 2006; Nicholson *et al.*, 2007). However, techniques such as PCR may introduce bias into the typing of a population. For example, two primer pairs both designed to detect all archaea indicated different population structures of methanogens to be present in the same rumen contents sample (Skillman *et al.*, 2006). Carefully selected primer usage and techniques such as Temporal Temperature Gel Electrophoresis (TTGE) have demonstrated a greater diversity of methanogens from the rumen ecosystem (Nicholson *et al.*, 2007).

1.7 Microbial Colonisation of the Rumen

Microbial colonisation of the digestive tract of calves was first studied in 1948 by Pounden and Hibbs. These authors described two main types of bacteria being present in the developing rumen soon after birth. Their study also found that young calves inoculated with rumen contents from mature animals developed bacterial populations faster than non-inoculated calves. More recently, Bryant *et al.* (1958) and Jayne-Williams (1979) isolated and described *Lactobacillus* spp., *Streptococcus* spp., *Clostridium* spp., *Eubacterium* spp., coliforms and other microbial species from the gut of young calves.

Other studies have shown successions of microbial species in establishment of gut ecosystem. Ziolecki and Briggs (1961) demonstrated that a *Streptococcus* sp. is replaced by other *Streptococcus* sp. in the rumen of young calves. Counts of this *Streptococcus* spp. and other microbes were found to increase rapidly after birth (Ziolecki and Briggs, 1961), and were higher than levels found in mature ruminants (Bryant *et al.*, 1958). After an initial increase, levels of microbes that colonise the rumen early began to decrease (Ziolecki and Briggs, 1961) and in some cases were deemed to be absent from the mature rumen (Hobson *et al.*, 1958). Later studies showed microbes similar to those previously isolated by Bryant *et al.* (1958) and Jayne-Williams (1979) can colonise the rumen within 24 hours of birth (Fonty *et al.*, 1987). Fonty *et al.* (1987) found that strictly anaerobic bacteria predominated two days after birth, and increased during the first week after solid feed ingestion. While anaerobic bacteria levels increased, facultative bacteria, *Streptococcus* spp. and *Escherichia coli* counts all decreased (Ziolecki and Briggs, 1961).

Overall, the information presented in these previous studies suggests a temporal ordered microbial colonisation in the young ruminant. Furthermore, the rumen ecosystem quickly establishes itself even though the rumen is not yet a functioning organ (Fonty *et al.*, 1986; Skillman *et al.*, 2004). Recent investigations into microbial colonisation of newborn animals have focused on lambs reared in sterile isolation (Fonty *et al.*, 1997; Faichney *et al.*, 1999; Chaucheyras-Durand and Fonty, 2001). As expected, lambs reared in sterile isolation showed a disrupted pattern of microbial colonisation (Fonty *et al.*, 1997). At eight days after birth no methanogens were observed in the gut of lambs reared in sterile isolation (Chaucheyras-Durand and Fonty, 2001). In contrast, the appearance of detectable methanogens in the developing rumen of lambs reared under natural conditions is as early as one day after birth (Skillman *et. al.*, 2004). Results of these studies indicate that when young ruminants were reared in sterile isolation there were negative consequences for microbial colonisation. Methanogen colonisation of the rumen may be related to the growth of other microbes colonising the rumen. For example, facultative microbes colonise first and are presumed to scavenge oxygen, providing conditions suitable for the colonisation of strictly anaerobic microbes, such as methanogens (Hobson, 1997).

Studies of methanogen colonisation of the lamb gut have shown the first detectable methanogens were first present between one and three days after birth (Morvan *et al.*, 1994; Skillman *et al.*, 2004). Methanogen appearance in calves was observed by culturing after 24 hours, but not before 20 hours by Minato *et al.* (1992). After colonisation, methanogen counts in a two-week old lamb fed a concentrate diet were found to be similar to those in mature adults (Fonty *et al.*, 1987). Similarly, Skillman *et al.* (2004) found that three weeks after birth of young lambs methanogens were at similar levels to those of adult sheep. Methanogens were found to be abundant by three weeks of age in newborn calves separated from their dams and hand-fed milk (Minato *et al.*, 1992). Methanogens in newborn ruminants are described collectively, rather than individual species by the current literature (Fonty *et al.*, 1986; Fonty *et al.*, 1987; Minato *et al.*, 1992; Morvan *et al.*, 1994). To date methanogen diversity in the rumen has only been described in adult ruminants, with the exception of Skillman *et al.* (2004). Investigation

of methanogens in rumen contents of lambs by PCR showed that species from the genus *Methanobacterium* or the genus *Methanobrevibacter* were the initial colonising methanogens (Skillman *et al.*, 2004). Skillman *et al.* (2004) also found no methanogens other than those from the order *Methanobacteriales* present in the developing rumen.

1.8 Research Aims

When this project was initiated there was little information available about the colonisation and establishment of methanogens in dairy calves. This paucity in information is partly due to problems associated with culturing methanogens. The aim of this study was to provide a greater understanding of methanogen colonisation in the developing rumen based on both culture and culture-independent techniques. The research began with a sampling trial of young calves to provide materials for isolation and characterisation of methanogens from the developing rumen. Methanogens from rumen contents were enumerated by culturing over the entire sampling period using decimal dilution series to determine their time of first colonisation and levels post-colonisation. Resulting cultures from the methanogen enumerations were subsequently used for the isolation, characterisation and identification of methanogen species. Also, DNA from the remainder of the rumen content samples was extracted and analysed by molecular techniques for determination of methanogen presence and identification.

Specific aims of this study were to discover:

- The time after birth of the first methanogen colonisation of the calf rumen.
- The densities that methanogens reach over a three week period in the developing rumen.
- The diversity of methanogens colonising the rumen of calves using culture techniques.
- The diversity of the representative methanogens cultured using molecular and phylogenetic techniques.

2 *Establishment and Enumeration of Methanogens*

2.1 Introduction

At birth the rumen is aerobic. It is first colonised by facultative microbes that scavenge oxygen making conditions suitable for the growth of strictly anaerobic microbes (Hobson, 1997). Facultative microbes can reach densities of 10^9 cells ml^{-1} within days of birth (Fonty *et al.*, 1987). Colonisation of the rumen by strictly anaerobic microbes in young ruminants happens after the rumen becomes anaerobic. Within days after birth anaerobic microbes will also reach densities of 10^9 cells ml^{-1}, similar to levels found in mature ruminants (Fonty *et al.*, 1987). Also, there appears to be a sequence of colonisation by microbes of the young ruminant (Bryant and Small, 1960). However, the removal of the newborn from its dam, a source of readily transferable microbes, will cause colonisation of the rumen to be disrupted and succession timing to be changed (Fonty *et al.*, 1986). After the initial colonisation, the rumen microbial ecosystem develops rapidly even though the rumen is not yet a functional organ (Fonty *et al.*, 1986).

Little is known about methanogen colonisation. Methanogens colonise the rumen between 24 and 48 hours after birth and reach similar densities to those found in mature ruminants within weeks after birth (Fonty *et al.*, 1986; Fonty *et al.*, 1987; Minato *et al.*, 1992; Morvan *et al.*, 1994; Skillman *et al.*, 2004). Methanogen densities at 24 hours or less after birth in all colonisation studies were found to be below 10^2 culturable cells ml^{-1}. By three weeks of age densities of culturable methanogens were between 10^6-10^9 cells ml^{-1} in all studies. These studies measured methanogen densities by serial decimal dilutions utilising a rumen fluid-based medium, under an atmosphere of H_2/CO_2.

The study of the establishment and enumeration of methanogens in young calves outlined in this chapter aimed to identify methanogens colonising the rumen, their pattern of establishment and densities reached during the three weeks after the birth of calves.

2.2 Methods

2.2.1 *Methanogen Colonisation Experiment*

Four newborn calves from a commercial farm at Massey University (Palmerston North, New Zealand) were sampled soon after birth using stomach intubation to remove contents from their rumen. The time after birth when the calves were first sampled was calculated based on the timeline in Figure 4.

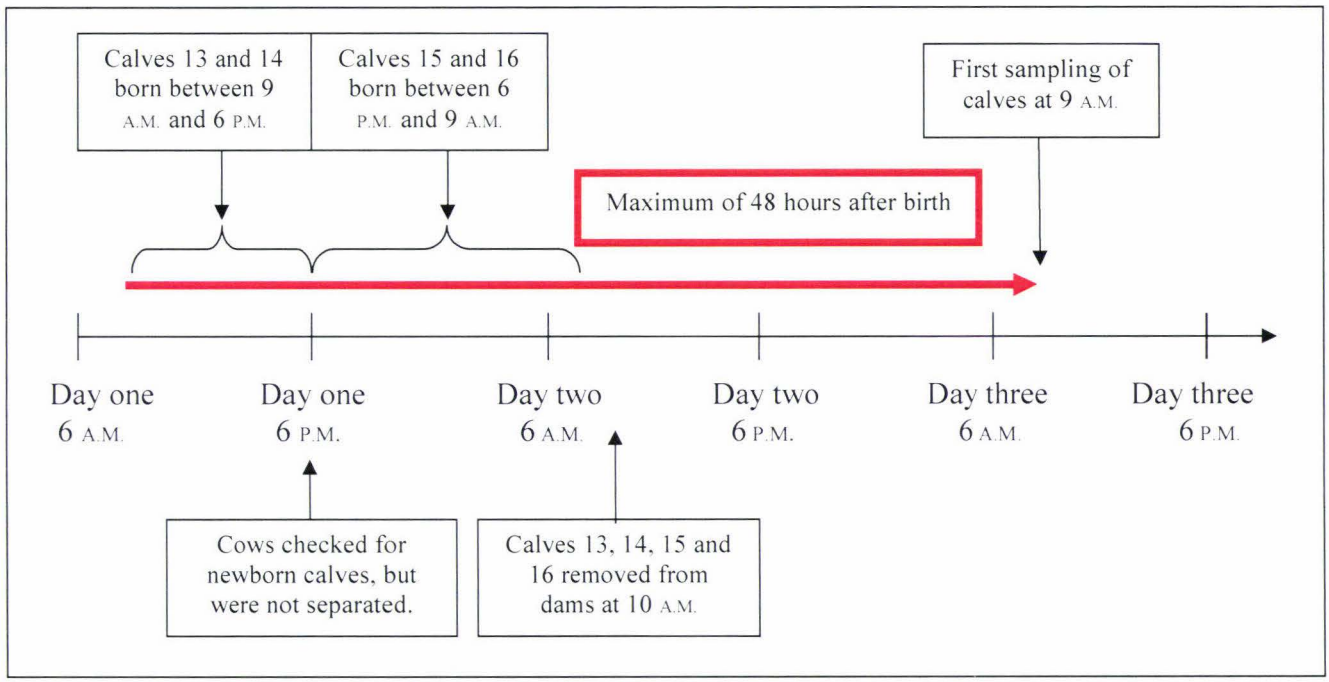


Figure 4: Timeline from Birth to First Sampling of Calves.

Each of the four calves was maintained with their dam until 10 A.M. on day two when the calves were separated from their dams and caged at a rearing unit for two weeks. The four calves were housed together in a covered rearing pen with flooring consisting of fine wood shavings. Hay, water and pellets were available *ad libitum* to calves at all times. For the third week after birth the calves were grazed on ryegrass pasture with 26 other calves of similar age. Hay, pellets and a limited amount of milk were always available throughout the sampling period. Calves were each fed four L of whole milk twice daily from a communal calf feeder throughout the sampling period. Rumen samples were collected from each calf by aspiration through a length of 10 mm diameter tubing using a 30 ml syringe. Samples were taken between 60 and 90 minutes after morning feeding.

The collected rumen contents were quickly transferred into anaerobic sterile CO_2-flushed serum bottles to avoid oxygenation. Following the first sampling, collections were made daily for five days, then on alternate days for eight days, and finally at 22 days after birth. This procedure had been repeated previously with another two calves, but sampling was discontinued because of inadvertent antibiotic contamination of the milk fed to these calves. However, samples collected from these two calves prior to the antibiotic contamination were kept and used for some analyses. The pH of all samples was measured using a PHM62 Standard pH meter (Radiometer, Denmark) and recorded (Table 21). The pH was recorded to ensure contents were actually from the rumen; if samples were overly acidic this would have suggested the samples were not from the rumen and methanogen viability may have been compromised. Following sample collection, one ml of all rumen contents collected from the six calves was cultured by inoculation aseptically and anaerobically into serial dilutions of rumen fluid medium (Section 2.2.2). In addition, one ml was removed for DNA extraction (Section 4.2.4). The remainder of rumen content samples were freeze-dried and stored at -85 °C.

2.2.2 Methanogen Culture Conditions

Methanogen enumeration by serial dilution was carried out in rumen fluid medium (RF medium). RF medium litre^{-1} consisted of: rumen fluid (300ml) centrifuged twice at 12,000 rpm in a GSA rotor using a Sorvall centrifuge (Sorvall, USA) for 15 minutes, salts solution A (170 ml) (Section 6.2), salts solution 2B (170 ml) (Section 6.3), yeast extract (2.0 g), $NaHCO_3$ (5.0 g) and resazurin (100 µl) and distilled water (360 ml). Components were heated to boiling, and then cooled to room temperature on ice under O_2-free CO_2 for 15 minutes. After cooling L-cysteine HCl (0.5 g) was added to the solution and mixed thoroughly until dissolved. Aliquots of 8.8 ml were removed by drawing into a CO_2-flushed pipette and dispensing into CO_2-filled Hungate tubes. Tubes were autoclaved for 20 minutes at 121 °C (103 kPa) and stored at room temperature. To the RF medium, 0.1 ml of each penicillin/streptomycin (Section 6.5) and vitamin (Section 6.1) solutions were added. From each rumen sample, one ml of contents was diluted to a final dilution of 10^{10} through duplicate Hungate tube series containing nine ml of RF medium. Cultures were then pressurised with a H_2/CO_2 (80%:20%) mixture to 172 kPa. The dilution of 10^{10} was

chosen as it was above the highest known culturable methanogen densities of 10^9 cells ml^{-1} observed in young ruminants (Skillman *et al.*, 2004). Because of methanogen sensitivity to oxygen serial dilutions were performed in duplicate to give a more rigorous estimate of cell number than a single decimal dilution series would provide. Cultures were incubated at 39 °C for two weeks. Methanogen growth was assessed by methods outlined in Section 2.2.3.

2.2.3 Assessment of Methanogen Growth

Growth of methanogens in culture tubes was determined by at least one of three methods. Methanogens use the H_2/CO_2 gas (headspace) in Hungate tubes to produce methane and cause a drop in excess headspace in tubes relative to negative controls of uninoculated Hungate tubes. So initial determination of growth was measured by excess gas headspace pressure in Hungate tubes using a syringe 30 ml syringe fitted with a hypodermic needle. However, some non-methanogens are also able to cause a drop in tube excess gas headspace (Morvan *et al.*, 1994), so more definitive methods for assessment of methanogen presence were required. Determination of the presence of methane is specific for methanogen growth. Methane was measured in the gas headspace of Hungate tubes using a gas chromatograph (Aerograph 660, Varian Associates, USA), which confirmed the presence or absence of methanogens. Presence or absence of methanogens was also determined by epifluorescence microscopy under ultraviolet light. Methanogen cells epifluoresce under ultraviolet light because of the presence of the methanogenesis cofactor, F_{420} (Eirich *et al.*, 1978). Methanogens were visualised using a Vanox AHBT3 microscope (Olympus America Ltd., USA) fitted with a mercury burner and appropriate filters to excite the F_{420} cofactor.

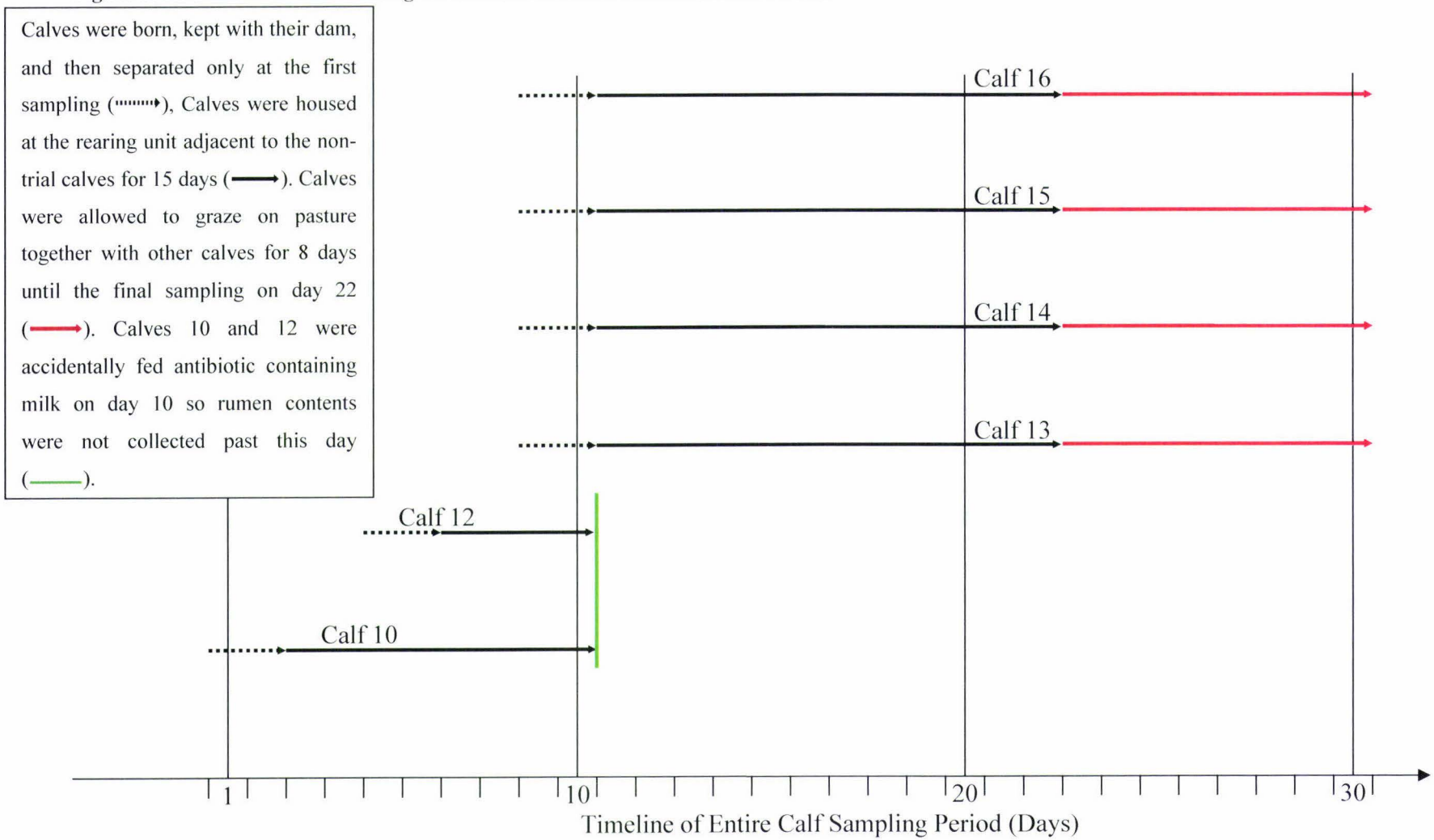
2.3 Results

2.3.1 Calf Trial

Two Friesian heifer dairy calves were born at the Massey University Number One Dairy Unit. The two calves were born over a period of 24 hours and sampled at a maximum of 48 hours after birth (Section 2.2.1). The calves were reared in a manner normally observed on a New Zealand dairy farm. Calves were born to grazing cows and maintained on pasture with their dam for a period before separation from their dam. Calves were housed in a calf-rearing unit, separate from other non-trial newborn calves. Initially, calf 10 (born day one, Figure 5) and calf 12 (born day five, Figure 5) were to be sampled over a three week period. However, on day 10 (Figure 5) these calves were fed inadvertently with milk contaminated by the antibiotic lincomycin, so were not sampled after this date. A limited analysis was performed on samples collected from calves 10 and 12 before antibiotic contamination. Then, four Friesian heifer dairy calves were born at the Massey farm over a 24 hour period (Figure 4) and sampled at a maximum of 48 hours after birth. These four calves replaced the two antibiotic-contaminated calves and were reared in the same manner as the previous two calves. Calves 13 and 14 were born in the daytime preceding the night when calves 15 and 16 were born. Consequently calves 13 and 14 were slightly older than calves 15 and 16, but all calves were considered to have been born at the same time (Figure 5). The four calves were first sampled on day 11 relative to the start of the trial with calf 10 (Figure 5), then sampled regularly by the method outlined in Section 2.2.1 and for the last time on day 31 (Figure 5). After calves 10 and 12 were contaminated with antibiotics and removed from the calf-rearing unit they only came into contact with calves 13, 14, 15 and 16 after all calves were transferred to pasture.

Rumen samples were successfully obtained from all calves on all attempts. Volumes of rumen contents obtained varied between calves on the same day of sampling and between samplings of the same calves on different days. Methanogens in freshly collected samples were enumerated as outlined in Section 2.2.2. One ml of rumen contents was set aside for DNA extraction (Section 4.2.4). After processing, rumen contents remained from 20 of the 44 samples.

Figure 5: Relative Birth and Housing Timeline of Newborn Friesian Heifer Calves.



2.3.2 Methanogen Culture Enumerations

Enumeration of methanogens by serial decimal dilutions was used to estimate the number of viable methanogens in rumen contents. The order of magnitude was estimated by the serial dilution of one ml of rumen contents into nine ml of RF medium through a series of Hungate tubes (Section 2.2.2). Overall, a total of 44 sets of duplicate dilutions from sample collections from calves 13, 14, 15, and 16 over 11 sampling days were prepared, inoculated, incubated, and analysed. Inoculation, incubation and analysis of rumen content serial dilutions in Hungate tubes revealed the presence of methanogens in all six calves by the first sampling, two days after birth, except in calf 10 (Table 1). The time of birth to first rumen sampling was a maximum 48 hours for all calves (Section 2.3.1). For all samples, two dilutions above the highest dilution that showed the presence of methanogens were incubated for a further two weeks at 39°C to check for further growth of methanogens. In all cases, no growth of methanogens was observed.

Table 1: Population Density of Methanogens in Young Calves. For example, methanogen density for Calf 13 six days after birth was $10^4/10^4$ represents a duplicate series that had methanogen growth to 10^4 and 10^4 cells per ml^{-1}. Data presented for each sampling day and calf is the order of magnitude for duplicate dilution series.

Days after Birth	Methanogen Density of Duplicate Samples (cell number/ml)					
	Calf 10	Calf 12	Calf 13	Calf 14	Calf 15	Calf 16
2	ND/ND	$10^1/10^1$	10^1/ ND	$10^1/10^1$	$10^2/10^1$	10^1/ND
3	$10^2/10^1$	$10^1/10^1$	10^1/ND	$10^1/10^1$	$10^3/10^2$	$10^2/10^1$
4	$10^2/10^3$	$10^1/10^1$	ND/ND	$10^1/10^1$	$10^1/10^1$	$10^1/10^1$
5	$10^4/10^5$	$10^3/10^2$	10^1/ ND	10^1/ ND	$10^1/10^1$	$10^2/10^2$
6	$10^5/10^6$	NC	$10^4/10^4$	ND/ND	ND/ND	$10^2/10^1$
7	$10^6/10^6$	NC	$10^2/10^2$	ND/ND	ND/ND	$10^2/10^2$
9	$10^7/10^7$	NC	$10^4/10^3$	ND/ND	ND/ND	$10^3/10^1$
11	NC	NC	$10^7/10^6$	ND/ND	ND/ND	$10^6/10^6$
13	NC	NC	$10^8/10^8$	$10^2/10^2$	$10^5/10^6$	$10^7/10^7$
15	NC	NC	$10^9/10^9$	10^2/ND	$10^4/10^5$	$10^8/10^6$
22	NC	NC	$10^7/10^7$	$10^7/10^7$	$10^8/10^8$	$10^9/10^6$

NC: Calves not sampled due to antibiotic contamination, ND: Methanogens not detected.

In the rumen of calf 10, methanogens were not detected until three days after birth (Table 1). However, tube headspace utilisation without methane production was observed in Hungate tubes in the duplicate dilution series from this calf at the first sampling. There was no evidence of tube headspace utilisation without methane production from enumeration tubes in calf 10 from the second and third sampling at days three and four after birth. In all other calves methanogens were present on the first day of sampling and were present at densities of 10^1 to 10^2 cells ml^{-1}. Headspace utilisation without methane production was also observed in calves 14 and 15 between six and 11 days after birth (Table 1).

Methanogen numbers may be underestimated in enumeration tubes because of their sensitivity to oxygen (Smith and Hungate, 1958). An example of a result involving possible methanogen sensitivity to oxygen is demonstrated by dilutions from calf 16 on day 22. The duplicate dilution series from produced estimates of 10^6 and 10^9 methanogen cells ml^{-1} from the same rumen contents sample, a difference in cell numbers of 10^3-fold. Other examples, such as calf 16 on days nine and 15, are evident between duplicate dilution series but do not differ more than 10^2-fold. Other duplicate serial dilutions produced only 10-fold differences between duplicate dilution series. In some cases methanogens were present at 10^1 cells ml^{-1} in only one of the two dilution series and not detectable in the duplicate dilution series. The data presented in Table 1 was graphed and is shown in Figure 6 as averages of four calves and as individuals in Figure 7 from the highest enumeration dilution showing methanogen growth in Table 2. Data from calves 10 and 12 were not included in Figure 6 or Figure 7 because too few samples were collected before these calves were fed antibiotic contaminated milk.

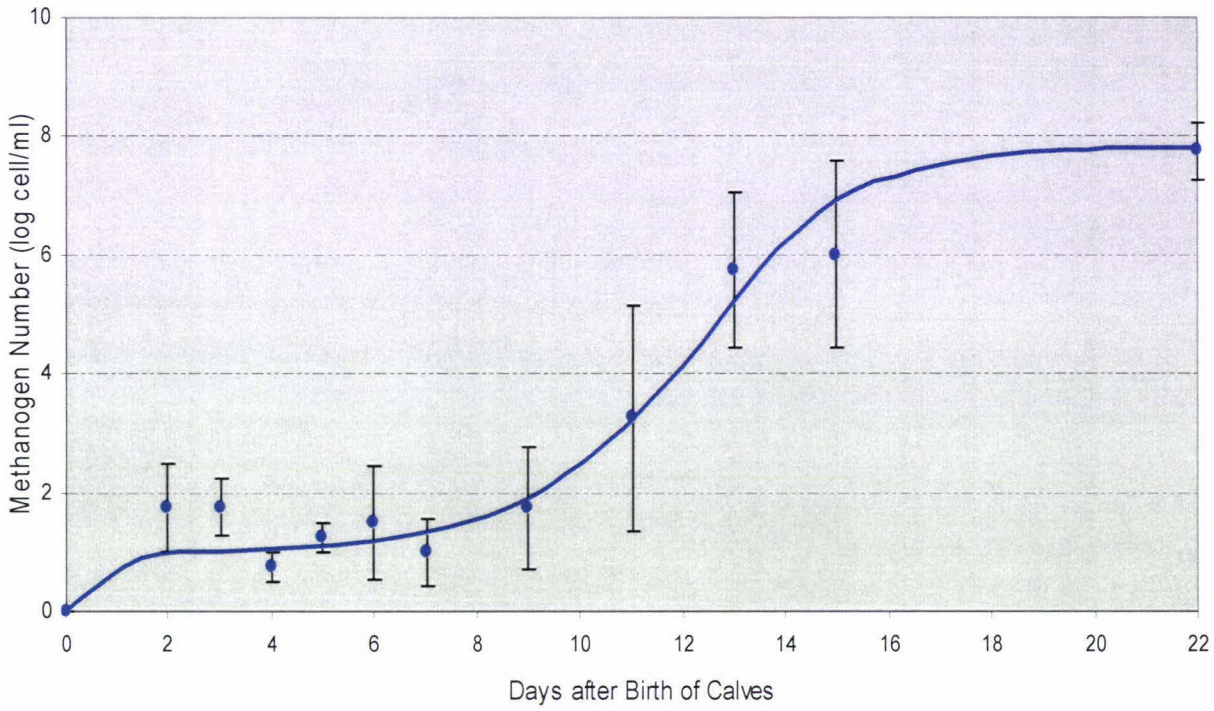


Figure 6: Methanogen Colonisation of Combined Data for Calves. Log value of the order of magnitude for the highest dilution positive for methanogen growth from Table 1 data of the four calves 13, 14, 15 and 16 is shown with a calculated line of best fit (———). The average methanogen cell density is expressed as the log cell number ml^{-1}. Error bars represent the standard error of the mean for each time point.

Newborn ruminants are born with a sterile gut with microbes colonising soon after birth (Fonty *et al.*, 1987). Therefore, Figure 6 shows that no methanogens were likely to be present in the rumen at birth. In all four calves (13, 14, 15 and 16) the initial colonisation had taken place before the first sampling at two days after birth. Following the initial colonisation the results support that a plateau period existed where culturable methanogens were relatively static at densities between 10^1 and 10^2 cells ml^{-1} of rumen contents between days two and nine. After the initial plateau period there was a rapid increase in methanogen numbers beginning on day nine and levelling off on day 13 (Figure 6). After the rapid increase, a slow but not significant increase in methanogen densities from about 10^7 to 10^8 cells ml^{-1} between days 15 to 22 was observed. When data from each individual calf was compared (Figure 7), it is apparent that the patterns of methanogen colonisation varied markedly between the individuals. Two distinctive

trends were observed in individual calves after day five. Methanogen densities in calves 14 and 15 declined and methanogens were not detected from days six to 11. After this period methanogen densities in calves 14 and 15 increased to 10^7 and 10^8 cells ml^{-1} respectively and were nearly equivalent to those of calves 13 and 16 at 22 days. In contrast to calves 14 and 15, calves 13 and 16 did not show methanogen disappearance between days six and 11. In these calves methanogen densities increased from 10^2-10^4 to 10^6-10^7 cells ml^{-1}. After day 11 methanogen densities in calves 13 and 16 increased steadily to 10^7 and 10^9 cells ml^{-1} by 22 days after birth.

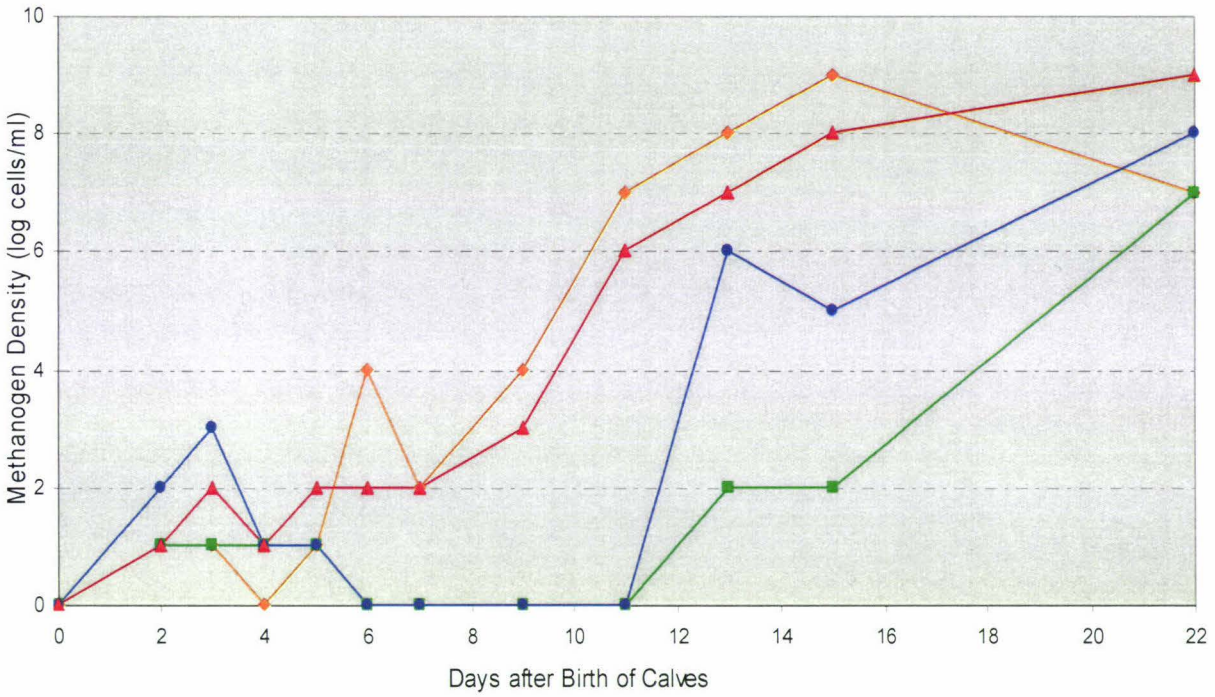


Figure 7: Individual Methanogen Colonisation Patterns of Newborn Calves. Methanogen densities from the rumen of calves 13(——), 14(——), 15(——), and 16(——) expressed as log of cell number ml^{-1}.

Thus, a feature of the colonisation by methanogens of calves 14 and 15 was the initial colonisation (Figure 7), followed by their apparent disappearance from days six to 11 and then subsequent re-colonisation. Based on this observation the data from the four calves can be divided into two groups (Figure 8).

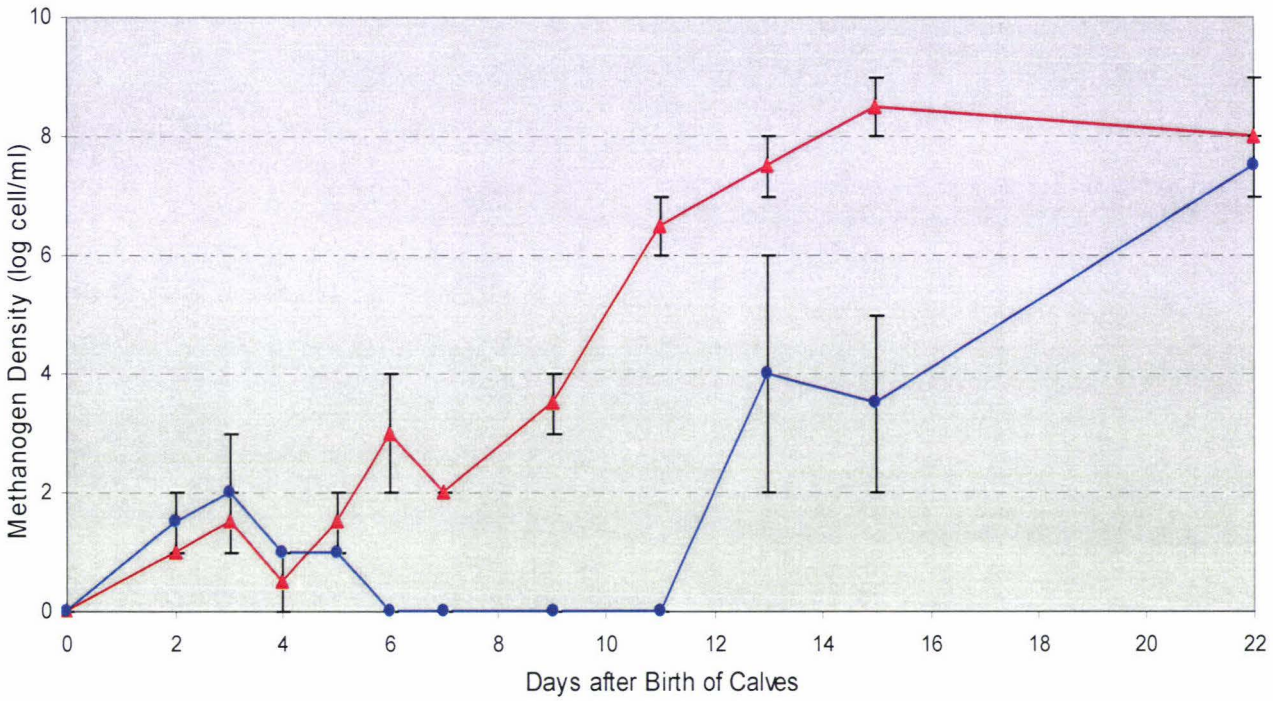


Figure 8: Methanogen Colonisation of Calves 13/16 and 14/15. For each set of calves, 13/16 (——) and 14/15 (——), the log of the highest dilution showing methanogen growth for each duplicate dilution series of each calf was selected. These dilutions were averaged for each pair of calves. Error bars indicate the standard error of the mean for each time point.

For the first five days after birth methanogen densities were similar between both pairs of calves (Figure 8). After this point there was a separation of methanogen densities of the two calf pairings. Between days six and 11 culturable methanogens disappeared from calves 14 and 15. But in calves 13 and 16, after an initial increase between days five and six, cell numbers increased to 10^7 cells ml^{-1} by day 11. After 15 days methanogen densities plateau at 10^9 cells ml^{-1}. In calves 14 and 15 after day 11 methanogen density rapidly increased to 10^4 cells ml^{-1} on average and reached 10^8 cells ml^{-1} by day 22. By 22 days of age methanogen densities in both pairs of calves were similar at levels of about 10^8 cells ml^{-1}. Methanogen disappearances from the rumen in calves 14 and 15 correlated with the appearance of what appeared to be a non-methanogenic hydrogenotroph. This unknown microbe produced a decrease in measurable headspace, without producing methane, and there was no fluorescence under epifluorescence microscopy confirming an

absence of methanogens. The identities of the hydrogen-utilising non-methanogenic microbes remain unknown. These isolates have been cryo-preserved for future study. Densities of the non-methanogenic hydrogenotrophs and methanogens in calves 14 and 15 are shown in Figure 9.

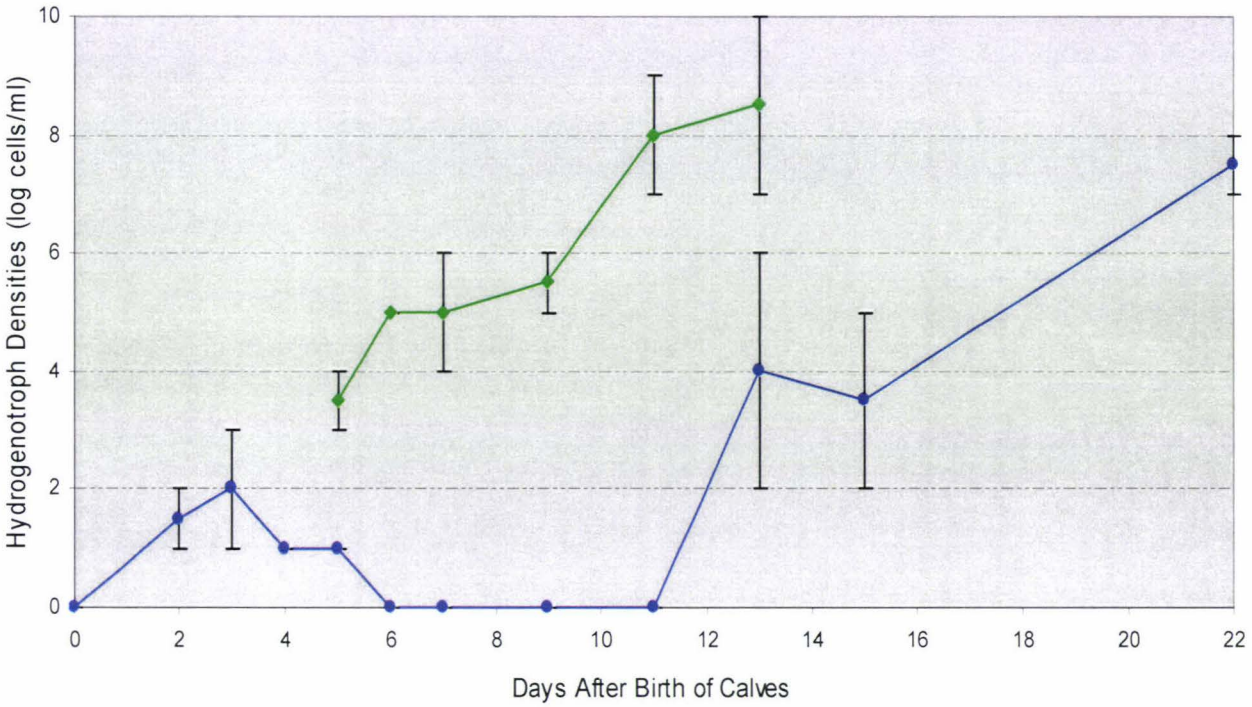


Figure 9: **Densities of Hydrogenotrophic Microbes in Calves 14 and 15.** Methanogenic hydrogenotrophs (——) and non-methanogenic hydrogenotrophs (——). Error bars indicate the standard error of the mean for each time point. Points indicate average hydrogenotroph densities in calves 14 and 15.

The non-methanogenic hydrogenotrophs were detected by headspace volume and gas chromatography analysis. These microbes could only be detected when they were in Hungate tube dilutions greater than the highest Hungate tube with positive methanogen growth. If methanogen growth was greater than non-methanogenic hydrogenotroph growth then methane production by methanogens masked any headspace utilisation by non-methanogens. Headspace utilisation by the non-methanogenic hydrogenotrophs was only detected between days 5 and 13 (Figure 9). Both headspace utilisation with methane production and headspace utilisation without methane production were detected in the same dilution series on days five and 13. In dilution series from days 5 and 13, headspace

utilisation without methane presence was observed in a higher dilution tube than tubes with headspace utilisation and methane presence. No growth of non-methanogenic hydrogenotrophs was detected between days zero to four and 15 to 22. The density of non-methanogen hydrogenotrophic cells increased from 10^4 cells ml^{-1} on day five when they were first detected to 10^8 cells ml^{-1} when they were last detected on day 13.

2.4 Discussion

Four heifer calves, calves 13, 14, 15 and 16, were sampled but not before two other heifer calves, calves 10 and 12, had been sampled and subsequently contaminated with the antibiotic lincomycin. Calves 10 and 12 were not sampled past day 10 (Figure 5) because of contamination with lincomycin. Lincomycin is not known to inhibit methanogen cells directly, but it can have an effect on bacteria, including the hydrogen-producing bacteria. The action of lincomycin on hydrogen producing bacteria is bacteriostatic, binding reversibly to the 50S ribosomal subunit, thus inhibiting protein synthesis (Merck, 1999). A decrease in the production of hydrogen used for methanogenesis may have caused a loss of methanogens from the developing rumen in calves 10 and 12. In a mature rumen 82% of methane production is said to be from microbe-derived hydrogen (Hungate *et al.*, 1970). Consequently, a reduction in hydrogen availability will play an important role in altering patterns of methanogen establishment in the rumen. Samples from calves 10 and 12 before antibiotic contamination were still included in some analyses with calves 13, 14, 15, and 16 (Chapters 4 and 5).

The pH values of all samples were above pH 5.0 (Table 21, Chapter 7), this value was used as minimum value for methanogen viability. However, lower pH values were observed in calves 14 and 15 between six and 11 days after birth compared to other samples from these calves. These low values were similar to values observed in other calves when methanogens were cultured. For example, methanogens were cultured from calf 16 nine days after birth from a sample with a pH of 5.8 and was equivalent to the pH of 5.8 of calf 15 on day nine when no methanogens were cultured.

Methanogens were first detected at two days after birth in calves 10, 13, 14, 15 and 16. Methanogens did not colonise the rumen of calf 12 until three days after birth. Minato *et al.* (1992) was able to culture methanogens from calves at 24 hours after birth. Skillman *et al.* (2004) also demonstrated methanogen colonisation by 24 hours in lambs. Morvan *et al.* (1994) found that methanogens had colonised before 30 hours of age in lambs. The time of first sampling in this study was greater than times of the first sampling in other studies because calves were not available to be sampled until 48 hours after birth (Section 2.2.1). Consequently an exact time to first methanogen colonisation in this study could not be determined. Uncertainties about the age of calves (Section 2.2.1) may mean that the first methanogen colonisation was not greatly different to those found by Minato *et al.* (1992), Morvan *et al.* (1994) and Skillman *et al.* (2004). Further experiments to determine more precisely the time to first colonisation did not seem necessary, as little new information would be gained from sampling nearer to birth. It is likely that the methanogens initially colonising the rumen were those detected two days after birth.

The biphasic trend of methanogen colonisation of calves observed in this study was different to the trends observed in calves of Minato *et al.* (1992) and lambs of Skillman *et al.* (2004). Minato *et al.* (1992) found that methanogens, once established by 24 hours after birth, colonised slowly and were at similar levels to those found in calves of this study at three weeks of age. Methanogens enumerated by Skillman *et al.* (2004) showed a trend similar to methanogens in calves 13, 14, 15 and 16 from 13 to 22 days of birth (Figure 10). The trend described by Skillman *et al.* (2004) was also similar to studies of methanogen colonisation in lambs of Fonty *et al.* (1986), Fonty *et al.* (1987) and Morvan *et al.* (1994). The constantly increasing methanogen colonisation trends from Minato *et al.* (1992) and Skillman *et al.* (2004) are different to the trend observed for this study, especially in calves 14 and 15 (Figure 10). The lambs of Skillman *et al.* (2004) were run with their dam, a potential source of methanogens transfer to the lambs. Calves from this study and calves of Minato *et al.* (1992) were all reared indoors separate from their respective dams and both showed a similar decreased level of colonisation by methanogens soon after birth when compared to lambs (Figure 10). The separation from their respective dams and being housed indoors is likely to contribute to the decreased

methanogen colonisation rate in both sets of calves. Calves 13, 14, 15 and 16 were housed together but separate from non-trial calves, whereas calves in the study by Minato *et al.* (1992) were housed both separate from each other and from non-trial calves. This may help explain the greater densities of methanogens observed in calves 13, 14, 15 and 16 from day nine onwards (Figure 10).

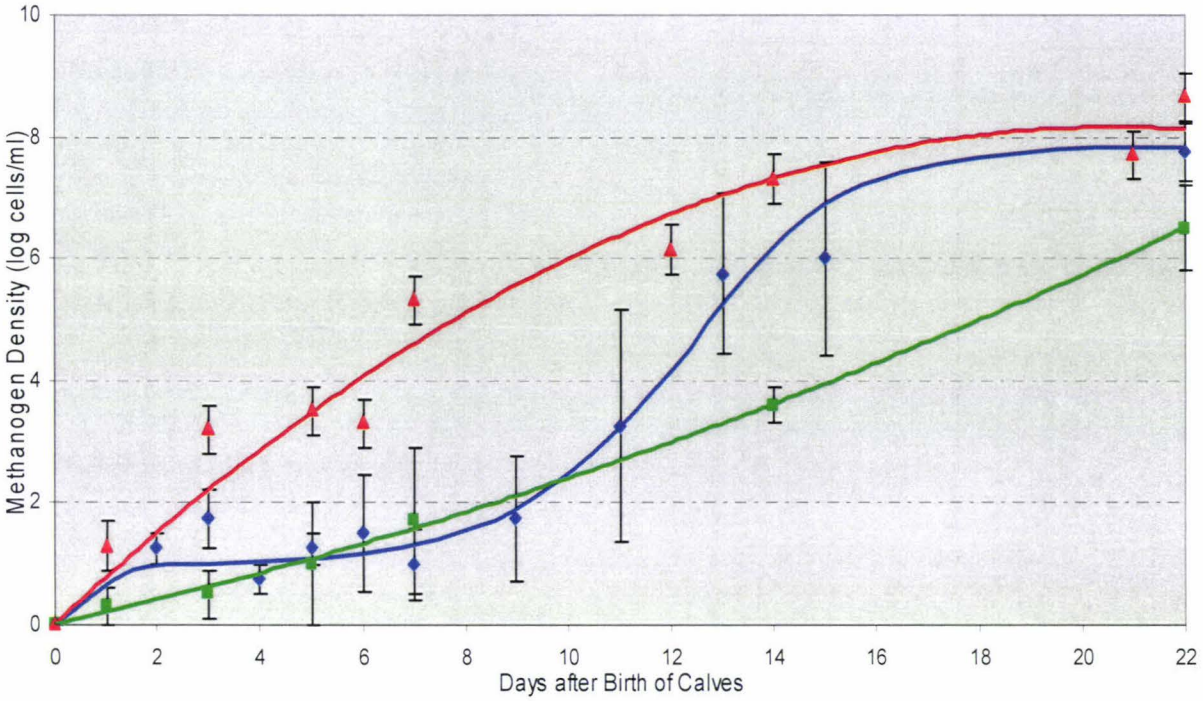


Figure 10: Comparison of Methanogen Colonisation of Calves and Lambs. Methanogen colonisation trends from calves 13, 14, 15 and 16 grouped (——) are compared with lambs from Skillman *et al.* (2004) (——) and calves of Minato *et al.* (1992) (——). Error bars represent the standard error of the mean for each time point.

However, the biphasic colonisation pattern in calves 13, 14, 15 and 16, illustrated in Figure 8, shows the average methanogen density of the four calves is exaggerated by the apparent reduction in colonisation between days six and 11 of calves 14 and 15. The non-detection of methanogens in enumeration tubes from calves 14 and 15 between days six and 11 is in contrast to the detection by PCR of archaea and sometimes methanogens from remaining gut contents shown in Section 4.3.4. Oxygenation of the samples may be one explanation, but the chances of oxygenating only samples from calves 14 and 15 and not from calves 13 and 16 over the six to 11 day period are small. A more likely cause

was the growth of the non-methanogenic hydrogenotrophs in culture tubes before methanogens were able to grow.

The identities of the non-methanogenic hydrogenotrophs found in calves 14 and 15 have not been determined. Likely candidates for this hydrogenotroph are acetogenic or sulphate-reducing bacteria. Some sulphate-reducing bacteria use hydrogen as an electron donor for the reduction of sulphate to H_2S (Gibson, 1990). Minato *et al.* (1992) was able to culture sulphate-reducing bacteria from the rumens of newborn calves 1 day after birth. In the rumen of newborn lambs Morvan *et al.* (1994) did not detect sulphate-reducing bacteria until 8 days after birth, at which point they were at densities of 10^5 cells ml^{-1}. In contrast, levels of sulphate-reducing bacteria in mature sheep are low, less than 10^3 cells ml^{-1}, and have been described as non-detectable in cattle (Morvan *et al.*, 1996). Low levels of sulphate reducing bacteria found in mature ruminants are likely to be a consequence of low sulphur levels available for sulphate reduction in the mature rumen (Gibson, 1990).

Acetogenic bacteria use hydrogen as an electron donor for the reduction of carbon dioxide to acetate (Daniel *et al.*, 1990). To date acetogens have been isolated from both mature (Leedle and Greening, 1988) and young ruminants (Rieu-Lesme *et al.*, 1996). Acetogen densities in young ruminants have been identified as being at 10^6 cells ml^{-1} at 20 hours after birth in lambs (Morvan *et al.*, 1994). To date, only four species of ruminal acetogens from young ruminants have been purified (Joblin, 1999). Acetogen populations in adult ruminants have been found to be at densities of 10^8 cell ml^{-1} (Leedle and Greening, 1988). However, lower and variable densities in adult ruminants have been observed ranging from barely detectable numbers to 10^6 cells ml^{-1} (Joblin, 1999). These results suggest that acetogens and/or sulphate-reducing bacteria are likely to be the non-methanogenic microbes that used the hydrogen in enumeration tubes.

The later appearance of methanogens in calf 10 compared to other calves (Table 1) and appearance of non-methanogenic hydrogenotrophs was similar to events observed in calves 14 and 15. In the rumens of calves 14 and 15 disappearances and appearances of

methanogen and non-methanogenic bacteria between days six and 11 were observed (Figure 8). This disappearance of methanogens corresponded with an increase in non-methanogenic hydrogenotrophs (Figure 9). After the disappearance of culturable methanogens in calves 14 and 15 a large increase (10^6 cells ml^{-1}) in methanogens was observed in calf 15 with a smaller increase observed in calf 14 (Figure 7). In contrast to the non-detection by culture methods, methanogens were detected by PCR on day 11 in the gut contents of calf 15 (Table 19, Chapter 4).

Differences in the levels of cultivated methanogens between calves were not evident at the final sampling with all four calves having similar levels of about 10^8 $cells^{-1}$ ml rumen contents. This result is similar to the studies of Skillman *et al.* (2004) and Morvan *et al.* (1994) who determined methanogen densities to be about 10^8 or 10^9 cells ml^{-1} in young lambs at three weeks of age. All of these densities are higher than the 10^6 to 10^7 cells ml^{-1} found in calves between three and 10 weeks after birth by Minato *et al.* (1992), but are unlikely to be significantly different. Densities found in this study are all comparable with densities of 10^9 cells ml^{-1} detected in culture studies from mature ruminants (Smith and Hungate, 1958; Miller *et al.*, 1986).

3 *Isolation of Methanogens from Rumen Contents*

3.1 Introduction

Methanogens inhabit environments that are highly anaerobic. The conditions prevailing in anaerobic environments vary widely, and offer a large diversity of conditions suitable for methanogen growth and survival. Even though methanogens inhabit a wide variety of anaerobic environments and over 83 species have been isolated, methanogens are known to only utilise relatively few substrates for their energy requirements (Garcia *et al.*, 2000). Because of their specialist requirements for habitat, substrates (Biavati *et al.*, 1988) and undefined growth factors (Jarvis *et al.*, 2000) methanogens have been, and still are, difficult to culture. The time taken to grow and isolate methanogens is often a difficulty in culture studies. In the case of ruminal methanogens, single colonies on agar have been observed to take up to 16 days to appear after inoculation (Jarvis *et al.*, 2000). Also, methanogens are known to lose viability without an obvious cause during both culturing and isolation (Jarvis *et al.*, 2000). Once cultured, isolation of methanogens to purity can also be difficult. Bryant *et al.* (1967) separated *Methanobacillus omelianskii* (later *Methanobacterium bryantii* M.O.H (Balch and Wolfe, 1981)) from close association with a eubacterium 27 years after it was first described as pure by Barker (1940).

Previous to the specialised techniques that were developed for the culture of strict anaerobes relatively few methanogens were cultivated (Hungate, 1966). These improved anaerobic techniques have allowed a much greater number of methanogen species to be isolated and characterised. The use of hydrogen and carbon dioxide as a substrate by methanogens is initially used as a selection method for methanogen growth. The combination of hydrogen and carbon dioxide as a growth substrate and selected antibiotics against eubacteria potentially discriminates against eubacteria, eukaryotes and other non-methanogenic archaea.

The isolation of cultured methanogens from serial decimal dilution tubes in this study aimed to demonstrate anaerobic culture techniques and to provide isolated methanogens for later identification purposes.

3.2 Methods

3.2.1 Media

Media used for the isolation of methanogens consisted of RF medium (Section 2.2.2) and agar roll tubes (Section 3.2.2). An additional medium used for the isolation of methanogens was MET medium. MET medium litre^{-1} consisted of: NaOH (4.0 g), yeast extract (2.0 g), tryptone (2.0 g), mercaptoethanesulfonic acid (500 mg), NH_4Cl (1.0 g), $K_2HPO_4 \cdot 3H_2O$ (0.2 g), $MgCl_2 \cdot 6H_2O$ (1.0 g), $CaCl_2 \cdot 2H_2O$ (0.4 g), Balch's trace elements (10 ml) (Section 6.8), hemin (0.1% solution) (1.0 ml) and distilled H_2O (990 ml). Initially, NaOH was dissolved in distilled H_2O under O_2-free CO_2. All other components were added to this solution once the NaOH had dissolved and were mixed until dissolved under O_2-free CO_2. The solution was heated until boiling, and then cooled to room temperature on ice under O_2-free CO_2 for 15 minutes. After cooling the pH was adjusted to 7.2 with NaOH. Aliquots of 4.5 ml were removed by drawing up into a CO_2-flushed pipette and dispensed into CO_2-filled Hungate tubes. Tubes were autoclaved for 20 minutes at 121 °C (103 kPa) and stored at room temperature. Once autoclaved 0.2 ml of $Na_2S \cdot 9H_2O$ (Section 6.7), 0.1 ml of 4 M tri-methylamine solution and 0.1 ml of 5 M sodium acetate solution were added anaerobically and aseptically by syringe. A variation of MET medium, MR, was also used for the isolation of methanogens. MR medium was similar to MET medium but contained 10 ml of centrifuged rumen fluid (Section 2.2.2) litre^{-1}. Also, the tryptone and yeast extract were removed. MR medium was prepared by the same method as MET medium.

In order to check the purity of the putatively pure methanogen cultures, PYG medium was used to select for non-methanogens. PYG medium litre^{-1} consisted of: tryptone (5.0 g), peptone (5.0 g), yeast extract (10.0 g), beef extract (5.0 g), glucose (5.0 g), K_2HPO_4 (2.0 g), Tween 80 (1.0 ml), resazurin (100 µl), salts solution A (Section 6.2) (20 ml), salts solution 2B (Section 6.3) (20 ml), hemin (0.05% solution) (10 ml), distilled H_2O (950 ml), vitamin K_1 solution (0.5% solution) (200 µl) and L-cysteine HCl (0.5 g). All components except hemin, vitamin K_1, L-cysteine HCl were added to the distilled H_2O. Components were heated until boiling, and then cooled to room temperature on ice under

O_2-free CO_2 for 15 minutes. After cooling hemin, vitamin K_1 and L-cysteine HCl were added to the solution and mixed thoroughly until dissolved. Aliquots of nine ml were removed by drawing up into a CO_2-flushed pipette and dispensed into CO_2-filled Hungate tubes. Tubes were autoclaved for 20 minutes at 121 °C (103 kPa) and stored at room temperature.

3.2.2 Roll Tube Isolations

After the isolation of methanogens using RF medium from serial dilutions, isolates were purified on agar roll tubes. Agar roll tubes were prepared by the addition of 15 g of bacteriological agar (Danisco, New Zealand) litre^{-1} to RF medium (Section 2.2.2). Instead of cooling on ice after boiling, the agar RF medium was cooled to 50 °C in a water bath and L-cysteine HCl (0.5 g litre^{-1}) added and dissolved. Aliquots of 4.5 ml were removed by drawing into a CO_2-flushed pipette and dispensed into CO_2-filled Hungate tubes. The medium was autoclaved for 20 minutes at 121 °C (103 kPa) and stored at room temperature. Roll tubes were placed in boiling water to melt the agar, cooled to 43 °C, and 0.1 ml of vitamin solution added to each molten agar tube. From each serial decimal dilution (Section 2.2.2), 0.5 ml of culture from the highest dilution tube showing growth was inoculated into the molten agar roll tubes and the tubes rolled under cold water. Molten agar was diluted 10-fold through four roll tube dilutions to obtain single methanogen colonies. The roll tubes were pressurised with H_2/CO_2 (80%:20%) and incubated for three weeks at 39 °C. Methanogen growth was analysed by methods outlined in Section 2.2.3. Representative colony morphologies were identified using a dissecting microscope from agar roll tubes positive for methane. Bent Pasteur pipettes were used to pick representative colonies from roll tubes under a stream of O_2-free CO_2 using the method of Hungate (1966) and sub-cultured into Hungate tubes containing nine ml of RF medium. Picked colonies were incubated at 39 °C for two weeks. After two weeks incubation headspace analysis of culture tubes by gas chromatography confirmed which isolates had formed methane. Cultures not showing methane formation were incubated for a further week at 39 °C to show further methane positive cultures. Isolates were stored based on headspace utilisation. Those tubes showing methane formation or headspace utilisation without methane formation were stored with DMSO (Section 6.10)

cryo-protectant (5% final concentration) at -85 °C. Selected methanogens were later removed from -85 °C storage for purification. Tubes showing no headspace utilisation continued to be incubated at 39 °C and checked periodically for methane formation.

3.2.3 Microscopy

Methanogen cultures were observed using a Reichart Diavar (Austria) phase contrast microscope and also Gram stained. The highest magnification used was 1000x using immersion glycerol lenses. Methanogen growth was also observed using a Vanox AHBT3 microscope (Olympus America Ltd., USA) to observe fluorescent cells when cultures were excited at 420 nm (1000x magnification using immersion oil). Fluorescence microscopy is an important technique for the observation of methanogens. Fluorescence of methanogen cells is primarily due to a cofactor, F_{420}, in the cell wall (Eirich *et al.*, 1978). Co-factor F_{420} is involved in the addition of hydrogen ions of the terminal step in methanogenesis. Because F_{420} is only involved with methanogenesis it is unique to methanogens (Deppenmeier *et al.*, 1999) and allows methanogen cells to be differentiated from non-methanogen cells using fluorescence microscopy. Photomicrographs of microbes were taken using a Camedia C-5050ZOOM digital camera (Olympus America Ltd., USA).

3.3 Results

The highest dilution tubes that produced methane (Table 1) for all calves on all days were sub-cultured into agar roll tubes and pressurised with H_2/CO_2. After incubation of tubes at 39 °C for three weeks, colonies were picked from the surface by the method of Hungate (1966). In total, 110 colony picks were re-inoculated into broth culture. These 110 isolates represented the majority of sampling days for all calves. After three weeks incubation at 39 °C the 110 cultures were analysed for the presence of methanogens by analysis of excess tube headspace volume, gas chromatography for methane, and epifluorescence (Section 2.2.3). Of the 110 isolates, 31 were positive for methane. Phase contrast microscopy of some of the 31 methane positive cultures revealed the presence of more than one cell phenotype. Epifluorescence microscopy had revealed only one type of methanogen phenotype in the same cultures.

From the 31 methanogen-enriched cultures, 11 were selected for purification on the basis that these represented a range of samples and calves. Cultures of interest were those from the first day of sampling (day two from all calves, Figure 6 and Figure 8). Others of interest were those present in calves 14 or 15 just before the disappearance of culturable methanogens between days six and 11. The most numerous methanogen species present at the final sampling was also of interest from calves 13, 14, 15 and 16. Methanogens cultured from calves 10 and 12 before antibiotic contamination, were also of interest. All methanogens identified for isolation by methods outlined in Section 3.2.2 are listed in Table 2.

A further 28 isolates of the 110 showed excess hydrogen tube headspace utilisation but no methane was produced and there was no cell epifluorescence. This result suggested that non-methanogenic hydrogenotrophs observed in culture enumerations (Section 2.3) were present among the colonies picked. These 28 non-methanogenic hydrogenotrophs plus the remaining methanogens, not selected for purification, were stored frozen at -85 °C with DMSO (Section 6.10) (5% final concentration) for future analyses.

Table 2: Selected Methanogen Isolates. The 11 methanogens of interest were initially characterised and tabulated. Methanogens (M), irregular cocci (IC), long rods (LR), short rods (SR), small cocci (SC), large cocci (LC) and coccus (C) were present.

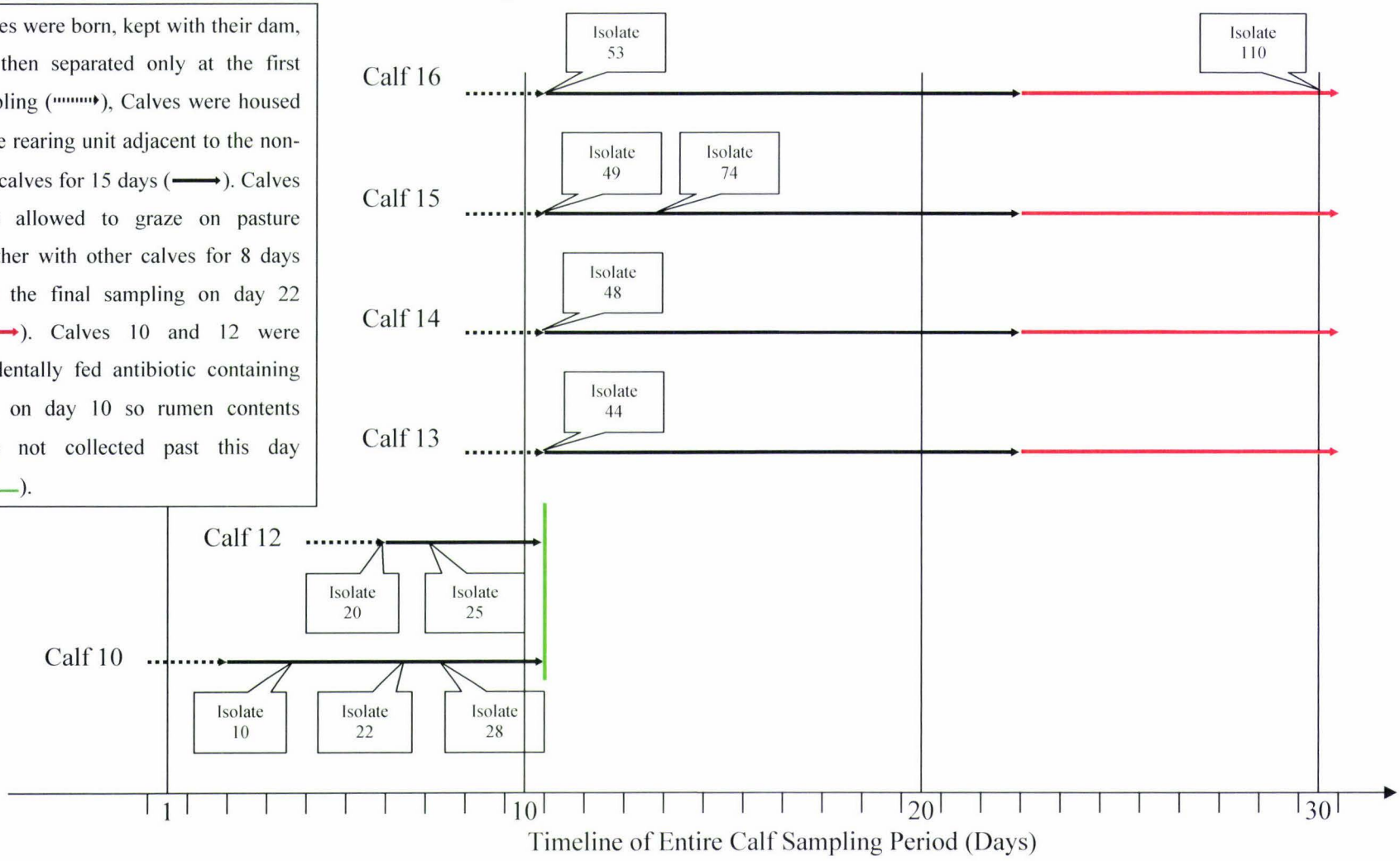
No. of Colony Pick	Calf Number	Days after Birth	Tube Dilution	Microbes Initially Present
10	10	4	-2	IC (M^2), SR
20	12	2	-1	IC (M^2), SR, SC
22	10	7	-5	IC (M^2), SR, SC
25	12	3	-1	LR (M^2), SR, SC
28	10	9	-7	LR (M^2), SR, SC
44	13	2	-1	IC (M^2), SC, SR, LR
48[1]	14	2	-1	IC (M^2), SC, SR, LR
49	15	2	-2	IC (M^2), SC, SR, LR
53	16	2	-1	IC (M^2), SC, SR, LR
74	15	5	-1	LR (M^2), SR, SC, LC
110[1]	16	21	-9	C (M^2), SR, LR

[1]Initially cultured from colony picks but lost viability upon sub-culturing. [2]Methanogen identification by epifluorescence microscopy examination.

Figure 11 describes the timeline when calves were born in relation to each other, adapted from Figure 5, overlaid with the times when methanogens from Table 2 were cultured. Methanogens of interest were those first colonising the rumen of calves; methanogen isolates 20, 44, 48, 49 and 53 were all obtained from the first sampling of calves after birth. Methanogen isolates 10, 22, 25, 28 and 74 were selected in an effort to represent a possible succession of the methanogens colonising the developing rumen. The final methanogen selected for isolation was colony pick 110 because it was cultured on the last sampling day.

Figure 11: Calf Trial Period Overlaid with Methanogen Isolates.

Calves were born, kept with their dam, and then separated only at the first sampling (·····▸), Calves were housed at the rearing unit adjacent to the non-trial calves for 15 days (——▸). Calves were allowed to graze on pasture together with other calves for 8 days until the final sampling on day 22 (——▸). Calves 10 and 12 were accidentally fed antibiotic containing milk on day 10 so rumen contents were not collected past this day (——).



Of the 31 methane-positive isolates cultured, only one, isolate 110 from calf 16 day 22 was obtained from calves nine days or greater after birth. However, isolate 110 did not grow until at least four weeks of incubation had elapsed and subsequently lost viability during purification. A major problem encountered with the cultured isolates was the contamination of tubes with non-methanogens. Examination using phase contrast and ultra violet-light microscopy examination (Section 3.2.3) revealed microbes that were non-methanogenic. The contamination with non-methanogens was a continual source of technical difficulty due to an apparent close association between methanogens and non-methanogens.

To establish a method for purifying methanogens from these enrichments, isolate 74 was investigated (Table 2). Microscopic examination of isolate 74 revealed non-methanogen microbes were present (Table 2). A single cell-type fluoresced when isolate 74 was examined using epifluorescence microscopy. The auto-fluorescing cells were long rods. They were either single cells, or chains of several cells. No other cell shapes auto-fluoresced, suggesting that only one type of methanogen was present. In an effort to purify this methanogen an inoculum from isolate 74 was diluted through RF medium. After one-week of incubation at 39 °C, both non-methanogens and methanogens were found in the highest dilution showing growth. A range of antibiotics were tested for their ability to inhibit growth of bacteria in the mixture. After incubation for one week, an inoculum of isolate 74 was diluted through RF medium containing one of the antibiotics clindamycin, rifamycin, hygromycin B or ampicillin at concentrations listed in Section 6.6. Cultures lacking antibiotics or an inoculum of isolate 74 were included as controls. All tubes were pressurised with H_2/CO_2 and incubated at 39 °C for three days. Examination of the clindamycin, rifamycin and hygromycin-B-treated cultures revealed that non-methanogens were still present in the highest dilutions also showing methanogen growth (Table 3). Use of these antibiotics was discontinued.

Table 3: Attempted Antibiotic Selection of Methanogens. Tubes with no microbes present were recorded as 'No Growth'. Gas volumes of 15 ml were found in control tubes without antibiotics and/or inoculum. Morphologies of microbes were recorded as long rods (LR), short rods (SR), large cocci (LC) or small cocci (SC).

	10^{-1} dilution	10^{-2} dilution	10^{-3} dilution	10^{-4} dilution	10^{-5} dilution	10^{-6} dilution	10^{-7} dilution	10^{-8} dilution	10^{-9} dilution	10^{-10} dilution
Clindamycin (20 μg ml^{-1})	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	No Growth	No Growth	No Growth
Excess Gas Volume	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	15 ml	15 ml	15ml
Methane	+	+	+	+	+	+	+	-	-	-
Rifamycin (20 μg ml^{-1})	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	No Growth	No Growth	No Growth
Excess Gas Volume	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	15 ml	15 ml	15ml
Methane	+	+	+	+	+	+	+	-	-	-
Hygromycin B (20 μg ml^{-1})	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	No Growth	No Growth	No Growth
Excess Gas Volume	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	15 ml	15 ml	15ml
Methane	+	+	+	+	+	+	+	-	-	-
Ampicillin (50 μg ml^{-1})	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, LC, SC	LR, LC, SC	LR, LC, SC	LR, LC, SC	LR, LC, SC	No Growth	No Growth
Excess Gas Volume	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	15 ml	15ml
Methane	+	+	+	+	+	+	+	+	-	-

Table 4: Attempted Antibiotic Selection of Methanogens with Ampicillin. Tubes with no microbes present were recorded as 'No Growth'. Gas volumes of 15 ml were found in control tubes without antibiotics and/or inoculum. Morphologies of microbes were recorded as long rods (LR), short rods (SR), large cocci (LC) or small cocci (SC).

Dilution	10^{-1} dilution	10^{-2} dilution	10^{-3} dilution	10^{-4} dilution	10^{-5} dilution	10^{-6} dilution	10^{-7} dilution	10^{-8} dilution	10^{-9} dilution	10^{-10} dilution
Ampicillin (500 μg ml^{-1})	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, SR, LC, SC	LR, LC, SC	LR, LC, SC	LR, LC, SC	No Growth	No Growth	No Growth
Excess Gas Volume	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml	15 ml	15 ml	15 ml
Methane	+	+	+	+	+	+	+	-	-	-

However, ampicillin did have an effect, with the disappearance of the short rods from the 10^4 to 10^8 dilutions relative to other antibiotic treated dilutions where long rods were seen to be present (Table 3). The effect of ampicillin in suppressing the short rods suggested that an increase in ampicillin concentration could possibly better inhibit the contaminating bacteria. Therefore ampicillin at 10-times the previous concentration (Table 3) was added to RF medium and tubes were inoculated with the 10^8 dilution from the ampicillin-treated dilution series (Table 3). Cultures were pressurised with H_2/CO_2 and incubated at 39 °C for three days. After incubation the 10-times concentration ampicillin culture was serially diluted 10-fold into a RF medium dilution series, pressurised with H_2/CO_2 and incubated at 39 °C for three days. The results showed that the microbial compositions of the 10-times concentration ampicillin dilution series were similar to those of the 1-time ampicillin concentration series (Table 3). In the 10-times ampicillin series (Table 4) non-methanogenic short rods were present to the 10^{-4} dilution, with methanogens and other non-methanogens showing growth to the 10^{-7} dilution. The reappearance of short rods in the dilution series (Table 4) after being inoculated from a tube apparently free of short rods indicated that the short rods were not sensitive to a high ampicillin concentration. It appears that the short rods present at a low level were not easily detectable by microscopy.

Following the partial success of ampicillin, further efforts were made to remove non-methanogens from the mixed cultures. Phase contrast microscopy suggested that the large coccus may be a ruminal mycoplasma. Information from Naylor (1998) suggested that mycoplasmas can be sensitive to the antibiotics tetracycline and lincomycin. Isolate 74 was therefore challenged with tetracycline and lincomycin at concentrations listed in Section 6.6. RF medium containing tetracycline or lincomycin was inoculated with the 10^{-7} dilution from the ampicillin-treated dilution series from Table 4. The culture were pressurised with H_2/CO_2 and incubated at 39 °C for three days. The results of this incubation are shown in Table 5.

Table 5: Antibiotic Selection of Methanogens over Non-Methanogens. The morphologies of microbes present in cultures were recorded as long rods (LR), short rods (SR), large cocci (LC) or small cocci (SC). Tubes with no microbes present were identified as 'No Growth'.

	10^{-1} dilution	10^{-2} dilution	10^{-3} dilution	10^{-4} dilution	10^{-5} dilution	10^{-6} dilution	10^{-7} dilution	10^{-8} dilution	10^{-9} dilution	10^{-10} dilution
Tetracycline	LR, SR, LC	LR, SR, LC	LR, SR, LC	LR, SR, LC	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth
Headspace Volume	4 ml	4 ml	4 ml	4 ml	15 ml	15 ml	15 ml	15 ml	15 ml	15 ml
Methane	+	+	+	+	-	-	-	-	-	-
Lincomycin	LR only	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth
Headspace Volume	4 ml	15 ml	15 ml	15 ml	15 ml	15 ml	15 ml	15 ml	15 ml	15 ml
Methane	+	-	-	-	-	-	-	-	-	-

Table 6: Agar Roll Tube Series from Rumen Fluid Media. Densities of colonies from each agar roll tube were recorded as Lawn (too dense to pick), many colonies (too dense to pick), single colonies (could be picked from). Tubes with no colonies evident were identified as No Growth.

	10^{-1} dilution	10^{-2} dilution	10^{-3} dilution	10^{-4} dilution	10^{-5} dilution	10^{-6} dilution
Colony Morphology	Small brown irregular	Small brown irregular	Small brown irregular	Small brown irregular	Small brown irregular	No growth
Methane (+/-)	+	+	+	+	+	-
Colony Density	Lawn	Lawn	Many Colonies	Single Colonies	Single Colonies	No growth

Table 7: Morphologies of Re-Picked Colonies of Isolate 74. The morphologies of microbes present in cultures were recorded as long rods (LR), short rods (SR) or large cocci (LC). Tubes with no microbes present were identified as 'No Growth'.

	Colony Pick 1	Colony Pick 2	Colony Pick 3	Colony Pick 4	Colony Pick 5	Colony Pick 6	Colony Pick 7
Phenotypes Present	LR, SR	LR, SR	LR, SR	LR, SR	No Growth	No Growth	No Growth
Headspace Volume	4 ml	4 ml	4 ml	4 ml	15 ml	15 ml	15 ml
Methane	+	+	+	+	-	-	-

Table 8: Agar Roll Tube Series from MET Media. Densities of colonies from each agar roll tube were recorded as Lawn (too dense to pick), many colonies (too dense to pick), single colonies (could be picked from). Tubes with no colonies evident were identified as 'No Growth'.

	10^{-1} dilution	10^{-2} dilution	10^{-3} dilution	10^{-4} dilution	10^{-5} dilution	10^{-6} dilution
Colony Morphology	Small brown irregular	Small brown irregular	Small brown irregular	Small brown irregular	Small brown irregular	No growth
Methane (+/-)	+	+	+	+	+	-
Colony Density	Lawn	Lawn	Many Colonies	Single Colonies	Single Colonies	No growth

Microscopic examination and headspace analysis of dilution series from the tetracycline-treated culture showed growth of methanogens and bacteria to the 10^{-4} dilution (Table 5). The long rod, short rod and large coccus were present in the highest dilution showing growth while the small coccus was absent. In contrast, the lincomycin-treated cultures showed growth only in the 10^{-1} dilution tube. The presence of methane in the 10^{-1} dilution tube indicated the presence of a methanogen. The absence of the short rod, large coccus, and small coccus, suggested that the long rod was the methanogen already identified. The long rod was affirmed as a methanogen when the cells fluoresced under epifluorescence (Table 2). The methanogen in the 10^{-1} dilution tube was diluted 10-fold in agar RF medium to obtain single methanogen colonies to pick from the agar surface. From the 10^{5} dilution tube of agar RF medium seven colonies were picked into separate Hungate tubes containing RF medium. Tubes were pressurised with H_2/CO_2 and incubated at 39 °C for 14 days. Microscopic examination of RF medium containing picked colonies revealed the presence of a short rod in addition to the long rod-shaped methanogen.

There appeared to be an association between the long rod methanogen and the short rod bacterium. Another possibility was that the bacterium may have only been growing on the rumen fluid in the RF medium. To exclude this, methanogen MET medium was selected to isolate the long rod methanogen from the bacteria, as it contains no rumen fluid (Section 3.2.1). The first colony pick from the seven colony picks in Table 7 was inoculated into MET medium, pressurised with H_2/CO_2 and incubated at 39 °C for three days. Microscopic examination of the MET tube showed that the short rod was present at high levels. Thus, a further attempt to purify the methanogen, isolate 74, was made using MR medium (Section 3.2.1). The removal of yeast extract, tryptone and the addition of rumen fluid to a final concentration of only 1.0% (v/v) are the differences between the MET and MR media. Further sub-culturing and culturing of isolate 74 in MR broth culture was continued for a further five rounds. A methane-positive tube from the final MR tube sub-culture was used to inoculate an agar RF medium dilution series. The results of the RF medium dilution series are shown in Table 8. After 10 days incubation small brown irregular colonies, positive for methane, were visible. From these tubes four colonies from each of the fourth and fifth dilutions were picked into RF medium,

pressurised with H_2/CO_2 and incubated at 39 °C for 21 days. Inoculum from the five culture tubes positive for methane was transferred to duplicate tubes containing RF and PYG medium (Section 3.2.1) with or without vancomycin. The cultured tubes were pressurised with H_2/CO_2 and incubated at 39 °C for three days, and analysed for methane; the results are given in Table 9. All five isolate 74 cultures were negative for bacterial growth in PYG medium, with or without vancomycin. Methane-producing long rods were the only cell type present in the RF medium with or without vancomycin.

Table 9: Verification of Methanogen Isolate 74 Purity. Morphology was recorded as long rods (LR). Tubes with no microbes present were identified as 'No Growth'.

Colony Pick	1	2	3	4	5
RFM[1] + Vancomycin	LR	LR	LR	LR	LR
Methane (+/-)	+	+	+	+	+
RFM[1] -Vancomycin	LR	LR	LR	LR	LR
Methane (+/-)	+	+	+	+	+
PYG +Vancomycin	No Growth	No Growth	No Growth	No Growth	No Growth
Methane (+/-)	-	-	-	-	-
PYG -Vancomycin	No Growth	No Growth	No Growth	No Growth	No Growth
Methane (+/-)	-	-	-	-	-

[1]RF Medium

Following the successful purification of Isolate 74 using MR medium this procedure was repeated to remove antibiotic resistant bacteria from other methanogen isolates. The methanogen isolates 44, 49 and 53 were all purified using the same methods as colony pick 74 on MR media by selection with lincomycin and vancomycin, continual dilution, incubation and sub-culturing into agar roll tubes. However, despite extensive efforts over a six month period these methods were not sufficient to purify methanogen isolates 10, 20, 22, 25 and 28. These methanogens have been stored at -85 °C as impure cultures. In addition, further methanogen isolates from the calves were not able to be purified. For example, isolates 48 and 110 did not retain viability during subculture in RF, MET or MR mediums and were lost.

3.4 Discussion

From the 11 methanogen colonies selected (Table 2) four cultures were ultimately purified and there are several reasons for the somewhat poor recovery. Firstly, difficulties in culturing methanogens were evident in that only 31 of the original 110 colonies were found to be methanogenic. Once grown, the isolation of methanogens was also difficult. This was also exemplified by isolate 110. This isolate did not show the presence of methane and hydrogen utilisation until four weeks after initial checks for methanogen growth were made and it subsequently lost viability upon subculture. Similar results were reported by Jarvis *et al.* (2000) who also found that methanogen isolates often lost viability upon subculture. Hydrogen utilisation by non-methanogenic hydrogenotrophs may inhibit the growth of methanogens during culture through competition with methanogens for hydrogen, or, by utilisation of hydrogen before the methanogens. In support of this, non-methanogenic hydrogenotrophs are known to compete with methanogens for hydrogen and predominate in ecosystems like the termite gut (Leadbetter *et al.*, 1998). In total, 59 colony picks showed some hydrogenotrophic activity. These 59 colonies represent just over half the 110 colony picks cultivated and demonstrate the difficulties of culturing not only methanogens, but hydrogenotrophic anaerobes in general. Even though only 31 of the 110 colony picks showed methanogenic activity this was sufficient to demonstrate that the anaerobic techniques as described by Hungate (1966) and Balch *et al.* (1979) had been successfully applied. The identities of the non-methanogenic hydrogenotrophs remain unknown, but are likely to be acetogenic or sulphate-reducing bacteria. These non-methanogenic microbes were cultured unintentionally from colony picks because they could not be distinguished from methanogens by colony morphology on agar roll tubes. Colonies were selected as being representative of the colony morphologies observed on the agar surface of methane-positive Hungate tubes. However, this did not guarantee the picked colonies to be methanogenic, or pure.

Methanogens representative of a progression of colonisation were cultured from calves 13, 14, 15 and 16 and showed a change in the predominant species isolated over the 22 day sampling period. However, methanogens were not able to be isolated from all calves at all time points. For instance, no methanogens were cultured from calves 13, 14, 15 and 16 between days seven and 21. The reason why methanogens were not cultured, as mentioned earlier, is likely due to loss of viability from oxygen exposure (Smith and Hungate, 1958) or for other unknown reasons (Jarvis *et al.*, 2000). In addition, methanogens may have lost viability sometime during the two weeks of culturing before subculture onto agar RF medium. It is unlikely that poor media preparation caused the non-growth of methanogen as the same batch of media was used to enumerate methanogens between days seven and 22.

From the 11 colony picks of interest selected for isolation (Table 2), both methanogens and non-methanogens, were found to be present. Non-methanogens were removed from some methanogenic isolates by dilution in selective media and incubation with antibiotics. No attempt was made to identify non-methanogens because of time constraints. Methanogens of interest selected for isolation came from the four calves sampled for the entire trial period and those from calves 10 and 12 obtained prior to antibiotic contamination. Methanogens cultured from calves 10 and 12 are of interest as the calves were never in contact with calves 13, 14, 15, and 16.

4 Identification of Methanogens Colonising Calf Rumens

4.1 Introduction

Methanobrevibacter ruminantium has been shown to be the predominant methanogen in the bovine rumen (Smith and Hungate, 1958; Lovley *et al.*, 1984). However, other methanogens including *Methanosarcina barkeri* (Beijer, 1952), *Methanobacterium formicicum* (Oppermann *et al.*, 1957), *Methanomicrobium mobile* (Paynter and Hungate, 1968), *Methanosarcina* sp. (Patterson and Hespell, 1979), *Methanoculleus olentangyi* (Skillman *et al.*, 2001) and *Methanobrevibacter* spp. (Lovley *et al.*, 1984; Miller *et al.*, 1986) have also been isolated.

Methanogens can be identified by both phenotypic and molecular methods. However, a definitive identification by phenotypic methods alone is not always possible. Phenotypic differences between strains of *Methanobrevibacter* species include bile tolerance, formate utilisation and acetate requirements for growth but these characteristics are insufficient to determine relationships (Lin and Miller, 1998). In recent years, a greater diversity of ruminal methanogens have been revealed by molecular ecology studies based on 16S rRNA gene sequences using sequence comparison programs. ARB (Ludwig *et al.*, 2004) is one such program that has been used to determine phylogenetic relationships from 16S rRNA gene sequences. Use of the ARB phylogeny program demonstrated that the bovine rumen contains species of methanogen yet to be cultivated (Nicholson *et al.*, 2007).

To date all published ruminal methanogen isolates have been isolated from mature ruminants. Very little information exists about the species of methanogens found in the developing rumen of newborn ruminants. Only species of methanogen belonging to the *Methanobacterium* or *Methanobrevibacter* genera have been detected in newborn lambs (Skillman *et al.*, 2004). No evidence for the presence of methanogens belonging to the orders *Methanomicrobiales*, *Methanococcales* and *Methanosarcinales* exists at present.

The present study was carried out with the aim of identifying those methanogens cultured from the developing rumens of young calves using both culture and non-culture methods.

4.2 Methods

4.2.1 Media

Methanogen isolates (Chapter 3) were cultured in RF medium (Section 2.2.2) with H_2/CO_2, formate, acetate, ethanol, methanol/H_2, 2-propanol/CO_2, 2-butanol/CO_2, cyclopentanol/ CO_2, or methylamine (Section 4.2.3) as substrates. A second, non-rumen fluid medium, Medium I, was also used to culture methanogens with the above additives (Section 4.2.1). Medium I (litre^{-1}) consisted of K_2HPO_4 (255 mg), KH_2PO_4 (255 mg), $(NH_4)_2SO_4$ (255 mg), NaCl (250 mg), $MgSO_4 \cdot 7H_2O$ (100 mg), $CaCl_2 \cdot 2H_2O$ (70 mg), sodium acetate (5.0 g), $FeSO_4 \cdot 7H_2O$ (2 mg), $Na_2WO_4 \cdot 2H_20$ (0.33 mg), Balch's trace element solution (Section 6.8) (10 ml), $NaHCO_3$ (6.0 g) and distilled water (990 ml). Components were heated until boiling, and cooled to room temperature on ice under O_2-free N_2 for 15 minutes. After cooling L-cysteine HCl (500 mg) and $Na_2S \cdot 9H_2O$ stock solution (5.0 ml) (Section 6.7) were added to the medium and mixed thoroughly until dissolved. Aliquots of nine ml were removed by drawing up into a N_2-flushed pipette and dispensing into N_2-filled Hungate tubes. Tubes were autoclaved for 20 minutes at 121 °C (103 kPa) and stored at room temperature.

4.2.2 Microbial Strains

The microbes listed in Table 10 were grown to provide genomic DNA for use as PCR controls (Table 18, Table 19, Figure 17, and Figure 22).

Table 10: Sources of DNA used in PCR Studies as Controls.

Species	Strain
Methanobrevibacter smithii	SM9
Methanobrevibacter ruminantium	NT7
Methanosarcina barkeri	CM1
Methanococcus voltae	DSM 1537
Methanoculleus olentangyi	DM2
Methanobacterium formicicum	BRM9
Streptococcus bovis	NCFB 2476

4.2.3 Methanogen Substrate Test

The four methanogen isolates 44, 49, 53 and 74 (Chapter 3) were tested for growth in RF medium and Medium I with the substrates H_2/CO_2, formate, acetate, ethanol, methanol/H_2, 2-propanol/CO_2, 2-butanol/CO_2, cyclopentanol/ CO_2, or methylamine at concentrations listed in Section 6.9. Quadruplicate tubes containing nine ml of RF medium or Medium I with substrate additives were inoculated with 0.5 ml cultures and incubated without shaking for three weeks at 39 °C. Positive substrate utilisation was determined by the presence of methane in at least one of the four replicate tubes.

4.2.4 DNA Extraction and Precipitation

Genomic DNA was extracted by a modified method of Stahl *et al.* (1988). For all extractions either one ml of bacterial culture, fresh gut contents or 100 mg of freeze-dried gut contents were used. Gut-content material was weighed and added to two ml screw capped tubes containing one g of zirconia beads plus 0.75 ml of TE buffer (10 mM Tris, 1.0 mM EDTA, pH 8.0) and mixed thoroughly by vortexing. Samples were centrifuged at 10,000 rpm in an Eppendorf F45-12-11 rotor for one minute, the supernatants removed and one ml of saline EDTA (150 mM NaCl, 100 mM EDTA, pH 8.0) added. Samples were mixed thoroughly and centrifuged at 10,000 rpm in an Eppendorf F45-12-11 rotor for one minute to pellet contents. Supernatants were removed and 50 µl of 20% SDS, 500 µl of buffer-equilibrated phenol, and 700 µl of saline EDTA added. Pellets were resuspended and homogenised for two minutes in a FastPrep bead beater (QBiogene Inc., USA) and incubated for three minutes at room temperature, 10 minutes at 60 °C and three minutes at room temperature. The homogenates were centrifuged for five minutes at 10,000 rpm, and the supernatants transferred to fresh 1.5 ml tubes with 500 µl of equilibrated phenol. The supernatants were centrifuged again under the previous centrifugation conditions for five minutes. Supernatants were transferred to fresh 1.5 ml tubes and extracted three times; twice with 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1) and once with 500 µl of chloroform, respectively. After the final centrifugation, supernatants were transferred to fresh 1.5 ml tubes. One ml of 100% ethanol and 50 µl of 20 M ammonium acetate were added to the supernatants and the tubes stored at -20 °C for one hour. Tubes were centrifuged at 11,000 rpm at 4 °C for 20 minutes, the

supernatants decanted and 100 µl of 70% ethanol added, and then decanted. The tubes were left vertically uncapped for one hour at 39 °C to dry the pellet, and 50 µl of TE and one µl of RNase added. Pellets in capped tubes were stored at 4 °C.

4.2.5 Agarose Gel Electrophoresis

DNA samples were separated on 1.2% (w/v) MP agarose gels (Roche Diagnostics GmBH, Germany) in 1x TAE buffer (Section 6.11). DNA samples (20 µl) were mixed with two µl of gel loading buffer. Agarose gels were run at 10 V cm^{-1} for 45 minutes, stained in 5.0 µg ml^{-1} ethidium bromide for 15 minutes and destained for 30 minutes in water. DNA bands were viewed on a UV-transilluminator, and photographed with the Gel Logic 200 gel documentation computer software (Kodak, USA).

4.2.6 PCR Amplification

Each PCR reaction contained one µl of 50 mM $MgCl_2$, 2µl 10x PCR mastermix (Invitrogen Scientific, New Zealand), one µl of 20 µM forward primer, one µl of 20 µM reverse primer, two µl of 2 mM dNTP mix, 0.1 µl of five units/µl Hot Start® Platinum Taq polymerase (Invitrogen Scientific, New Zealand), 11.9 µl distilled water and one µl of template DNA. Each PCR reaction mixture was sealed in 20 µl capillaries and run on a FTS-1 PCR thermocycler instrument (Corbett Research, Australia). Cycling conditions were 95 °C denaturation for three minutes, followed by six cycles of 95 °C denaturation for 30 s, annealing for 15 s at the required temperature and 72 °C for 30 s, followed by 25 cycles of 95 °C denaturation for 15 s, annealing for five s at the required temperature and 72 °C for 30 s, followed by elongation at 72 °C for three minutes and cooling at 25 °C for one minute.

4.2.7 Methanogen-Specific PCR Primers

The methanogen selective PCR primers used for DNA amplification are listed in Table 11. The identification method used was a top-down nested PCR approach based on primers targeted at specific groups of methanogens. Total archaeal primers, Arch f364/Arch r1386 detect all methanogens. Three of the four methanogen orders within the Archaea, the *Methanomicrobiales*, the *Methanosarcinales* and the *Methanococcales*, are detected by the primer set, Arch f2/Arch r1386. The order *Methanobacteriales* is detected

by the set Mbac f331/Arch r1386. The individual orders *Methanosarcinales* and *Methanococcales* are detected using the primer sets Msar r589/Arch f364 and Mcoc r1045/Arch f364, respectively (Skillman *et al.*, 2004). Methanogens belonging to the *Methanobrevibacter* and *Methanobacterium* genera of the *Methanobacteriales* can be detected with primer sets Mbb1/Arch r1386 and Mbium/Arch r1386, respectively (Skillman *et al.*, 2004). The *Methanobrevibacter* genus contains several species and primers Mbb-g1-f/Arch r934 and Mbb-g2-f/Arch r934 will detect *Methanobrevibacter ruminantium*-like and *Methanobrevibacter smithii*-like spp. (Skillman *et al.*, 2004).

Table 11: Targeted PCR Primers for Methanogen DNA Amplification.

Target	Primer Name	Associated Primer	Annealing Temp (°C)
Domain Archaea	Arch r1386[1]	Arch f364	58
Order *Methanobacteriales*	Mbac f331[1]	Arch r1386	64
Orders Mmb/Msc/Mcc[3]	Arch f2[1]	Arch r1386	65
Order *Methanosarcinales*	Msar r589[1]	Arch f364	65
Order *Methanococcales*	Mcoc r1045[1]	Arch f364	65
Genus *Methanobrevibacter*	Mbb1[1]	Arch r1386	62
Genus *Methanobacterium*	Mbium[1]	Arch r1386	64
Species *Mbb. Ruminantium*-like	Mbb-g1-f[2]	Arch r934[1]	57
Species *Mbb. Smithii*-like	Mbb-g2-f[2]	Arch r934[1]	53
Domain Eubacteria	fd1	rd1	55

[1]Skillman *et al.*, (2004); [2]Skillman *et al.*, (2006); [3]Orders *Methanomicrobiales*, *Methanosarcinales* and *Methanococcales*.

4.2.8 Identification of Methanogens from 16S rRNA Gene Sequence

The 11 methanogen isolates from newborn calves (Table 2) were each identified by sequencing of PCR products generated by amplification of the 16S rRNA gene. For the four purified methanogens (Chapter 3), 16S rDNA products were isolated using primer sets f18/r1492, f69/r1492 or f109/r1492 (Table 12). Internal sequencing primers (Table 12) were used to give at least two-fold coverage of the PCR product. The phenotypes of methanogens identified in Table 2 suggested that only one type of methanogen was likely to be present in each culture tube. The semi-purified methanogens from Table 2 were amplified with primers f364 and r1386. No internal primers were used to increase the coverage of the PCR fragment. All PCR products were purified using a column purification kit (Qiagen, Germany). DNA was bound to a silica matrix under high salt concentration and eluted according to the manufacturer's instructions. For sequencing,

purified PCR products (one ng of DNA per 100 base pairs) were premixed with 3.2 pmol of primer in a 15 µl total volume with distilled H_2O. Premixed DNA was sequenced using the BigDye® Terminator v3.1 kit (Applied Biosystems, USA) products and visualised with an ABI 3730 Genetic Analyzer (Applied Biosystems, USA) by the AWC Genome Service.

Table 12: Methanogen 16S rRNA gene Sequencing Primers.

Primer Name	Targets
f18[4]	Eubacteria and Archaea
f69[1]	Eubacteria and Archaea
f109[3]	Eubacteria and Archaea
f364[2]	Eubacteria and Archaea
f530[5]	Eubacteria and Archaea
r530[5]	Eubacteria and Archaea
f915[6]	Archaea
r934[2]	Archaea
r1386[2]	Archaea
r1492[4]	Eubacteria and Archaea

[1]Leadbetter *et al.*, (1998); [2]Skillman *et al.*, (2004); [3]Grosskopf *et al.*, (1998); [4]Jarvis *et al.* (2000); [5]Lane (1991); [6]Watanabe *et al.* (2002).

4.2.9 Sequence Alignment

DNA sequences were checked for chimeric artefacts with the RDP database chimera check program (Cole *et al.*, 2003) to determine if sequences were fusions of more than one PCR product. Sequences were edited manually and assembled into contiguous sequences using Vector NTI Contig Express and Align X programs (Invitrogen, New Zealand). Editing was needed to varying degrees for sequences from all of the isolates. Apparent ambiguities in sequencing reactions were resolved by a visual inspection of the sequencing chromatograms using Contig Express. Where ambiguities could not be resolved, the sequence was left ambiguous. Editing and alignment of individual fragments produced a consensus 16S rDNA sequence of about 1.4 kb (f109/r1492) for each of the four purified methanogens with two-fold coverage over the entire fragment. Consensus sequences of about 1.0 kb (f364/r1386) were produced for the seven non-purified isolates. The consensus sequence for the non-purified isolates varied between one and two times coverage over the entire PCR fragment. All assembled fragments were saved in FASTA format for phylogenetic analysis.

4.2.10 Sequence Identification and Phylogenetic Placement of Isolates

Consensus sequences were imported into ARB and added to the main database which contained about 25,000 16S rRNA gene sequences. Database sequences clustering closely with isolates from this study and relevant methanogen outgroups were selected for further analysis. Similarity matrices were constructed using the Kimura-2 correction parameter method (Kimura, 1980). Phylogenetic positions of isolate and database sequences were determined by PHYLIP (Felsenstein, 1993) with the programs DNADIST, NEIGHBOR, DNAML and DNAPARS. Bootstrapping confidence analyses were performed using the SEQBOOT of the PHYLIP package (Felsenstein, 1993). Dendograms with bootstrapping values were exported into, and viewed with, the drawing package XFig© Version 3.2.4 (Lawrence Berkeley National Laboratory, USA).

4.2.11 Electron Microscopy

To obtain information on cell morphology, cells of isolates 44 and 74 were grown for seven days on H_2/CO_2 and visualised by scanning electron microscopy (SEM). Pelleted cells were fixed in a mixture of 3% (v/v) glutaraldehyde and 2% (v/v) formaldehyde in 0.1 M phosphate buffer, pH 7.2. Cells were dehydrated and dried to critical point with liquid CO_2. Specimens were mounted on aluminium stubs, sputter-coated with gold and examined using a Cambridge Model 250 MkIII Scanning Electron Microscope (Cambridge Instruments, UK) by the HortResearch Electron Microscopy Unit.

4.2.12 Gram Staining

Samples were removed from each culture with an inoculation loop and air dried on a microscope slide. The air dried inoculum was fixed with a Bunsen flame, crystal violet solution was applied to the samples and allowed to stain for one minute. The crystal violet was rinsed with water for a maximum of 5 s, and Gram's iodine solution applied for one minute. The slides were rinsed with a 50:50 mixture of acetone and ethanol and then immediately rinsed with water. Safranin was applied, and left for one minute, and excess removed by rinsing after one minute. The slides were air dried and visualised under bright field microscopy using a Reichert Divar microscope (Reichert, Austria).

4.3 Results

4.3.1 Phenotypic Characteristics of Purified Methanogen Isolates

Some phenotypic characteristics of the purified methanogen isolates 44, 49, 53 and 74 (Chapter 3) were determined. Gram stain reactions (Section 4.2.12) were assessed and morphologies were determined from epifluorescence (Section 2.2.3) and electron microscopy (Section 4.2.12). The Gram stains of isolates 44, 49, 53 and 74 are given in Figure 12, and show that 44, 49 and 53 are Gram negative cocci with a dark staining margin. Isolate 74 is a Gram variable rod, that was sometimes concatenated (Figure 12).

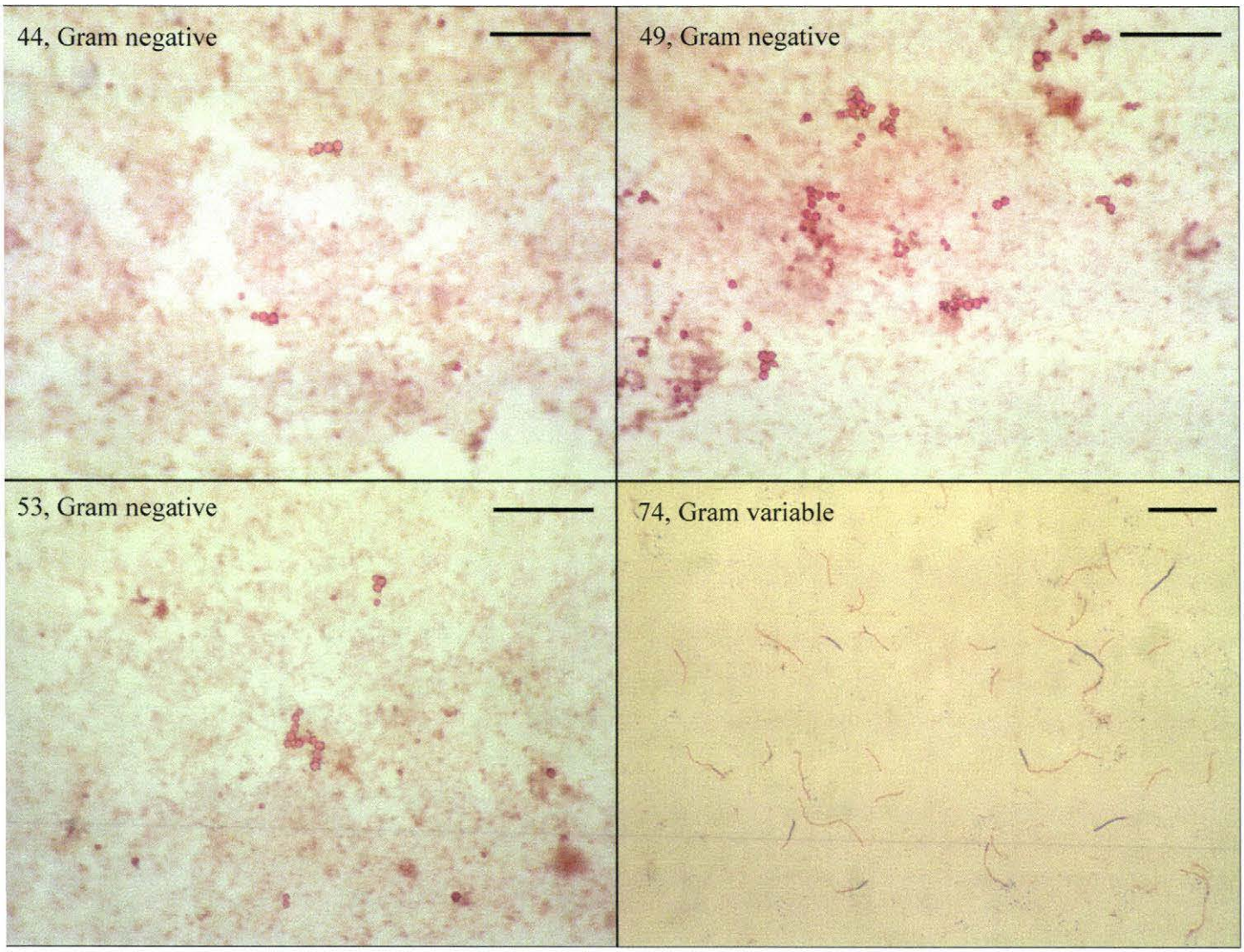


Figure 12: Gram Stains of Methanogen Isolates. Scale bar is 10 μm.

Further tests were performed on isolate 44 to determine if the dark staining cell margin was an artefact of the staining procedure by a comparison with known Gram-negative (*Escherichia coli*) and Gram-positive (*Enterococcus faecium*) species (Figure 13). Results showed that isolate 44 was not Gram positive when compared to *Enterococcus faecium* (Figure 13A), and a different shade of pink compared to the Gram negative *Escherichia coli* cells (Figure 13B). However, based on the overall appearance the cells were deemed to be Gram negative.

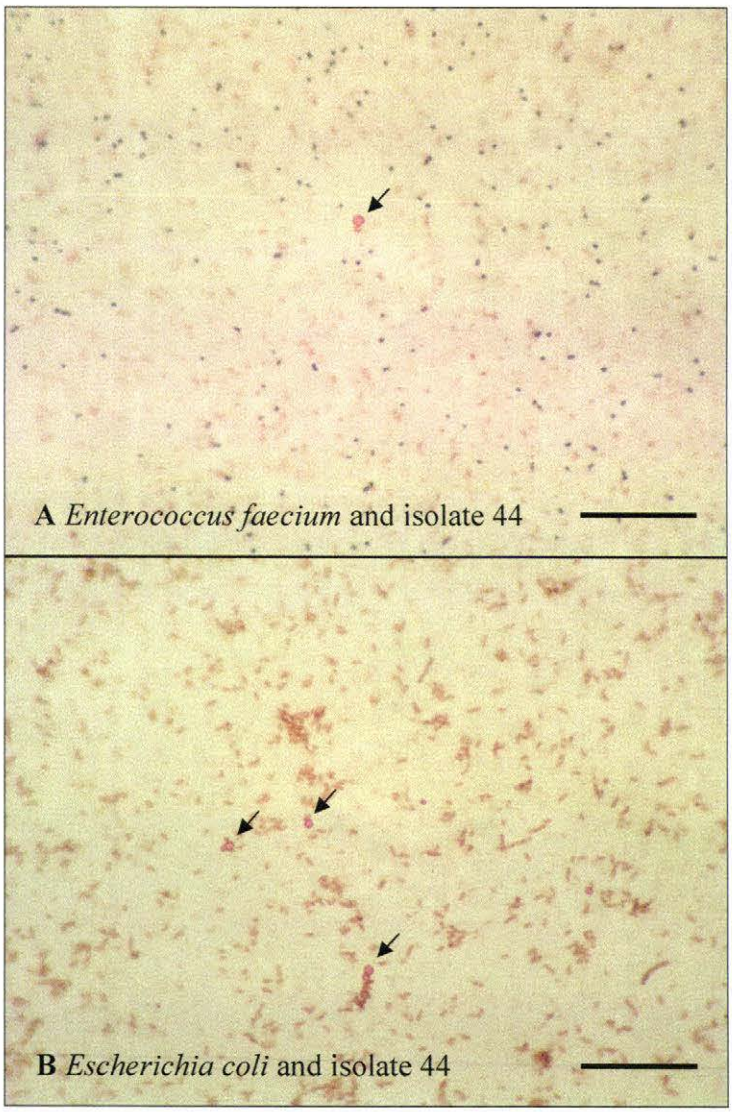


Figure 13: Gram stains of isolate 44. With (**A**) *Enterococcus faecium* and (**B**) *Escherichia coli*. Arrows indicate isolate 44 cells. Scale bar is 10 μm.

Microscope slide wet mounts of the four purified methanogen isolates were examined under epifluorescence. This revealed that in each culture only one methanogen type was present. Isolates 44, 49 and 53 were small cocci and isolate 74 was a concatenated rod (Figure 14). These findings confirm those found using Gram stains.

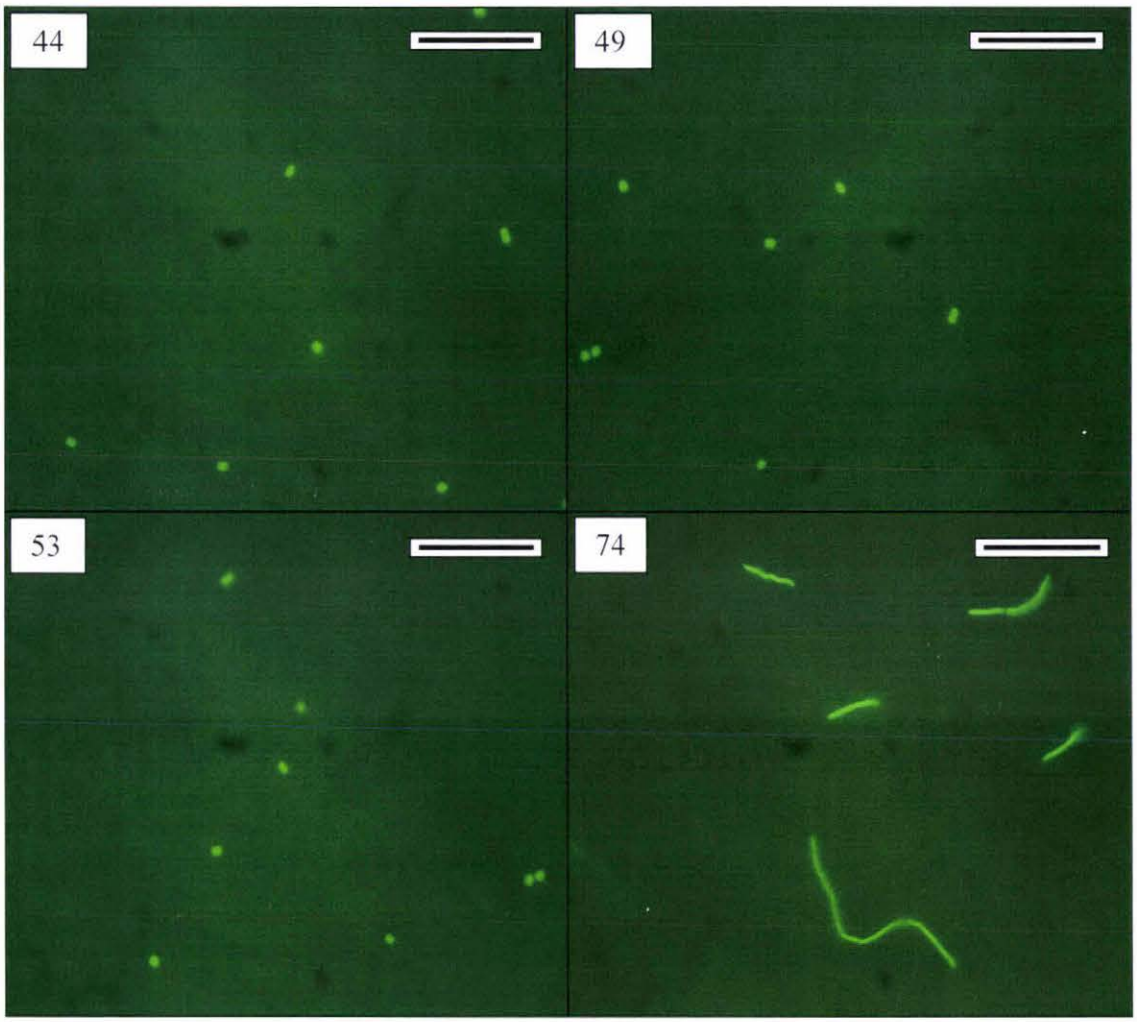


Figure 14: Epifluorescence of Methanogen Isolates. Scale bar is 10 μm.

The cell dimensions of the four purified methanogen isolates were determined by scanning electron microscopy (SEM). Electron micrographs of isolates 44 and 74 are given in Figure 15. Isolates 49 and 53 are assumed to be similar to isolate 44. Isolate 44 was found to be a 0.75-1.0 μm irregular coccus (A, Figure 15), while isolate 74 was a rod at least 4.0 μm long and 0.75 μm wide (B, Figure 15). Lengths of isolate 74 rods varied between 4.0 and 8.0 μm.

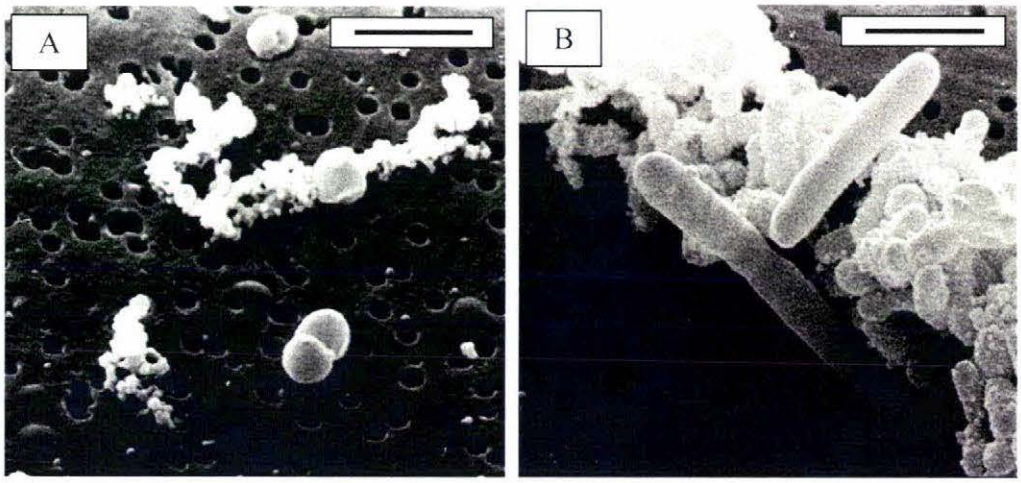


Figure 15: SEM of Methanogen Isolates. Scale bar is two μm.

Tests for substrate utilisation were carried out as described in Section 4.2.3. The results of these tests are presented in Table 13. Of the 9 substrates tested only formate and H_2/CO_2 supported methanogen growth in RF medium. Formate was used by isolates 44, 49 and 53, but not by isolate 74. All four isolates were able to grow on H_2/CO_2 which is consistent with the method used in the initial isolation of these methanogens. These growth tests were repeated to ensure negatives were indeed negative, and the same results as those in Table 13 were found. Zellner *et al.* (1990) was able to grow methanogens on substrates other than H_2/CO_2 and formate using the non-rumen-fluid medium, Medium I (Section 4.2.1). Isolates 44, 49, 53 and 74 were inoculated into Medium I containing a range of potential growth substrates, and after two weeks incubation without shaking at 39 °C isolates 44, 49 and 53 utilised H_2/CO_2 and formate, while isolate 74 only utilised H_2/CO_2 (Table 14).

Table 13: Substrate Utilisation by Isolates. Growth on RF medium

Substrate	Isolate 44	Isolate 49	Isolate 53	Isolate 74
H_2/CO_2	+	+	+	+
Formate	+	+	+	-
Acetate	-	-	-	-
Ethanol	-	-	-	-
Methanol	-	-	-	-
2-Proponal/CO_2	-	-	-	-
2-Butanol/CO_2	-	-	-	-
Cyclopentanol/CO_2	-	-	-	-
Methylamine	-	-	-	-

Table 14: Substrate Utilisation by Isolates. Growth on Medium I

Substrate	Isolate 44	Isolate 49	Isolate 53	Isolate 74
H_2/CO_2	+	+	+	+
Formate	+	+	+	-
Acetate	-	-	-	-
Ethanol	-	-	-	-
Methanol	-	-	-	-
2-Proponal/CO_2	-	-	-	-
2-Butanol/CO_2	-	-	-	-
Cyclopentanol/CO_2	-	-	-	-
Methylamine	-	-	-	-

4.3.2 Identification of Methanogen Isolates by 16S rDNA Analysis

16S rDNA was amplified from each of the 11 methanogen isolates by PCR. PCR products from isolates 44, 49, 53 and 74 were produced using primers f18 and r1492 (Table 12). For the non-purified methanogen isolates 10, 20, 22, 25, 28, 48 and 110, DNA was amplified using primers f364 and r1386 (Table 12). Half of the PCR products were visualised by gel electrophoresis methods (Section 4.2.5) and are shown in Figure 17. The remaining PCR products were retained for later purification and sequencing. No Archaeal DNA was detected in the PCR mixture only, or in the negative control (lanes 1 and 2). Archaeal DNA was detected in the NT7 positive control (lane 3). All semi-purified methanogen isolates produced appropriate sized PCR products with the f364/r1386 primer set (lanes 4 to 10). Archaeal DNA was not detectable in the PCR mixture or negative control (lanes 11 and 12). Archaea were detectable in the NT7 positive control (lane 13). The four pure methanogen isolates produced appropriate sized PCR products with the f18/r1492 primer combination (lanes 13 to 17). Primer dimer is visible in all lanes (1 to 17) at about the 100 base pair marker size.

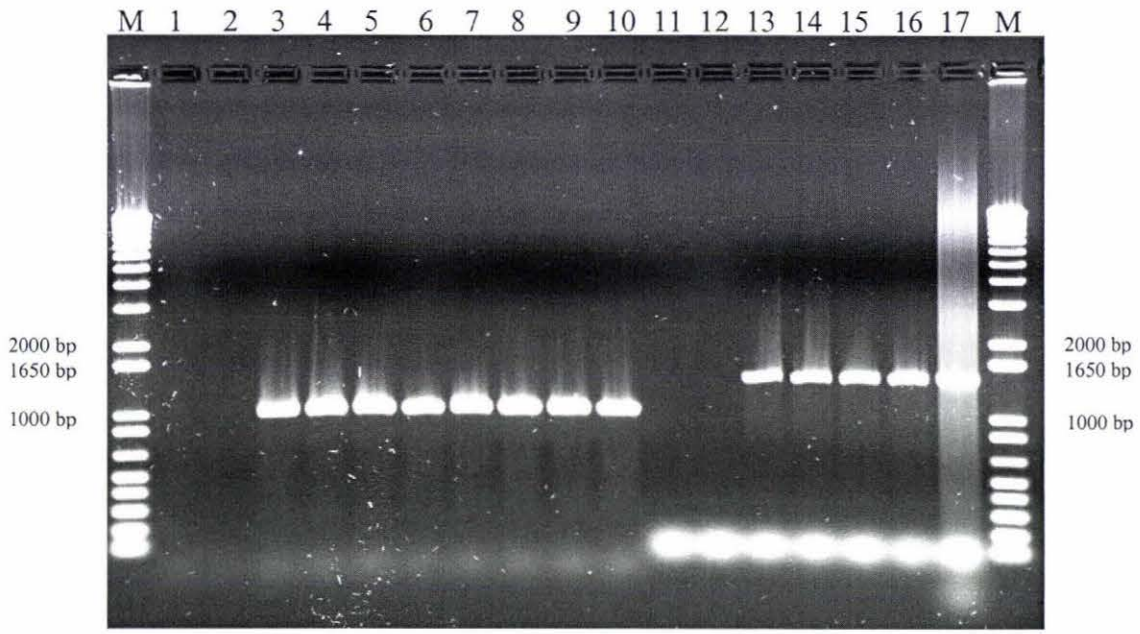


Figure 17: PCR Detection of Methanogen 16S rRNA Genes for Sequencing.

Lane M: 1 Kb^+ Ladder,

Lane 1: Negative Control (Water only) (f364/r1386),

Lane 2: Negative Control (*Streptococcus bovis* NCFB 2476) (f364/r1386),

Lane 3: Positive Control (*Methanobrevibacter ruminantium* NT7) (f364/r1386),

Lane 4: Isolate 10 (f364/r1386),

Lane 5: Isolate 20 (f364/r1386),

Lane 6: Isolate 22 (f364/r1386),

Lane 7: Isolate 25 (f364/r1386),

Lane 8: Isolate 28 (f364/r1386),

Lane 9: Isolate 48 (f364/r1386),

Lane 10: Isolate 110 (f364/r1386),

Lane 11: Negative Control (Water only) (f18/r1492),

Lane 12: Negative Control (*S. bovis* NCFB 2476) (f18/r1492),

Lane 13: Positive Control (*M. ruminantium* NT7) (f18/r1492),

Lane 14: Isolate 44 (f18/r1492),

Lane 15: Isolate 49 (f18/r1492),

Lane 17: Isolate 53 (f18/r1492),

Lane 18: Isolate 74 (f18/r1492),

Lane M: 1 Kb^+ Ladder.

The remaining PCR products not visualised on the agarose gel (Figure 17) were purified by column purification kit and sequenced (Section 4.2.8) with contiguous sequences assembled (Section 4.2.9). Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997) searches of sequences from semi-purified methanogen isolates 10, 20 and 22 showed a high level of similarity (99%) to *Methanoculleus palmolei* INZUZ. Methanogen isolates 25 and 28 were 99% identical to *Methanobacterium bryantii* RiH2. Isolate 48 was 98% identical to *Methanosarcina barkeri* CM1 and isolate 110 was 98% identical to *Methanobrevibacter thaueri* CW. BLAST searches of contiguous sequences from the purified methanogens revealed that these had the greatest identity to the eubacteria *Stenotrophomonas* sp. JRL-2 (Table 15).

Table 15: BLAST Results of Sequenced Methanogen 16S rRNA Genes.

Isolate[1]	Greatest BLAST Identity[2]	Identity (%)
10	*Methanoculleus palmolei* INZUZ	99
20	*Methanoculleus palmolei* INZUZ	99
22	*Methanoculleus palmolei* INZUZ	99
25	*Methanobacterium bryantii* RiH2	99
28	*Methanobacterium bryantii* RiH2	99
44	*Stenotrophomonas* sp. JRL-2	99
48	*Methanosarcina barkeri* CM1	98
49	*Stenotrophomonas* sp. JRL-2	99
53	*Stenotrophomonas* sp. JRL-2	99
74	*Stenotrophomonas* sp. JRL-2	99
110	*Methanobrevibacter thaueri* CW	98

[1]Methanogen isolates 10, 20, 22, 25, 28, 48 and 110 were sequenced with f364/r1386 primers. Methanogen isolates 44, 49, 53 and 74 were sequenced with f18/r1492 primers. [2]Identification of isolates was performed by BLAST searches of contiguous 16S rRNA gene sequences.

The presence of *Stenotrophomonas* sp. JRL-2 in the original DNA extracts suggests contamination of the DNA extracts from isolates 44, 49, 53 and 74. Primer sets for eubacteria (fd1/rd1) and archaea (f364/r1386) were used to amplify the original DNA extracts from isolates 44, 49, 53 and 74 (Table 16) to confirm eubacterial contamination. Freshly-extracted DNA from isolates 44, 49, 53 and 74 were also PCR amplified as eubacteria negative controls along with DNA from *Methanobrevibacter ruminantium* NT7 (Table 16). DNA from *Streptococcus bovis* NCFB 2476 served as a eubacterial positive control.

Table 16: PCR Results of Original and New DNA Extractions.

Isolate of Interest	DNA Extract	Eubacteria[1]	Archaea[1]
No added DNA	-	-	-
M. ruminantium	-	-	+
S. bovis	-	+	-
44	Original extract	+	+
49	Original extract	+	+
53	Original extract	+	+
74	Original extract	+	+
44	New extract	-	+
49	New extract	-	+
53	New extract	-	+
74	New extract	-	+

[1]Eubacterial and archaeal DNA in each DNA extract was determined by the presence or absence of a DNA band for the primers fd1/rd1 (eubacterial), f18/r1492 (archaeal) and f364/r1386 (archaeal).

The presence of eubacterial DNA in original DNA extracts but not new extracts (Table 16) indicated that the original DNA extracts were likely contaminated by *Stenotrophomonas* sp. JRL-2 (Table 15). The eubacterial primer set, fd1/rd1, amplified the *S. bovis* NCFB 2476 DNA but not *M. ruminantium* NT7 DNA as expected. The Archaea-specific primers f364/r1386 amplified the *M. ruminantium* NT7 DNA but not *S. bovis* NCFB 2476 DNA as expected. Archaeal DNA was detected in both original and new DNA extracts. The PCR negative control with no added DNA produced no amplicons (Table 16).

The new DNA extracts were amplified with primer set f18/r1492 using an annealing temperature of 59 °C but this produced specific and non-specific bands so the annealing temperature was raised to 60 °C. At 60 °C, although the methanogen positive control amplified, no amplicons from fresh DNA extracts of the four isolates were obtained. The primer pair was changed to the Archaea-specific pair f69/r1492 of Leadbetter and Breznak (1996) and the DNA was amplified at an annealing temperature of 59 °C. Appropriate sized PCR products were detected for methanogen isolate 74 and the *M. ruminantium* NT7 positive control. However, no PCR products were detected for isolates 44, 49 and 53. The annealing temperature was lowered to 50 °C, this produced no PCR products also. The primer set f69/r1492 was not used any further and another Archaea-

specific primer set, f109/r1492 (Grosskopf *et al.*, 1998), was subsequently used successfully to amplify 16S rRNA genes of 44, 49, 53 and 74 without non-specific bands. The flanking primers f109/r1492, and internal primers f530, r530, f915 and r934 (Table 12) were used to obtain sequences which were assembled into contiguous sequences (Section 4.2.9). Contiguous sequences of the 11 isolates are listed in Section 7.3. BLAST searches showed that isolates 44, 49, and 53 had identities 96% similar to that of *Methanofollis liminatans* GKZPZ. Isolate 74 had an identity of 99% to *Methanobacterium bryantii* RiH2 (Table 17).

Table 17: BLAST Results of Sequenced Methanogen 16S rRNA Genes.

Methanogen Isolate	Closest BLAST Match	Identity (%)
44	*Methanofollis liminatans* GKZPZ	96
49	*Methanofollis liminatans* GKZPZ	96
53	*Methanofollis liminatans* GKZPZ	96
74	*Methanobacterium bryantii* RiH2	99

Methanogen isolates 44, 49, 53 and 74 sequenced with f109/r1492. Identification of isolates was performed by BLAST searches of contiguous 16S rRNA gene sequences.

4.3.3 *Methanogen Phylogeny*

Non-chimeric contiguous 16S rDNA sequences from methanogen isolates 10, 20, 22, 25, 28, 44, 48, 49, 53, 74, and 110 obtained in Section 4.2.19 have been deposited in the Genbank database and assigned accession numbers EF112186 to EF112196. The sequences were compared to database sequences by the methods outlined in Section 4.2.10 and similarity matrices constructed using the Kimura-2 correction parameter (Tables 23-28). No differences in tree topologies were observed when NEIGHBOR, DNAML or DNAPARS were used. The Neighbour-joining trees are given as dendograms of choice together with confidence values from 1000 bootstrapping iterations in Figures 16 to 21. In these figures numbers in parentheses are the Genbank accession numbers, bootstrapping values of less than 50% are not indicated and bars represent 2% sequence divergence. The aligned sequences from isolates 44, 49, 53 and 74 were 1350 bases in length while aligned sequences for isolates 10, 20, 22, 25, 28, 48 and 110 were 1000 bases in length.

Phylogenetic analysis of sequences from isolates 44, 49 and 53 showed the sequences all grouped within the *Methanofollis* genus (Figure 16), with all three isolates being almost identical to each other (99.9-100%) (Table 23). The database sequence with the greatest identity to isolates 44, 49 and 53 was *Methanofollis liminatans* GKZPZ at 96.0%. Other *Methanofollis* species had identities of 95.4% (*Methanofollis tationis*), 95.6% (*Methanofollis formosanus*), 95.1% (*Methanofollis aquaemaris*) and 95.9% (*Methanofollis liminatans* BM1). While *Methanoculleus palmolei* and *Methanocalculus halotolerans* had identities of approximately 92.0% and 85.0% to isolates 44, 49 and 53 respectively.

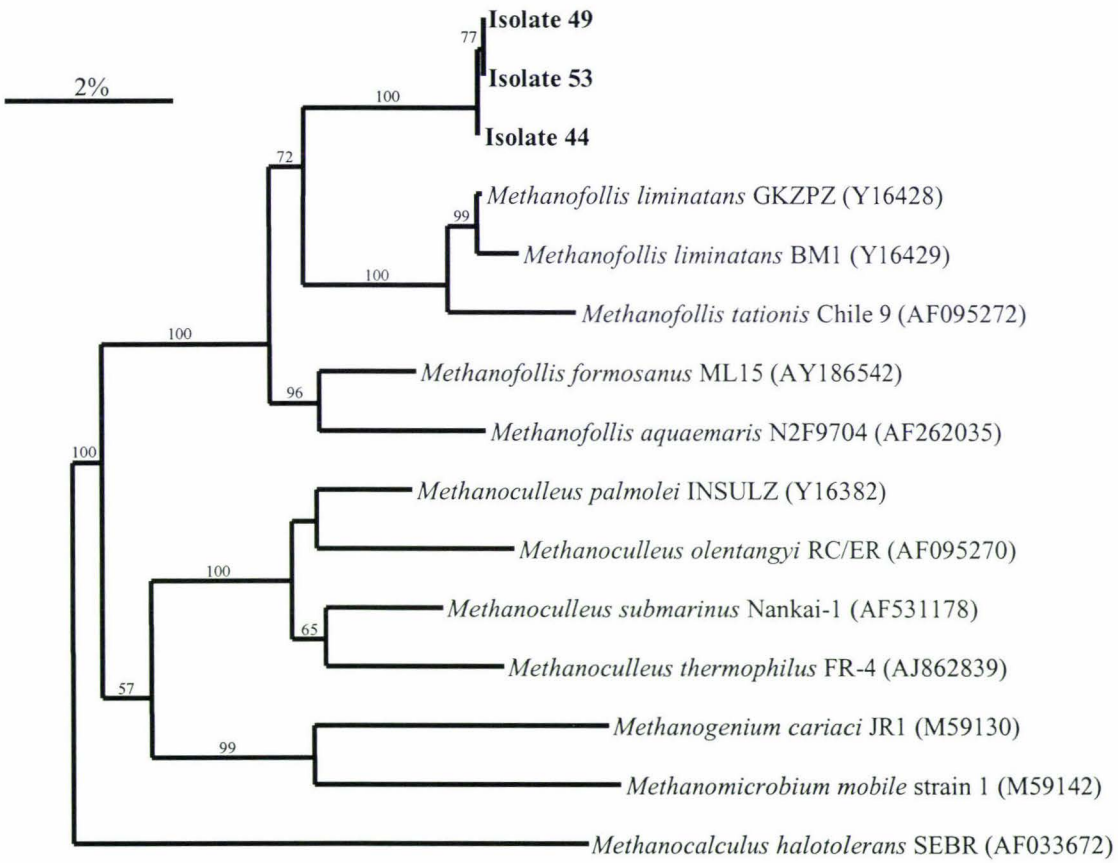


Figure 16: Neighbour-Joining Dendogram Showing the Phylogenetic Relationships of Isolates 44, 49 and 53.

In the case of isolate 74 the neighbour-joining dendogram is given in Figure 17. The inferred relationship showed that isolate 74 was most identical to *Methanobacterium bryantii* RiH2 at 98.5%, with *Methanobacterium bryantii* MOH being 99.4% identical. Isolate 74 was less related to *Methanobacterium oryzae* FPi (96.4%) or *Methanobacterium formicicum* MF (94.4%). *Methanosphaera stadtmanae* MCB-3 and *Methanobrevibacter smithii* SM9 had identities of 89.4% and 90.1%, respectively, to isolate 74. The similarity matrix of isolate 74 and database sequences are listed in Table 24.

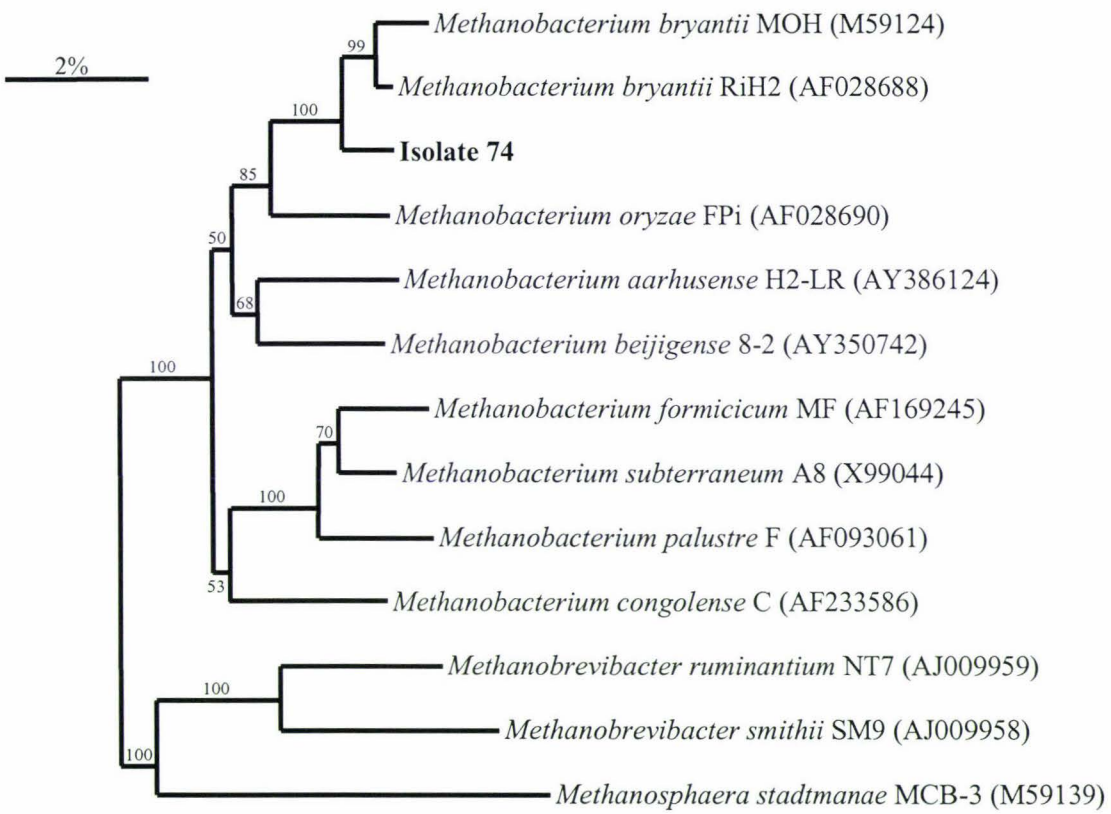


Figure 17: Neighbour-Joining Dendogram Showing the Phylogenetic Relationships of Isolate 74.

Phylogenetic analysis of 16S rDNA sequences from isolates 10, 20 and 22 revealed that these had identities of 99.5, 99.5 and 99.2%, respectively, with that of *Methanoculleus palmolei*. The neighbour-joining dendogram and similarity matrix showing the inferred relationships are given in Figure 18 and Table 25, respectively. Isolates 10 and 20 were found to be 100% identical to each other, while isolate 22 was 99.7% similar to isolates 10 and 20. *Methanoculleus* species that more distantly related to isolates 22, 20 and 10 included *Methanoculleus marisnigri* JR1 at 97.5, 97.5, and 97.9%, respectively; *Methanoculleus thermophilicus* TCI at 96.6, 96.9, and 96.9%, respectively; *Methanoculleus olentangyi* RC/ER at 97.7, 97.7 and 98.1%, respectively. Other closely related non-*Methanoculleus* species included *Methanofollis liminatans* GKZPZ at 92.7, 93.1 and 93.1% identity respectively, and *Methanogenium organophilum* CV at 93.1, 93.4 and 93.4% identity, respectively.

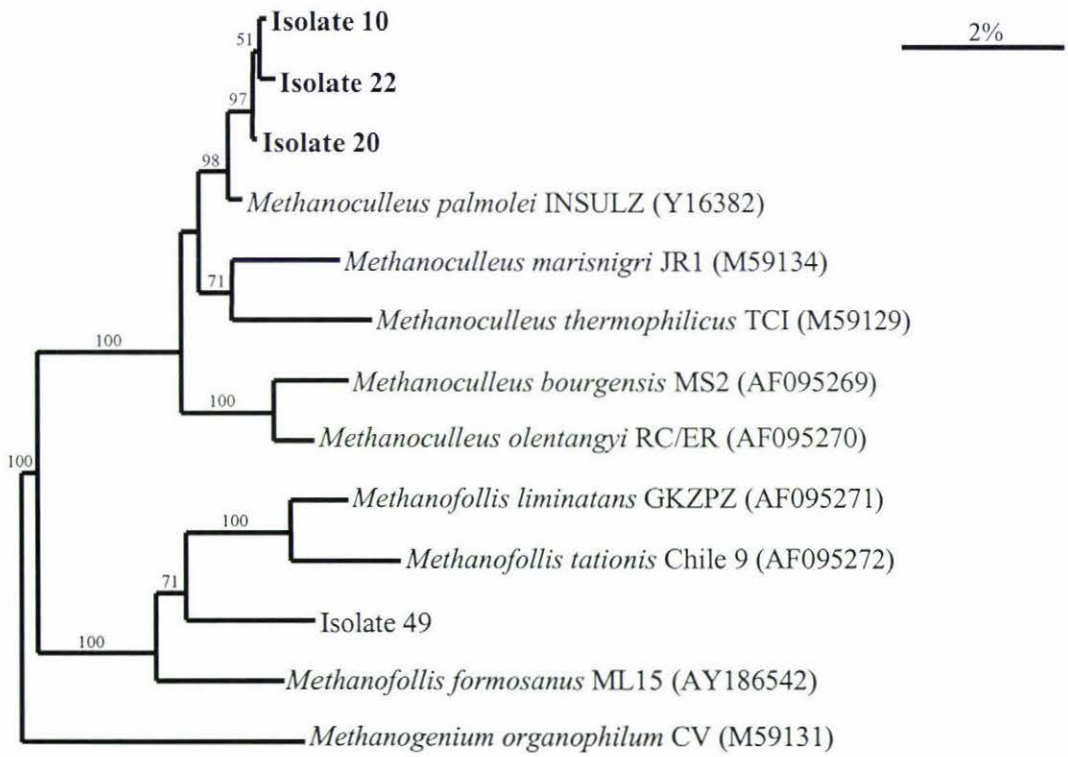


Figure 18: Neighbour-Joining Dendogram Showing the Phylogenetic Relationships of Isolates 10, 20 and 22.

Phylogenetic analysis of 16S rDNA sequences from isolates 25 and 28 revealed that both had identities of 98.9% respectively to that of *Methanobacterium bryantii* RiH2. The neighbour-joining dendogram and similarity matrix showing the inferred relationships are given in Figure 19 and Table 26 respectively. Isolates 25 and 28 were found to be 100% identical to each other. *Methanobacterium* species that less related to isolates 25 and 28 than *M. bryantii* RiH2 included *Methanobacterium bryantii* MOH at 98.5% identity; *Methanobacterium beijingense* 8-2 at 96.0% identity; *Methanobacterium formicicum* MF at 95.1% identity. Other closely related non-*Methanobacterium* species included *Methanobrevibacter ruminantium* NT7 at 92.4% identity and *Methanosphaera stadtmanae* MCB-3 at 90.3% identity.

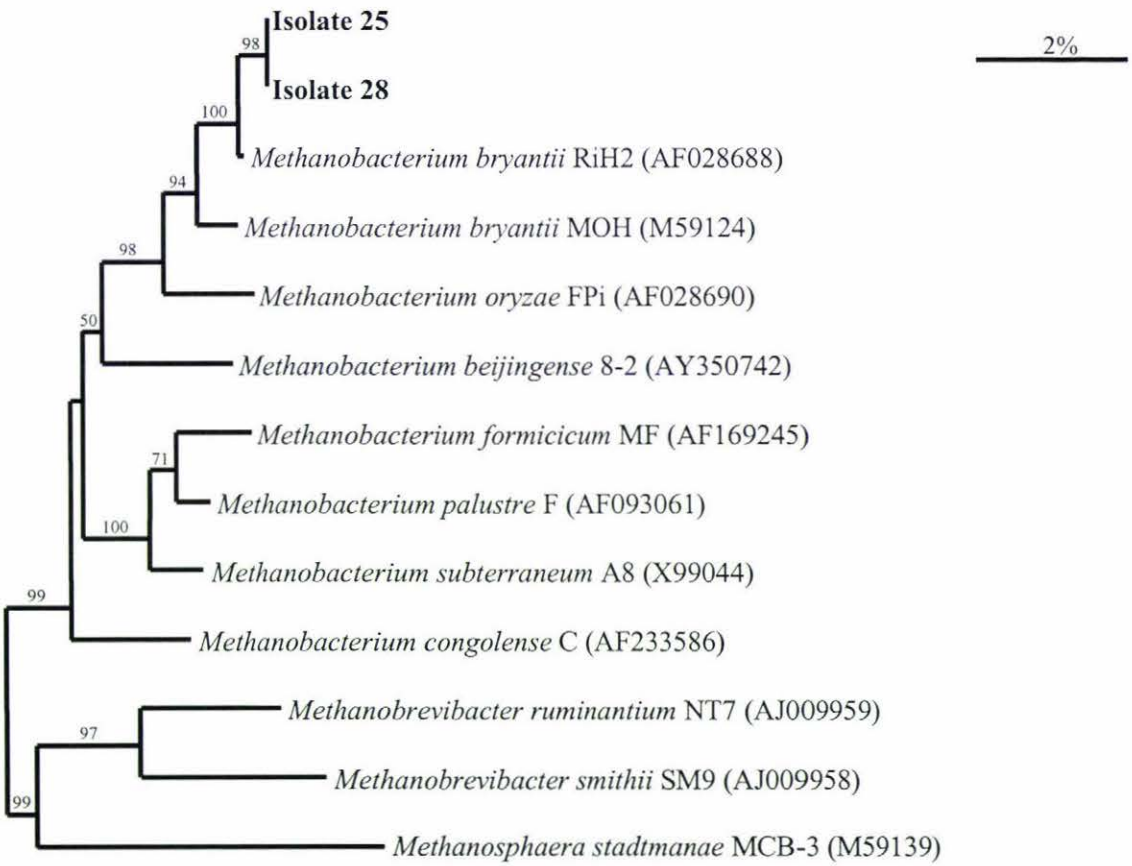


Figure 19: Neighbour-Joining Dendogram Showing the Phylogenetic Relationships of Isolates 25 and 28.

Phylogenetic analysis of 16S rDNA sequences from isolate 48 revealed an identity of 99.2% with that of *Methanosarcina barkeri* Sar. The neighbour-joining dendogram and similarity matrix showing the inferred relationships are given in Figure 20 and Table 27 respectively. Isolate 48 was found to be 99.1% identical to the ruminal strain *Methanosarcina barkeri* CM1. *Methanosarcina* sp. that were less related to isolate 48 than *M. barkeri* Sar and *M. barkeri* CM1 included *Methanosarcina acetivorans* C2A at 98.8% identity; *Methanosarcina mazei* C16 at 98.1% identity; *Methanosarcina lacustera* ZS at 96.6% identity. Other closely related non-*Methanosarcina* species included *Methanohalophilus mahii* SLP at 93.0% identity and *Methanimicrococcus blatticola* PA at 88.4% identity.

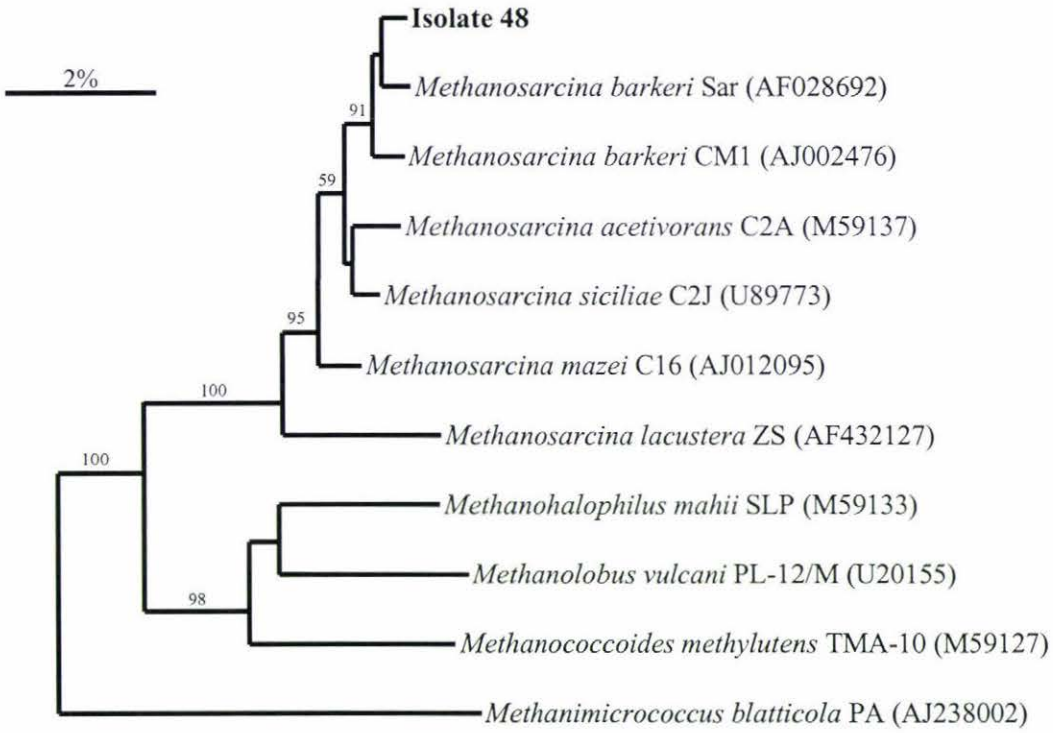


Figure 20: Neighbour-Joining Dendogram Showing the Phylogenetic Relationships of Isolate 48.

Phylogenetic analysis of 16S rDNA sequences from isolate 110 revealed an identity of 99.1 % with that of *Methanobrevibacter thaueri* CW. The neighbour-joining dendogram and similarity matrix showing the inferred relationships are given in Figure 21 and Table 28 respectively. *Methanobrevibacter* species that were less related to isolate 48 than *M. thaueri* CW included *Methanobrevibacter gottschalkii* HO at 98.1 % identity; *Methanobrevibacter smithii* SM9 at 98.6 % identity; *Methanobrevibacter ruminantium* NT7 at 93.2 % identity. Other closely related non-*Methanobrevibacter* species included *Methanobacterium bryantii* RiH2 at 90.2 % identity and *Methanobacterium formicicum* MF at 88.4 % identity.

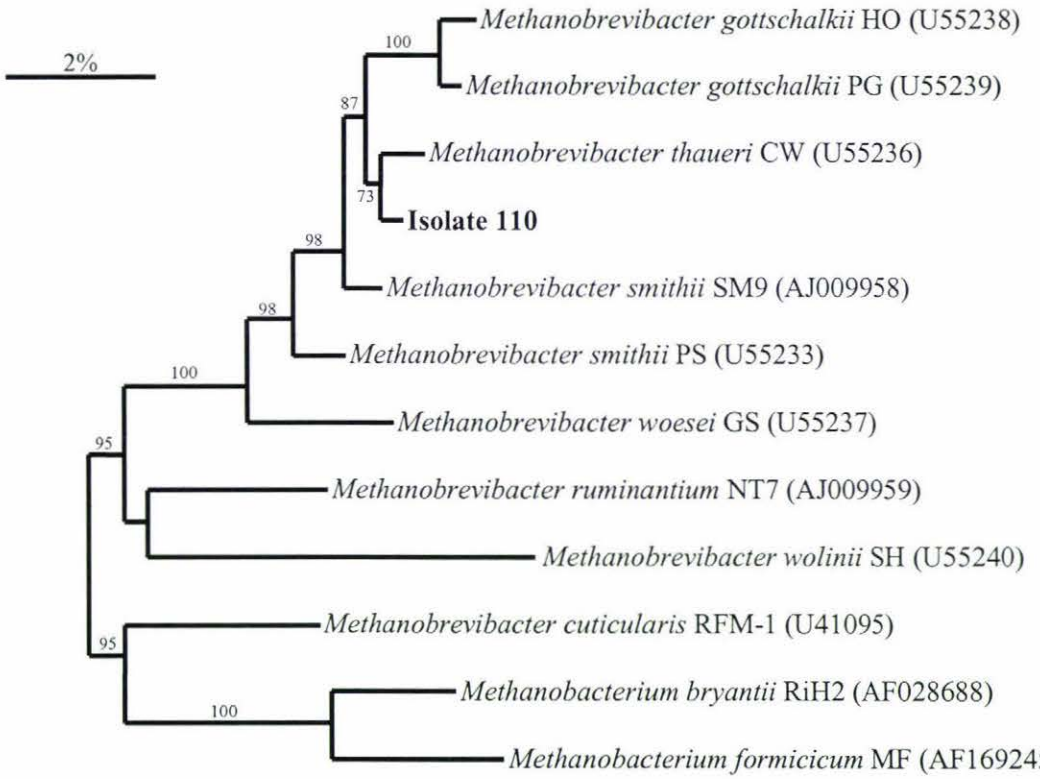


Figure 21: Neighbour-Joining Dendogram Showing the Phylogenetic Relationships of Isolate 110.

4.3.4 Diversity of Methanogens in the Calf Rumen

Methanogens that had colonised the developing calf rumen were also identified from PCR analysis of DNA extracts from rumen samples collected from calves at regular intervals after birth. The PCR detections used a total of nine nested primer sets. The specific primers and their methanogen targets are shown in Section 4.2.7. PCR products from each primer set were run and visualised by the gel electrophoresis methods outlined in Section 4.2.5. Data from calves 13, 14, 15, and 16 were analysed with the nine primer sets and the results are presented in Table 18 and Table 19. Calves 10 and 12 were not included because their sampling was limited to a shorter period. The presence of targeted methanogens in samples was indicated by the amplification of a PCR product of the appropriate size for each primer pair. Appropriate methanogen DNAs (Section 4.2.2) were used as positive controls and were included with each primer set test. Rumen samples that did not give PCR amplicons were recorded as not detected. In these cases, PCR was repeated to minimise false negatives and revealed 10 new positive results. Those samples still negative for targeted methanogens after the second PCR were designated as negative (Tables 18 and 19). No Archaea were detected in four of the 44 rumen samples tested. An example of this is calf 14 on day 13 when no Archaea were detected (Table 19).

Table 19: PCR Detection of Methanogens in the rumens of calves: Days 9 – 22 after birth.

Calf	Day	Arch	3 orders	Mcoc	Msar	Mbac	Mbct	Mbvb	Mrum	Msmi
13	9	+	-	-	-	+	+	-	-	-
14	9	+	-	-	-	+	+	-	-	-
15	9	+	-	-	-	+	+	-	-	-
16	9	+	-	-	-	+	+	+	-	-
13	11	+	-	-	-	+	+	+	-	+
14	11	+	-	-	-	+	+	-	-	-
15	11	+	-	-	-	+	+	-	-	-
16	11	+	-	-	-	+	+	+	-	-
13	13	+	-	-	-	+	+	+	-	+
14	13	-	-	-	-	-	-	-	-	-
15	13	+	-	-	-	+	-	+	+	+
16	13	+	-	-	-	+	+	+	-	+
13	15	+	-	-	-	-	-	-	-	-
14	15	+	-	-	-	+	+	-	-	-
15	15	+	-	-	-	+	-	+	-	+
16	15	+	-	-	-	+	+	+	-	+
13	22	+	-	-	-	+	+	+	+	+
14	22	+	-	-	-	+	+	+	+	+
15	22	+	-	-	-	+	+	+	+	+
16	22	+	-	-	-	+	+	+	+	+

Primer targets were Arch (total Archaea), 3 orders (orders *Methanomicrobiales*, *Methanosarcinales*, and *Methanococcales*), Mcoc (order *Methanococcales*), Msar (order *Methanosarcinales*), Mbac (order *Methanobacteriales*), Mbct (genus *Methanobacterium*), Mbvb (genus *Methanobrevibacter*), Mrum (*Methanobrevibacter ruminantium*-like sp.) and Msmi (*Methanobrevibacter smithii*-like sp.).

Samples negative for methanogens in Tables 18 and 19 were tested to confirm that the absence of a PCR product was likely due the absence of methanogens, rather than the non-detection due to PCR failure. In one case DNA from an archaea-negative sample from calf 13 on day two (Table 18) was combined (1:1) with samples positive for methanogen DNA from calf 14 and 16 also on day two. In another case, DNA from an archaea-negative sample of calf 13 on day three was combined (1:1) with DNA from methanogen positive samples from calf 14 and 16 also on day three. The results from the PCR amplification of these samples together with controls using archaeal primers, Arch f364/Arch r934, are shown in Figure 22. PCR products were visualised by gel electrophoresis methods outlined in Section 4.2.5. No archaea were detected in the PCR mixture control (lane one), or in the eubacterial control (lane two). Archaeal DNA was detected in the *M. ruminantium* NT7 positive control (lane three). As was found previously, archaea were not detectable in DNA from calf 13 on days two and three (lanes four and nine) but were detected in DNA from calves 14 and 16 on day two (lanes five and six) and calves 14 and 16 on day three (lanes 10 and 11) showed detectable archaea. When DNA from calf 13 was combined with DNA from calves 14 or 16 on day two (lanes seven and eight) a similar result was found to DNA from calves 14 and 16 on day three (lanes 12 and 13). These results indicate that there were no PCR inhibitors in DNA extracts from calf 13 on days 2 and three, and that methanogen DNA was amplifiable in this matrix. It appears that the archaea-negative results from calf 13 on days two and three are true indications of a lack of archaea in these samples. This appears also to be the case for archaea in calf 14 on day 13, when none were present.

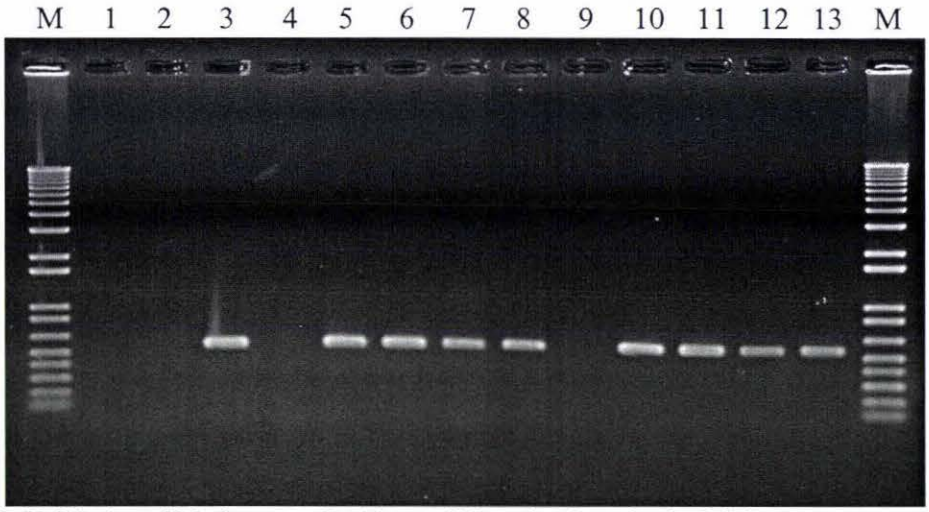


Figure 22: Validation of Methanogen Positive and Negative Samples by PCR.

M: 1 Kb^+ Ladder,

Lane 1: Negative Control,

Lane 2: Negative Control (*Streptococcus bovis* NFCB 2476),

Lane 3: Positive Control (*Methanobrevibacter ruminantium* NT7),

Lane 4: DNA from Calf 13 day 2,

Lane 5: DNA from Calf 14 day 2,

Lane 6: DNA from Calf 16 day 2,

Lane 7: DNA from Calf 13 and Calf 14 day 2,

Lane 8: DNA from Calf 13 and Calf 16 day 2,

Lane 9: DNA from Calf 13 day 3,

Lane 10: DNA from Calf 14 day 3,

Lane 11: DNA from Calf 16 day 3,

Lane 12: DNA from Calf 13 and Calf 14 day 3,

Lane 13: DNA from Calf 13 and Calf 16 day 3,

Lane M: 1 Kb^+ Ladder.

4.4 Discussion

The methanogen isolates investigated were all isolated or enriched from the highest methane positive dilution cultures. Therefore, are representative of the predominant culturable methanogens present in their host animals at a particular time after birth. Characterisation and identification of 11 methanogen isolates by sequence analysis from the developing rumen of the calves revealed that some, such as *Methanosarcina barkeri*, had been previously isolated from the rumen. However, many of the 11 have not been detected in, or isolated from, the rumen before. These included strains of *Methanobacterium bryantii*, *Methanoculleus palmolei* and *Methanobrevibacter thaueri*. Isolates 44, 49 and 53 may be a new species of methanogen.

Based on 16S rDNA sequences, the species with greatest identity to isolates 44, 49 and 53, isolated from two days after birth of calves 13, 14 and 16, respectively, was *Methanofollis liminatans* GKZPZ (Figure 16) at 96.0% (Table 23). Boone *et al.* (1993) considered that a 16S rDNA sequence with an identity of less than 98.0% was sufficient evidence for a separate species within the methanogens. On this basis the isolates 44, 49 and 53 are likely to be strains of a new species within the *Methanofollis* genus. Methanogens belonging to the *Methanofollis* genus have previously only been isolated from aquatic environments, such as fish ponds (*Methanofollis formosanus*) and industrial waste water (*M. liminatans* GKZPZ), but never from a rumen or gut environment. The morphology of isolate 44 was that of an irregular coccus that varied between 0.75-1.0 μm in diameter. The cell diameter of 0.75-1.0 μm for isolate 44 was smaller than the diameter and morphology of the irregular coccus *M. liminatans* described by Zellner *et al.* (1990) at 1.5 μm. No dimensions of isolates 49 and 53 were taken but appeared to be of a similar size to isolate 44. The Gram reaction of isolates 44, 49 and 53 were negative, which is consistent with all other *Methanofollis* spp. (Zabel *et al.*, 1984; Zellner *et al.*, 1990; Zellner *et al.*, 1999; Lai and Chen, 2001; Wu *et al.*, 2005). However, cells of the three isolates appeared to have a Gram positive margin (Figure 12). Methanogens have a different cell-wall composition to bacteria, but still react with Gram staining, with

interpretations of Gram negative or positive being sometimes variable. The substrate specificities of isolates 44, 49 and 53 were found to be different to those described by Zellner *et al.* (1990) for *M. liminatans*. Isolates 44, 49 and 53 were able to utilise only H_2/CO_2 and formate, known ruminal methanogenesis precursors (Hungate *et al.*, 1970). This contrasts with *M. liminatans* GPZKZ which is able to use the substrates formate and H_2/CO_2 plus 2-propanol/CO_2, 2-butanol/CO_2 and cyclopentanol/CO_2 to form methane (Zellner *et al.*, 1990). Other *Methanofollis* spp. have been shown to grow on only H_2/CO_2 and formate as substrates (Zabel *et al.*, 1984; Lai and Chen, 2001; Wu *et al.*, 2005). On the basis of morphology, substrate utilisation and phylogeny, isolates 44, 49 and 53 appear to be a new *Methanofollis* species.

Isolate 74, isolated five days after birth from calf 15, was determined to have the greatest identity to *Methanobacterium bryantii* RiH2 (Figure 17) at 98.5% (Table 24). The type strain of *M. bryantii* was originally isolated from sewage digester sludge (Bryant *et al.*, 1967) and this methanogen has not previously been isolated from the rumen or gut ecosystem. *Methanobacterium* species are easily identified by their longer slender rod shape. Cells of isolate 74 are rods are at least 4.0 μm long by 0.75 μm wide, often growing in chains of varying lengths (Figure 12). The Gram reaction of isolate 74 was variable (Figure 12). This is consistent with data presented for *Methanobacterium* spp. (Zellner and Boone, 2001). Isolate 74 was unable to utilise formate and used only H_2/CO_2 to form methane. However, some strains of *M. bryantii* are known to produce methane from formate (Benstead *et al.*, 1991). Phylogenetic comparisons showed isolates 25 and 28, from calves 12 and 10 on days three and eight respectively, were most similar to *M. bryantii* RiH2 (Figure 17) at 99.6% (Table 26).

Methanogen isolates 10, 20 and 22, from calves 10, 12 and 10 on days four, two and seven were identified as being most similar to *Methanoculleus palmolei* (Figure 18). Similarities of the three isolates to *M. palmolei* were 99.5, 99.5 and 99.2%, respectively (Table 25). *M. palmolei* has previously only been isolated from industrial waste water (Zellner *et al.*, 1998). The methanogen isolates 10, 20 and 22 visualised under epifluorescence, were found to be small cocci and were consistent with the description of *M. palmolei* of Zellner *et al.* (1998).

From phylogenetic comparisons, isolate 48, from calf 15 two days after birth, was identified as being most similar (99.3%, Table 27) to *Methanosarcina barkeri* Sar (Figure 20) and was highly similar (99.1%) to *M. barkeri* CM1 isolated from the rumen of cattle (Jarvis *et al.*, 2000). Isolate 48 subsequently lost viability upon subculture. Isolate 110 was identified as being most similar (98.1%, Table 28) to *Methanobrevibacter thaueri* (Figure 21). Both isolates 48 and 110 were irregular cocci and these methanogens were consistent with the morphologies of *M. barkeri* and *M. thaueri* as described by Miller (2001). *M. thaueri* has been previously isolated from the faeces of cattle (Miller *et al.*, 1986). Isolate 110 also subsequently lost viability upon subculture.

PCR analysis of methanogens in calf rumen contents using nested primers targeted at specific phylogenetic groupings (domain, order, genera, species) revealed trends associated with colonisation of the rumen (Tables 18 and 19). Nested PCR detection results are exemplified by the analysis of a sample from calf 15 two days after birth (Table 18). Methanogens from the domain Archaea, order *Methanobacteriales,* genus *Methanobacterium*, and genus *Methanobrevibacter* were present and both *M. ruminantium*-like and *M. smithii*-like spp. were also present. Methanogens from the orders *Methanococcales*, *Methanomicrobiales* and *Methanosarcinales* were not detected.

At the first sampling, two days after birth, methanogenic archaea were detected in three of the four calves (14, 15 and 16). Archaea were not detected in the rumen contents from calf 13 by PCR, this was consistent with a lack of detection of other methanogen groupings, such as the order *Methanobacteriales*, in this sample. No methanogens from the orders *Methanosarcina*, *Methanomicrobiales* or *Methanococcales* were detected in the rumen contents of the four calves two days after birth. This result is consistent with the findings of Skillman *et al.* (2004); these authors did not detect methanogens from these orders in young lambs. However, in this study methanogens with a high similarity to the *M. liminatans*-like sp. (isolates 44, 49 and 53) from the order *Methanomicrobiales* were isolated from calves 13, 14 and 16 two days after the birth Also, *M. barkeri* (isolate 48) from the order *Methanosarcinales* was cultured from calf 15 two days after birth (Chapter 3), but was not detected using nested PCR analysis (Table 18).

Between four and 11 days after birth in all calves, the types of methanogens detected by the nested primer sets were similar. No methanogens from the orders *Methanosarcina*, *Methanomicrobiales* or *Methanococcales* were detected, however, methanogens from the genus *Methanobacterium* were always detected. Methanogens belonging to the *Methanobacteriales* produced detectable amplicons in all of the 24 rumen samples in this timeframe. Also during this time, detection of *Methanobacterium* species was consistent with the isolation of a strain of *M. bryantii* (isolate 74) from calf 15, five days after birth. The large number of *Methanobacterium* detections over this period contrasts with only six detections of species from the *Methanobrevibacter* genus. *M. ruminantium*-like and *M. smithii*-like spp. were detected only once and twice, respectively, over this same period. These results indicate that species of *Methanobacterium* were always present and that species of *Methanobrevibacter* were sometimes present or always present but regularly below the level of detection by PCR. In contrast, clone library studies by Whitford *et al.*, (2001) Skillman *et al.*, (2006) from mature rumens have detected *Methanobrevibacter* species consistently, while only detecting *Methanobacterium* species sporadically.

Between 13 and 15 days after birth, archaea were detected in the rumen contents from all calves, except for calf 14 on day 13 with this sample yielding no amplicons. The number of *M. ruminantium*-like and *M. smithii*-like spp. detections over this period increased in comparison to detections between days four and 11. This change in detection frequency suggests that *Methanobrevibacter* species were increasing in numbers. *M. ruminantium*-like and *M. smithii*-like spp. are recognised as the predominant methanogen species in grazing ruminants (Miller *et al.*, 1986). At three weeks of age all calves had detectable levels of *Methanobacterium, Methanobacteriales*, *Methanobrevibacter*, *M. smithii*-like and *M. ruminantium*-like spp.. Confirmation of the presence of *Methanobrevibacter* species was obtained with the culture of the *M. smithii*-like methanogen *Methanobrevibacter thaueri* (isolate 110) from calf 16 on day 22. No methanogens from the orders *Methanosarcina*, *Methanomicrobiales* or *Methanococcales* were detected on day 22. It is likely that methanogen populations continue to fluctuate beyond three weeks of age. The predominant methanogen in calves at three weeks of age was *M. thaueri*. However, most studies indicate *M. ruminantium* is the predominant methanogen in mature cattle (Smith and Hungate, 1958; Miller *et al.*, 1986).

Also, inconsistencies existed with the nested PCR analysis. For example, archaea from calf 13 two days after birth were not detected by PCR (Figure 22) but isolate 44 was cultured from calf 13 in the same sample. Archaea were also not detected in rumen contents of calves 13 and 15 three days after birth and from calf 14 on day 13. From this result the sensitivity of PCR detection in the calf gut contents was investigated. Enumerated culturable methanogens from calf 13 were measured at 10^1 cells ml^{-1} (Table 2), but were not detected by nested PCR (Figure 22). Enumerated culturable methanogens from calf 14 on day two, the same day as calf 13, were also measured at 10^1 cells ml^{-1} (Table 2) and archaea were detected by nested PCR (Figure 22). These results suggest that the PCR detection limit of total archaea in calf rumen contents could be as low as 10^1 cells ml^{-1}, but is variable. Uncultured methanogens may raise the template copy number, thus more template is available above 10^1 cells ml^{-1}. Variations in detection limit may also be due to

the inhibition of PCR by compounds such as humic acids, phenolic compounds, or insufficient cell lysis (Wilson, 1997 and references within). This variation may also be a problem associated with low template concentration compounded by the presence of inhibitors. It is likely that archaea were not detected in calf 14 on day 13 because of PCR inhibitors, as culturable cell numbers were at densities of 10^2 cells ml^{-1}.

The pattern of methanogen establishment in the young calves was somewhat different to that found in young lambs (Skillman *et al.*, 2004). In young lambs, *Methanobacterium* species were not present during the first three weeks (Skillman *et al.*, 2004), whereas in this study, *Methanobacterium* species were present in the calves throughout the sampling period. There were also differences with the detection of *Methanobrevibacter* species. In calves, *Methanobrevibacter* species were present in only 19 of the 44 calf rumen samples and were not detectable in the second week after birth. In contrast, *Methanobrevibacter* species were detected in all samples from lambs including immediately after birth (Skillman *et al.*, 2004). These and other differences, probably arise from the different environments in which lambs and calves were raised. The lambs studied by Skillman *et al.* (2004) were raised with their dams in a grazing flock, whereas in this study the calves were raised separated from cows and had no contact with mature ruminants. Without inoculation from mature ruminants, it is probable that colonisation from *Methanobrevibacter* species was delayed or slowed. This may have allowed other species of methanogen e.g. *M. bryantii*, *M. liminatans*-like, *M. barkeri* or *M. palmolei* to colonise and predominate.

5 Discussion and Future Research

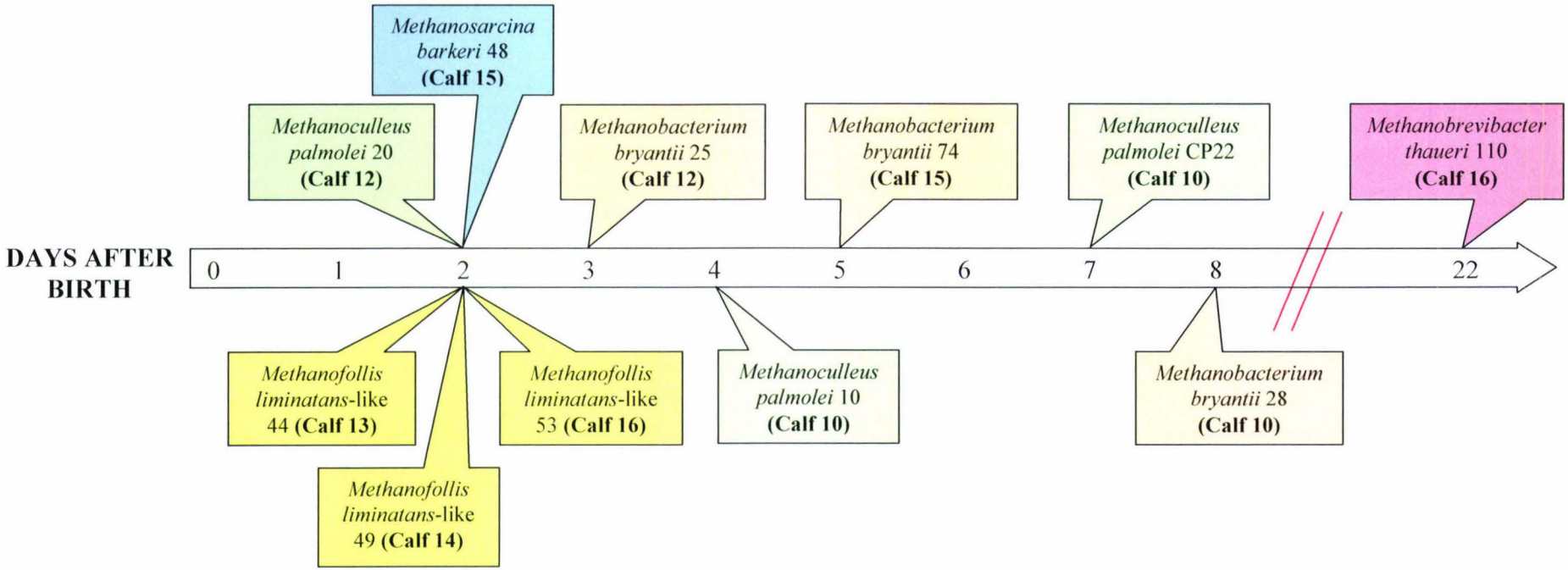
The initial aims of this study were to investigate the time at which methanogens were first present in the rumens of newborn calves; identify methanogen densities in the developing rumen of newborn calves over a three-week period after birth; culture representative methanogens from different times during the three week period; determine methanogen identities by phenotypic and phylogenetic characterisation. Calves were chosen for study because there have been no previous studies on methanogens in dairy calves in New Zealand and because the dairy industry is responsible for 37% of New Zealand's ruminant methane emissions (Joblin, 2006). This section summaries results on the colonisation of milk-fed, non-grazing, pre-ruminant calves and discusses possibilities for future research.

In five of the six calves sampled, methanogens colonised their developing rumens within two days of birth. The first samples were collected from calves at 48 hours after birth, so the precise time of first methanogen colonisation is not known. It is likely that the methanogens present at this time are those that first colonised the rumen. In calf 10, methanogens were not present in the first sample collected, but were present in the second sample suggesting that colonisation of calf 10 by methanogens took place between 48 and 72 hours after birth. The methanogen densities in the five calves at two days were similar to those found by other workers in young pre-ruminant calves (Fonty *et al.*, 1986; Fonty *et al.*, 1987; Minato *et al.*, 1992; Morvan *et al.*, 1994; Skillman *et al.*, 2004).

The initial analysis of culture enumerations showed that methanogen establishment had two phases. The establishment pattern, based upon mean population densities is given in Figure 6 (Chapter 2). Within two days of birth, mean methanogen densities were about 10^1-10^2 cells ml^{-1}, and remained relatively constant until day nine when densities increased to about 10^7 cells ml^{-1} by day 15, and about 10^8 cells ml^{-1} by day 22. Similar methanogen densities have been found in pre-ruminant lambs in New Zealand (Skillman

et al., 2004) and in pre-ruminant calves in Japan (Minato *et al.*, 1992). However, the overall biphasic pattern of colonisation found from the four calves contained two distinct patterns of colonisation, where two calves showed one pattern and the remaining two another pattern (Figure 8). In calves 14 and 15 there was a period (days six to 11) in which non-methanogenic hydrogenotrophs appear to have out-competed methanogens for the available hydrogen (Figure 9). These hydrogenotrophs are likely to be the reason for the lack of detection of methanogens in the rumen of calf 10 until three days after birth (24 hours later than other calves). This suggestion is based on evidence obtained from bacterial isolates 4, 5 and 6 (Table 22) purified from calf 10 two days after birth. These bacteria utilised hydrogen without producing methane. The identities of the non-methanogenic hydrogenotrophs found in calves 10, 14 and 15 have yet to be determined, but are likely to be acetogenic or sulphate-reducing bacteria which utilise hydrogen. Sulphate-reducing and acetogenic bacteria have previously been found in young ruminants soon after birth (Morvan *et al.*, 1994).

Figure 23: Timeline for Methanogens Obtained from the Rumens of Calves. The timeline shows times of appearance of 11 methanogen isolates obtained from six calves between birth and 22 days after birth. The numbers with each isolate refer to the isolate number (see Table 2). Despite intensive efforts no methanogen isolates were cultivated from enumeration tubes between nine and 21 days (red lines) after birth.



When isolates representative of the predominant culturable methanogens were placed on a timeline, a pattern of colonisation became apparent (Figure 23). The first methanogens to colonise the rumen included *Mcl. palmolei*, *Mfl. liminatans*-like sp., and *Msr. barkeri* two days after birth, with densities as low as 10^1-10^2 cells ml^{-1}. Between three and eight days after birth, the predominant culturable methanogens in calves 10, 12, and 15 included *Mcl. palmolei* or *Mbc. bryantii*. Despite extensive efforts, methanogens were unable to be cultured from enumeration cultures between days nine and 21. *Mbb. thaueri* from calf 16 was identified as the predominant methanogen 22 days after birth.

Further consideration of the results in Figure 23 revealed new information. The calves had been sampled in two batches with calves 10 and 12 entering the rearing facility together, and before calves 13, 14, 15 and 16 were introduced. When the methanogens colonising these groups were assessed (Figure 24) analysis showed that *Mcl. palmolei* was obtained only from calves 10 and 12, while *Mfl. liminatans*-like isolates were obtained only from calves 13, 14, and 16. *Msr. barkeri* was cultured from only calf 15. The results in Figure 24 suggested that whichever methanogen species colonised first, it would later be replaced by *Mbc. bryantii* as a predominant culturable species.

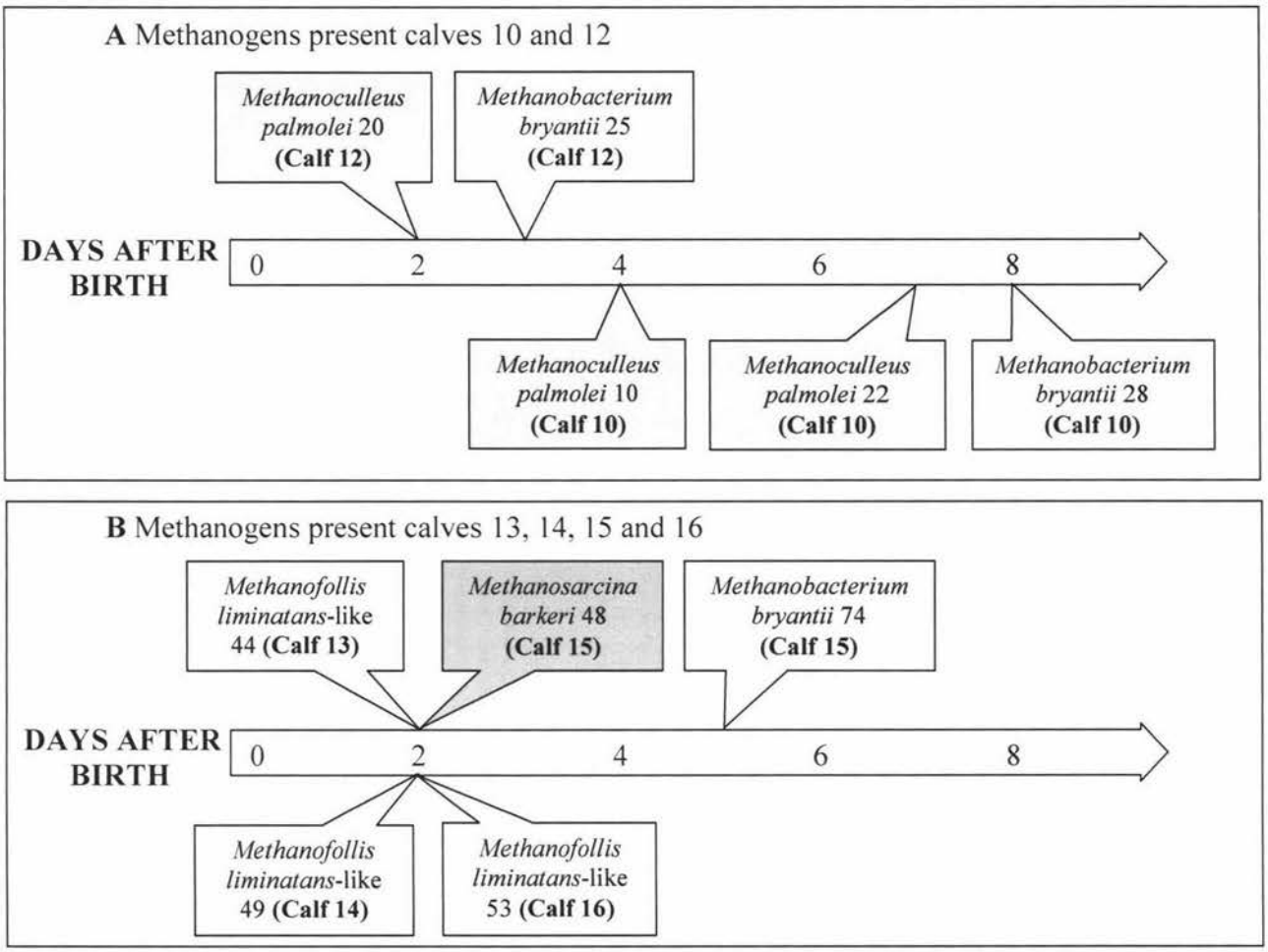


Figure 24: Timeline for Methanogens Colonising Calves in the First Eight Days after Birth.

These observations indicate that the first species to colonise the rumen of calves was likely to be the first methanogen to which calves were exposed. This first methanogen had a higher probability of being transferred between calves in close association or being introduced to all calves simultaneously. The results suggested that the first exposure to methanogens may have occurred outside the rearing unit, because calves 13, 14, 15 and 16 (Figure 24B) had different initial colonising methanogens to those of calves 10 and 12 (Figure 24A). The reasons why *Mcl. palmolei* and *Mfl. liminatans*-like sp. were the initial colonising methanogens are not known. Little is known about either species but both have been previously isolated from the terrestrial environment. *Mcl. palmolei* had been isolated from wastewater from a palm oil factory (Zellner *et al.*, 1998). *Mfl. liminatans*

had been isolated from wastewater of bioreactors (Zellner *et al.*, 1990, 1999). Clone sequences closely related to *Mcl. palmolei* and *Mfl. liminatans* spp. have only been detected in aquatic environments (Wantanabe *et al.*, 2002; Karr *et al.*, 2006). This study was the first to find these methanogens in the gut of animals and it is probable that they originated from soil or water (non-rumen sources). It is likely that the developing rumen provides a niche environment suited to a range of hydrogenotrophic methanogens and the niche is first occupied by the first methanogen ingested. In the present study, it was likely that the first colonising methanogen was from a non-rumen source because calves had been removed from mature ruminants.

Methanogens are difficult to culture (Smith and Hungate, 1958) or maintain in culture (Jarvis *et al.*, 2000) so the above analysis, based on cultures, is likely to be limiting. However, an advantage is that cultures are available for future study. To obtain further information on methanogen colonisation in the calves a culture-independent method was used. PCR analysis of DNA extracted from rumen contents was carried out with nested primer sets targeted at specific methanogen groups (Tables 18 and 19). A comparison of the PCR results from this study with results from a similar study of methanogens in newborn lambs in New Zealand (Skillman *et al.*, 2004) showed considerable differences in the methanogen colonisation patterns of the two species of ruminant. In lambs, *Methanobrevibacter* spp. were detectable at three days of age and persisted throughout the trial (48 days) whereas colonisation by *Methanobacterium* spp. was erratic (Skillman *et al.*, 2004). At the end of the lamb trial, *Methanobacterium* spp. were not detected. In contrast, in this study on calves, *Methanobacterium* spp. were detected by PCR throughout the trial (22 days), but *Methanobrevibacter* spp. were detected in less than half the samples. At the end of the trial (Table 19), the PCR analysis showed the presence of *Methanobacterium* and *Methanobrevibacter* spp. in all four calves. A major difference between the two studies was that lambs were raised with their dams while calves were raised with no mature ruminants present. With no mature ruminants to provide a source of *Methanobrevibacter* spp., the predominant species in mature ruminants (Smith and Hungate, 1958), poor colonisation by *Methanobrevibacter* spp. was not unexpected.

An examination of results from the PCR analysis, in light of the identities of isolates cultured from calves 13, 14, 15 and 16, revealed some consistencies and some inconsistencies. The presence of isolate *Mbc. bryantii* from calf 15 on day five is in agreement with the PCR analysis using the *Methanobacterium* primer set. Isolation of *Mbb. thaueri* from calf 16 on day 22 is in agreement with PCR results using the *Mbb. smithii*-like spp. primer set. However, although *Mfl. liminatans*-like sp. (isolates 44, 49 and 53) and a strain of *Msr. barkeri* (isolate 48) belonging to the orders *Methanomicrobiales* and *Methanosarcinales* respectively were isolated, PCR analysis of the same samples failed to detect methanogens belonging to these orders. The reasons for this are not known but may involve biases from DNA extraction or PCR amplification (von Wintzingerode *et al.*, 1997).

In conclusion, there are only five published studies on methanogens in newborn ruminants and this study has yielded a range of new information in an area of considerable interest to researchers searching for strategies to lower methane emissions from ruminants. The isolation, purification and identification of a range of methanogens from six calves, combined with a nested PCR analysis of gut samples, has provided the first information on species identification and on successional changes of colonising methanogens. This includes the isolation of a novel methanogen species, a *M. liminatans*-like species. The data obtained in this study is consistent with a model in which the first colonising methanogens, *Mfl. liminatans*-like sp., *Mcl. palmolei* or *Msr. barkeri* are displaced by a succession which included *Mbc. bryantii*. In turn *Mbc. bryantii* was replaced by *Methanobrevibacter* spp. which included *Mbb. thaueri*. *Mbb. thaueri* was present at densities of 10^9 cells ml^{-1}, a level similar to that observed in mature ruminants.

The investigation of methanogen colonisation and establishment in the developing rumen initiated by this study is ongoing. The continuing efforts include; a 16S rDNA clone library analysis of methane-positive cultures in enumerations to provide expanded information on changes in the methanogen successions during the first three weeks after the birth of calves. This analysis is of particular interest because few methanogens were cultured from enumeration tubes between five and 22 days after birth. Efforts are continuing to purify and identify methanogens (Chapter 3) and to further characterise the *Methanofollis liminatans*-like sp. as it is likely to be a new methanogen species. Work is in progress to isolate and identify hydrogenotrophs which interfered with the methanogens during enumerations in order to obtain cultures for testing of interactions between methanogens isolated in this study.

Future studies of methanogen colonisation of the rumen should include enumeration of methanogens by real-time PCR. This method would overcome culture problems experienced because of sensitivity of methanogens to oxygen and problems of competition with other microbes such as those experienced in the examination of samples from calves 14 and 15. Real-time PCR has been used to quantify methanogens in the gut ecosystem of humans (Stewart *et al.*, 2006) and these workers found that methanogens were present at levels between 7.4×10^5 and 4.9×10^7 cells ml^{-1}. To date there have been no similar studies on methanogens in the rumen.

Recently methanogen diversity in mature ruminants was investigated by a variation of DGGE, Temporal Temperature Gradient Electrophoresis (TTGE) (Nicholson *et al.*, 2007). This study of archaeal 16S rRNA gene sequences revealed a new lineage of methanogens from the rumen, as yet uncultured. It is suggested that future studies on methanogen colonisation of the rumen should use DGGE to obtain better information on methanogen diversity. DGGE/TTGE methods have the advantage over a targeted PCR analysis because the diversity of methanogens can be determined from the band fingerprints (Muyzer, 1999).

6 Appendix A- Media Components

6.1 Vitamin Solution Composition/ 1.0 L

Distilled H_2O was heated until boiling and then cooled to room temperature on ice under O_2-free CO_2 for 15 minutes. Components were added to cooled anaerobic distilled H_2O and mixed thoroughly until dissolved. Aliquots of 10 ml were removed from the solution by drawing up into a CO_2-flushed syringe and filter sterilised through a 0.22 μm filter into sterile capped CO_2-filled Hungate tubes. To each 10 ml tube of vitamin solution 0.1 ml of previously sterilised $Na_2S{\cdot}9H_2O$ reducing agent (Section 6.7) was added anaerobically, and aseptically. Prepared tubes were stored frozen at -20 °C until needed.

Pyridoxine HCl (Vitamin B_6)	10.0 mg
L-ascorbic acid (Vitamin C)	5.0 mg
Calcium pantothenate (pantothenic acid)	5.0 mg
Choline chloride	5.0 mg
Lipoic acid (D,L-6,8-thioctic acid)	5.0 mg
i-Inositol (myo-inositol)	5.0 mg
Nicotinamide	5.0 mg
Nicotinic acid	5.0 mg
p-Aminobenzoic acid	5.0 mg
Pyridoxal-HCl	5.0 mg
Riboflavin (Vitamin B_2)	5.0 mg
Thiamine-HCl (Vitamin B_1)	5.0 mg
D-biotin (Vitamin H)	2.0 mg
Folic acid	2.0 mg
Vitamin B_{12} (cyanocobolamin)	0.1 mg
Distilled H_2O	1000 ml

6.2 Salt Solution A Composition/ 2.0 L

All components were added to distilled H_2O and mixed thoroughly until dissolved.

NaCl	12.0 g
KH_2PO_4	6.0 g
$(NH_4)_2SO_4$	3.0 g
$CaCl_2 \cdot 2H_2O$	1.58 g
$MgSO_4 \cdot 7H_2O$	2.4 g
Distilled H_2O	2000 ml

6.3 Salt Solution 2B Composition/ 2.0 L

All components were added to distilled H_2O and mixed thoroughly until dissolved.

$K_2HPO_4 \cdot 3H_2O$	15.72 g
Distilled H_2O	2000 ml

6.4 Anaerobic Salts Solution Composition/ 1.0 L

Salts Solution A (Section 6.2), Salts Solution 2B (Section 6.3), $NaHCO_3$ and resazurin were added to distilled H_2O as listed below. These components were heated until boiling, and then cooled to room temperature on ice under O_2-free CO_2 for 15 minutes. After cooling, L-cysteine HCl was added to the solution and mixed thoroughly until dissolved. Aliquots of 10 ml were removed from the solution by drawing up into a CO_2-flushed syringe and dispensed into sterile CO_2-filled Hungate tubes. Tubes were autoclaved at 121 °C for 20 minutes under 103 kPa pressure and then stored at room temperature.

Salt Solution A	170 ml
Salt Solution 2B	170 ml
$NaHCO_3$	5.0 g
Resazurin (0.1% solution)	100 µl
L-cysteine HCl	0.5 g
Distilled H_2O	660 ml

6.5 Antibiotic (P/S) Stock Solution Composition/ 0.1 L

Salts Solution A (Section 6.2), Salts Solution 2B (Section 6.3), $NaHCO_3$ and resazurin were added to distilled H_2O. These components were heated until boiling, and then cooled ice under O_2-free CO_2 for 15 minutes. After cooling benzyl penicillin, streptomycin sulfate and L-cysteine HCl were added to the solution and mixed thoroughly until dissolved. Aliquots of 10 ml were removed from the solution by drawing up into a CO_2-flushed syringe and dispensed into sterile capped CO_2-filled Hungate tubes. To each 10 ml tube of vitamin solution 0.1 ml of previously sterilised $Na_2S{\cdot}9H_2O$ reducing agent (Section 6.7) was added anaerobically and aseptically. Prepared tubes were stored frozen at -20 °C until required. Working solution of P/S was prepared by adding six ml of P/S stock solution into nine ml of Anaerobic Salts Solution (Section 6.4).

Benzyl Penicillin (BP)	6.0 g (2 x 10^7 I.U.)
Streptomycin SO_4 (SS)	2.0 g
Salt solution A	17 ml
Salt solution 2B	17 ml
resazurin (0.1% solution)	10 µl
$NaHCO_3$	0.5 g
L-cysteine HCl	50 mg
Distilled H_2O	66 ml

6.6 Antibiotic Stock Solution Composition/ 0.01 L

Antibiotic solutions were prepared as stock solutions by adding known weights of dry antibiotics to 10 ml aliquots of the appropriate solvent.

Table 20: Antibiotic Concentrations: Adapted from Naylor (1998).

Antibiotic	Stock Solution	Solvent	Final Concentration[2]
Lincomycin	2 mg ml^{-1}	AS	20 μg ml^{-1}
Tetracycline	2 mg ml^{-1}	AS and Methanol[1]	20 μg ml^{-1}
Rifampicin	2 mg ml^{-1}	Methanol	20 μg ml^{-1}
Ampicillin	5 mg ml^{-1}	AS	50 μg ml^{-1}
Chloramphenicol	2 mg ml^{-1}	AS	20 μg ml^{-1}
Hygromycin B	2 mg ml^{-1}	AS	20 μg ml^{-1}
Clindamycin	2 mg ml^{-1}	AS	20 μg ml^{-1}

AS= Anaerobic Salts Solution, [1]Nine ml of AS and one ml of methanol, [2]Approximately

6.7 $Na_2S·9H_2O$ Stock Solution Composition/ 0.1 L

Distilled H_2O was heated until boiling, and then cooled on ice under O_2-free N_2 for 15 minutes. After cooling, and still under O_2-free N_2, $Na_2S·9H_2O$ was added to the solution and mixed thoroughly until dissolved. Aliquots of 10 ml were removed from the solution by drawing up into a N_2-flushed syringe and filter sterilised through a 0.22 μm filter into sterile capped N_2-filled Hungate tubes. Prepared tubes were stored at room temperature until needed.

$Na_2S·9H_2O$	1.0 g
Distilled H_2O	100 ml

6.8 Trace Elements Solution (Balch's) Composition/ 1.0 L

Nitrilotriacetic acid was added to 500 ml of distilled H_2O and the pH adjusted to 6.5 with KOH. Remaining salts were added one at a time with the remaining 500 ml of distilled H_2O added last and mixed thoroughly until salts dissolved. Aliquots of 200 ml were dispensed into plastic screw cap containers and stored frozen at -20 °C until needed.

Nitrilotriacetic acid	1.5 g
$MgSO_4 \cdot 7H_2O$	3.0 g
NaCl	1.0 g
$MnSO_4 \cdot H_2O$	0.5 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$CoCl_2 \cdot 6H_2O$	0.1 g
$CaCl_2$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
$CuSO_4 \cdot 5H_2O$	10 mg
$AlK(SO_4)_2 \cdot 12H_2O$	10 mg
H_3BO_3	10 mg
$Na_2MoO_4 \cdot 2H_2O$	10 mg
$NiSO_4 \cdot 6H_2O$	30 mg
Na_2SeO_3	20 mg
$Na_2WO_4 \cdot 2H_2O$	20 mg
Distilled H_2O	1000 ml

6.9 Substrates for Methanogen Growth

Stock solutions of 20% (v/v) acetate, formate, ethanol, methanol, 2-propanol, 2-butanol, cyclopentanol and methylamine were prepared in anaerobic salts solution (Section 6.4). Substrate solutions were equilibrated under a stream of O_2-free CO_2 for 30 minutes in Hungate tubes and then sealed anaerobically and aseptically. Substrate stock solutions were added to growth medium to give a final concentration of 0.5%. The growth substrate H_2/CO_2 was added to tubes of growth medium anaerobically at a pressure of 172 kPa.

6.10 50% Dimethylsulphoxide Solution/ 1.0 L

Salts Solution A (Section 6.2), Salts Solution 2B (Section 6.3), dimethylsulphoxide, $NaHCO_3$ and resazurin were added to distilled H_2O and dissolved. The solution was heated until boiling, and then cooled on ice under O_2-free CO_2 for 15 minutes. After cooling L-cysteine HCl was added to the solution and mixed thoroughly until dissolved. Aliquots of 10 ml were removed from the mixture by drawing up into a CO_2-flushed syringe and dispensed into sterile CO_2-filled Hungate tubes. Tubes were autoclaved at 121 °C for 20 minutes under 103 kPa pressure and then stored at room temperature.

Salt Solution A	170 ml
Salt Solution 2B	170 ml
Dimethylsulphoxide	500 ml
$NaHCO_3$	5.0 g
Resazurin (0.1% solution)	100 µl
L-cysteine HCl	0.5 g
Distilled H_2O	160 ml

6.11 10x TAE Buffer Solution/ 1.0 L

Tris-base, acetic acid and EDTA were added to distilled water and dissolved. The 10-times solution was diluted to 1-times concentration with distilled H_2O to obtain a working solution.

Tris-base	48.4 g
Acetic acid	11.40 ml
0.5 M EDTA (pH 8.0)	20 ml
Distilled H_2O	1000ml

7 Appendix B- Measurements and Raw Data

7.1 pH of Rumen Samples Collected from Newborn Calves

Table 21: pH of Rumen Samples Collected from Newborn Calves

Day	10	12	13	14	15	16
Day 2	6.2	6.0	6.3	6.3	6.7	6.2
Day 3	6.3	6.3	6.4	6.1	6.4	6.2
Day 4	6.2	6.3	6.3	6.2	6.4	6.2
Day 5	6.1	6.1	7.2	6.4	6.2	6.7
Day 6	5.9	N/S[1]	6.2	5.8	5.8	5.8
Day 7	5.8	N/S[1]	6.3	5.8	5.7	6.0
Day 9	6.5	N/S[1]	6.1	6.0	5.8	5.8
Day 11	6.0	N/S[1]	6.3	6.0	5.8	6.1
Day 13	N/S[1]	N/S[1]	5.8	6.4	5.7	5.9
Day 15	N/S[1]	N/S[1]	6.2	6.5	6.3	6.3
Day 22	N/S[1]	N/S[1]	6.0	6.7	6.2	6.4

[1]Calf was not sampled because of lincomycin contamination

7.2 Characteristics of Colony Picks from Enumeration Tubes

Table 22: Colonies Picks from Methanogen Enumeration Tubes

Calf No.	Age (Days)	Isolate	Description	Methane	Final tube Headspace (ml)
-	-	-	Control	N	12
Test	-	1	Small Round Brown	N	11
Test	-	2	Small Round Brown	N	11
Test	-	3	Small Feathery Brown	N	10
10	2	4	Small Round Brown	N	5
10	2	5	Small Round Brown	N	7
10	2	6	Small Round Brown	N	6
10	3	7	Small Round Brown	Y	4
10	3	8	Small Round Brown	N	12
10	4	9	Small Round Brown	N	10
10	4	10	Small Round Brown	Y	11
10	4	11	Small Round Brown	N	11
10	5	12	Large Round Brown	N	6
10	5	13	Large Brown Centre White Irregular	N	7
10	5	14	Small Round Brown	N	9
10	6	15	Small Brown Centre White Round	N	5
10	6	16	Small Round Brown	N	8
10	6	17	Large Brown White	N	8
12	2	18	Large White/Brown Feathery	N	3
12	2	19	Small Round Opaque Brown Centre	N	8
12	2	20	Small Round White	Y	1

Calf No.	Day	Isolate	Description	Methane	Headspace volume (ml)
10	7	21	Small Round Brown	Y	5
10	7	22	Small Round Dark Brown	Y	4
10	7	23	Small Round White	Y	4
12	3	24	Small Round Dark Brown	Y	3
12	3	25	Small Round White	Y	4
12	3	26	Large Irregular White Brown	Y	3
10	9	27	V. Large Round White	Y	4
10	9	28	Large Irregular White Brown Centre	Y	3
10	9	29	Large White Irregular	N	8
12	4	30	Large White Round	Y	4
12	4	31	Small Brown Round	Y	3
12	4	32	Small Brown Round	N	1
12	5	33	Irregular Feathery White Large	N	2
12	5	34	Irregular Dark Brown	N	12
12	5	35	Round White Large	Y	2
10	11	36	Irregular Dark Brown Large	N	12
10	11	37	Round Brown Medium	N	14
10	11	38	Irregular Brown Medium	N	13
13	2	39	Large Irregular Opaque Brown Centre	Y	5
13	2	40	Round White Large	Y	5
13	2	41	Round Brown Large	Y	4
13	2	42	Clear Round Large	Y	2
14	2	43	Irregular Opaque Brown Centre	Y	4
14	2	44	Granular Irregular Light Brown	Y	2
14	2	45	Small Round Brown	Y	3
15	2	46	Irregular Feathery White	Y	3
15	2	47	Small Round Brown	Y	1
15	2	48	Large Feathery Brown	Y	3
16	2	49	Small Round Brown	Y	5
16	2	50	Small Round White	Y	5
15	3	51	Clear Round Large	Y	4
15	3	52	Feathery White/ Brown Large	Y	1
15	3	53	Irregular White Large	Y	4
15	3	54	Large Irregular Opaque Brown Centre	Y	2
16	3	55	Medium Brown Round	Y	2
16	3	56	White Irregular Large	N	12
16	3	57	Irregular Opaque Large	N	12
13	3	58	White Irregular Large	Y	3
13	3	59	White Irregular Large	N	8
16	3	60	Large White Feathery	N	6
16	3	61	Small Light Brown Irregular	N	12
16	3	62	Small Dark Brown Irregular	N	6
15	3	63	V.V. Large White Brown Centre	N	12
15	3	64	Large White Irregular Brown Centre	N	5
16	5	65	Irregular Large Brown Centre	N	11
16	5	66	Irregular Round Light Brown Medium	N	11
16	5	67	Round Small Light Brown	N	11
14	5	68	Large Irregular White Brown Centre	N	12
14	5	69	Large Irregular White Brown Centre	N	4
15	5	70	Medium Round Brown/Opaque	N	12
15	5	71	Medium Dark Brown Irregular	Y	4

Calf No.	Day	Isolate	Description	Methane	Headspace volume (ml)
13	5	72	Medium Dark Brown Irregular	N	12
13	5	73	Medium Dark Brown Irregular	N	12
16	5	74	Very Small White/Light Brown	N	12
14	7	75	Circular Large Creamy Brown	N	4
14	9	76	Large Spreading Not Well Defined	N	4
14	9	77	Medium Round White Brown Centre	N	4
13	6	78	Medium Round White Brown Centre	N	10
13	6	79	Medium Round White Brown Centre	N	12
16	9	80	Large Irregular White Brown Centre	N	11
16	9	81	Large Irregular White Brown Centre	N	7
15	9	82	Large Creamy Brown Circular	N	5
15	9	83	Small Round Creamy Brown	N	2
14	7	84	Large White with Brown Centre	N	13
14	7	85	Irregular White Brown Centre	N	12
16	7	86	Large Round White Brown Centre	N	12
16	7	87	Large Irregular Brown	N	11
16	7	88	Small Round Light Brown	N	13
15	7	89	Small Round Light Brown	N	13
15	7	90	Large Feathery White	N	13
15	7	91	Large White Irregular Brown Centre	N	14
13	7	92	Large Dark Brown Round	N	14
13	7	93	Large Brown with Dark Brown Centre	N	13
13	7	94	Large Irregular White Brown Centre	N	13
14	6	95	Feathery Large White	N	12
14	6	96	Large Round White Brown Centre	N	10
14	6	97	Small Light Brown Oval	N	12
15	6	98	Large Feathery Dark Brown Centre	N	10
15	13	99	Large Feathery Dark Brown Centre	N	10
14	13	100	Large Feathery Dark Brown Centre	N	9
15	13	101	Large Irregular White Brown Centre	N	11
16	13	102	Medium Round Dark Brown	N	1
13	15	103	Medium Round White Brown Centre	N	5
14	15	104	Large Feathery Dark Brown Centre	N	12
15	15	105	Clear Opaque Round	N	12
16	15	106	White Medium Round Star-Like	N	11
13	22	107	Small Brown Round	N	12
14	22	108	Large Brown Orange Round	N	12
15	22	109	Medium White Brown Centre	N	12
16	22	110	Small Brown Round	Y	4

7.3 16S rDNA Sequences Obtained from Methanogen Isolates

Methanogen Isolate 10

cctccgggggcacagcgcgaaaactttacaatgcgggcaaccgtgataagggaacctcgagtgcctgtata
tgcaggctgtccaggtgtctaaaacacacttgaagaaagggccgggcaagaccggtgccagccgccgcggta
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Methanogen Isolate 20

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Methanogen Isolate 22

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Methanogen Isolate 25

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Methanogen Isolate 28

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Methanogen Isolate 44

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Methanogen Isolate 48

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Methanogen Isolate 49

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Methanogen Isolate 110

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Methanogen Isolate 53

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gaaaccgtgaaacgcgccgcctgggaagtacggtcgcaaggctgaaacttaaaggaattggcgggggagcac
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agccgggctgaagaccttgcttgatcagctgagaggaggtgcatggccgtcgtcagttcgtactgtgaagca
tcctgttaagtcaggcaacgagcgagacccacgccaacagttgccagcatgttctccggaatgatggggaca
ctgttgggaccgcctctgctaaagaggaggaaggaatgggcaacggtaggtcagcatgccccgaattacccg
ggctacacgcgggctacaatggacaggacaatgggtatcgacaccgaaaggtgaaggcaatctcctaaacct
gcccttagttcggattgtgggctgtaactcgcccacatgaagctggaatccgtagtaatcgcgtttcaaaat
agcgcggtgaatctgtccctgctccttgcacacaccgcccgtcaaaccacccgagtggggtttggatgaggc
tgcggttgttgccgtagtcgaatctaggttccgcaagggggttaagtcgtaacaaggtaaccgtaa

Methanogen Isolate 74

ttacggctcagtaacacgtggataacctgcccttaggactgggataaccctgggaaactggggataataccg
gatatttagaggttcctggaatggtcttctatttaaatgttcaggcgcctaaggatggatctgcggccgatt
aggtagttggtggggtaatggcccaccaagccttttatcggtacgggttgtgagagcaagagcccggagatg
gaacctgagacaaggttccaggccctacggggcgcagcaggcgcgaaacctccgcaatgcgagcaatcgcga
cgggggggaccccaagtgccactcttaacggggtggctttttttagagtgtaaaaagctttaagaataagagct
gggcaagaccggtgccagccgccgcggtaacaccggcagctcaagtggtggccatttttattgggcctaaag
cgttcgtagccggcttgataagtctctggtgaaatcccatagcttaactgtgggaattgctggagatactat
caggcttgaggccgggagaggttaggggtactcccagggtaggggtgaaatcctataatcctgggaggacca
cctgtggcgaaggcgcctaactggaacggacctgacggtgagtaacgaaagccaggggcgcgaaccggatta
gatacccgggtagtcctggccgtaaacgatgcggacttggtgttggaatggcttcgagctgctccagtgccg
aagggaagctgttaagtccgccgcctgggaagtacggtcgcaagactgaaacttaaaggaattggcgggggga
gcaccacaacgcgtggagcctgcggtttaattggattcaacgccggacatctcaccaggggcgacagcagaa
tgatggccaggttgacgaccttgcttgacaagctgagaggaggtgcatggccgccgtcagctcgtaccgtga
ggcgtcctgttaagtcaggcaacgagcgagacccacgcccttagttaccagcggatcctccgggatgccggg
cacactaaggggaccgccagtgataaactggaggaaggagtggacgacggtaggtccgtatgccccgaatcc
cctgggcaacacgcgggctacaatggctatgacaatgggttccgacactgaaaggtgaaggtaatctcctaa
acatagtcttagttcggatcgagggctgtaacccgccctcgtgaagctggaatgcgtagtaatcgcgtgtca
taatcgcgcggtgaatacgtccctgctccttgcacacaccgcccgtcacgccacccaaaaagggtttggatg
aggcctcaatcgttggttggggtcgaatctgggttctttgaggagggcgaagtcgtaacaaggtaaccgtaa

7.4 Similarity Matrices of 16S rDNA Sequences from Methanogen Isolates

Table 23: Similarity Matrix of 16S rDNA Sequences Including Isolates 44, 49 and 53 (% Identity)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*Methanocalculus halotolerans* SEBR (AF033672)	100.0	88.4	89.0	88.8	88.8	88.8	89.3	89.6	88.6	89.7	89.3	90.1	89.9	86.2	87.2
2	*Methanofollis aquaemaris* N2F9704 (AF262035)	88.4	100.0	97.0	95.1	95.1	95.1	95.1	95.5	94.5	91.5	90.5	91.1	91.6	88.9	90.9
3	*Methanofollis formosanus* ML15 (AY186542)	89.0	97.0	100.0	95.6	95.6	95.6	96.0	96.4	95.4	92.8	91.6	92.1	92.5	89.4	91.6
4	**Isolate 44**	88.8	95.1	95.6	100.0	99.9	99.9	95.9	96.0	95.4	92.0	90.7	91.4	92.0	88.5	90.5
5	**Isolate 49**	88.8	95.1	95.6	99.9	100.0	100.0	95.9	96.0	95.4	92.0	90.7	91.4	92.0	88.5	90.5
6	**Isolate 53**	88.8	95.1	95.6	99.9	100.0	100.0	95.9	96.0	95.4	92.0	90.7	91.4	92.0	88.5	90.5
7	*Methanofollis liminatans* BM1 (Y16429)	89.3	95.1	96.0	95.9	95.9	95.9	100.0	99.7	99.5	91.7	90.8	90.6	91.2	88.4	90.1
8	*Methanofollis liminatans* GKZPZ (Y16428)	89.6	95.5	96.4	96.0	96.0	96.0	99.7	100.0	99.2	92.2	91.2	91.0	91.6	88.6	90.6
9	*Methanofollis tationis* Chile 9 (AF095272)	88.6	94.5	95.4	95.4	95.4	95.4	99.5	99.2	100.0	91.2	90.2	90.0	90.6	88.0	89.5
10	*Methanoculleus palmolei* INSULZ (Y16382)	89.7	91.5	92.8	92.0	92.0	92.0	91.7	92.2	91.2	100.0	95.9	96.6	97.5	90.0	91.5
11	*Methanoculleus olentangyi* RC/ER (AF095270)	89.3	90.5	91.6	90.7	90.7	90.7	90.8	91.2	90.2	95.9	100.0	96.5	96.0	89.5	90.4
12	*Methanoculleus thermophilus* FR-4 (AJ862839)	90.1	91.1	92.1	91.4	91.4	91.4	90.6	91.0	90.0	96.6	96.5	100.0	97.3	89.5	92.4
13	*Methanoculleus submarinus* Nankai-1 (AF531178)	89.9	91.6	92.5	92.0	92.0	92.0	91.2	91.6	90.6	97.5	96.0	97.3	100.0	90.1	92.3
14	*Methanomicrobium mobile* strain 1 (M59142)	86.2	88.9	89.4	88.5	88.5	88.5	88.4	88.6	88.0	90.0	89.5	89.5	90.1	100.0	92.2
15	*Methanogenium cariaci* JR1 (M59130)	87.2	90.9	91.6	90.5	90.5	90.5	90.1	90.6	89.5	91.5	90.4	92.4	92.3	92.2	100.0

Table 24: Similarity Matrix of 16S rDNA Sequences Including Isolate 74 (% Identity)

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Methanobacterium palustre* F (AF093061)	100	95.7	95	90.8	89.3	94.9	95.5	95.3	95.6	95	97.8	98.1	90
2	*Methanobacterium congolense* C (AF233586)	95.7	100	95.1	90.8	89.1	94.8	94.8	94.6	95.3	95.2	94.8	95.1	89.6
3	**Isolate 74**	95	95.1	100	90.9	90.1	96.4	98.5	98.4	95.2	95.6	94.4	94.8	89.4
4	*Methanobrevibacter ruminantium* NT7 (AJ009959)	90.8	90.8	90.9	100	93.5	90.3	91.2	91.2	90.8	90.8	90.5	90.1	89.3
5	*Methanobrevibacter smithii* SM9 (AJ009958)	89.3	89.1	90.1	93.5	100	89.5	89.7	89.8	89.4	90.1	89.1	88.8	87.9
6	*Methanobacterium oryzae* FPi (AF028690)	94.9	94.8	96.4	90.3	89.5	100	96.6	96.5	95.5	95.8	94.5	95.2	89.4
7	*Methanobacterium bryantii* RiH2 (AF028688)	95.5	94.8	98.5	91.2	89.7	96.6	100	99.3	95.1	94.6	95.2	95.5	89.5
8	*Methanobacterium bryantii* MOH (M59124)	95.3	94.6	98.4	91.2	89.8	96.5	99.3	100	94.8	94.5	94.9	95.3	89.5
9	*Methanobacterium aarhusense* H2-LR (AY386124)	95.6	95.3	95.2	90.8	89.4	95.5	95.1	94.8	100	96.1	94.5	95.2	89.8
10	*Methanobacterium beijigense* 8-2 (AY350742)	95	95.2	95.6	90.8	90.1	95.8	94.6	94.5	96.1	100	93.8	94.7	88.7
11	*Methanobacterium formicicum* MF (AF169245)	97.8	94.8	94.4	90.5	89.1	94.5	95.2	94.9	94.5	93.8	100	97.9	89.8
12	*Methanobacterium subterraneum* A8 (X99044)	98.1	95.1	94.8	90.1	88.8	95.2	95.5	95.3	95.2	94.7	97.9	100	89.6
13	*Methanosphaera stadtmanae* MCB-3 (M59139)	90	89.6	89.4	89.3	87.9	89.4	89.5	89.5	89.8	88.7	89.8	89.6	100

Table 25: Similarity Matrix of 16S rDNA Sequences Including Isolates 10, 20 and 22 (% Identity)

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Methanoculleus bourgensis* MS2 (AF095269)	100.0	99.9	93.2	93.0	96.8	92.5	97.3	98.3	92.9	93.3	98.1	98.1	97.8
2	*Methanoculleus olentangyi* RC/ER (AF095270)	99.9	100.0	93.0	92.8	96.7	92.4	97.2	98.2	92.7	93.1	98.1	98.1	97.7
3	*Methanofollis liminatans* GKZPZ (AF095271)	93.2	93.0	100.0	99.8	92.1	92.8	91.7	93.2	96.9	96.8	93.1	93.1	92.7
4	*Methanofollis tationis* Chile 9 (AF095272)	93.0	92.8	99.8	100.0	91.7	92.7	91.5	92.9	96.9	96.6	92.7	92.7	92.4
5	*Methanoculleus thermophilicus* TCI (M59129)	96.8	96.7	92.1	91.7	100.0	92.2	97.1	97.2	91.3	92.1	96.9	96.9	96.6
6	*Methanogenium organophilum* CV (M59131)	92.5	92.4	92.8	92.7	92.2	100.0	92.5	93.3	92.9	92.6	93.4	93.4	93.1
7	*Methanoculleus marisnigri* JR1 (M59134)	97.3	97.2	91.7	91.5	97.1	92.5	100.0	98.0	91.8	91.9	97.9	97.9	97.5
8	*Methanoculleus palmolei* INSULZ (Y16382)	98.3	98.2	93.2	92.9	97.2	93.3	98.0	100.0	92.9	93.2	99.5	99.5	99.2
9	*Methanofollis formosanus* ML15 (AY186542)	92.9	92.7	96.9	96.9	91.3	92.9	91.8	92.9	100.0	96.4	92.8	92.8	92.4
10	Isolate 49	93.3	93.1	96.8	96.6	92.1	92.6	91.9	93.2	96.4	100.0	92.9	92.9	92.5
11	**Isolate 20**	98.1	98.1	93.1	92.7	96.9	93.4	97.9	99.5	92.8	92.9	100.0	100.0	99.7
12	**Isolate 10**	98.1	98.1	93.1	92.7	96.9	93.4	97.9	99.5	92.8	92.9	100.0	100.0	99.7
13	**Isolate 22**	97.8	97.7	92.7	92.4	96.6	93.1	97.5	99.2	92.4	92.5	99.7	99.7	100.0

Table 26: Similarity Matrix of 16S rDNA Sequences Including Isolates 25 and 28 (% Identity)

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Methanobacterium bryantii* MOH (M59124)	100.0	97.7	96.8	96.1	96.0	91.1	92.4	90.8	96.6	98.9	95.8	98.5	98.5
2	*Methanobacterium oryzae* FPi (AF028690)	97.7	100.0	96.0	95.3	96.0	91.2	91.8	90.2	95.9	97.9	96.3	97.4	97.4
3	*Methanobacterium palustre* F (AF093061)	96.8	96.0	100.0	98.8	96.8	91.4	92.7	91.5	98.6	96.3	96.5	95.9	95.9
4	*Methanobacterium formicicum* MF (AF169245)	96.1	95.3	98.8	100.0	96.2	90.7	92.0	90.8	98.2	95.4	95.4	95.1	95.1
5	*Methanobacterium congolense* C (AF233586)	96.0	96.0	96.8	96.2	100.0	91.0	93.0	91.1	96.4	96.1	96.3	95.6	95.6
6	*Methanobrevibacter smithii* SM9 (AJ009958)	91.1	91.2	91.4	90.7	91.0	100.0	93.5	87.7	91.0	91.6	91.3	91.3	91.3
7	*Methanobrevibacter ruminantium* NT7 (AJ009959)	92.4	91.8	92.7	92.0	93.0	93.5	100.0	90.5	92.1	92.6	92.4	92.4	92.4
8	*Methanosphaera stadtmanae* MCB-3 (M59139)	90.8	90.2	91.5	90.8	91.1	87.7	90.5	100.0	90.9	90.6	90.6	90.3	90.3
9	*Methanobacterium subterraneum* A8 (X99044)	96.6	95.9	98.6	98.2	96.4	91.0	92.1	90.9	100.0	96.0	96.5	95.5	95.5
10	*Methanobacterium bryantii* RiH2 (AF028688)	98.9	97.9	96.3	95.4	96.1	91.6	92.6	90.6	96.0	100.0	96.4	99.6	99.6
11	*Methanobacterium beijingense* 8-2 (AY350742)	95.8	96.3	96.5	95.4	96.3	91.3	92.4	90.6	96.5	96.4	100.0	96.0	96.0
12	**Isolate 25**	98.5	97.4	95.9	95.1	95.6	91.3	92.4	90.3	95.5	99.6	96.0	100.0	100.0
13	**Isolate 28**	98.5	97.4	95.9	95.1	95.6	91.3	92.4	90.3	95.5	99.6	96.0	100.0	100.0

Table 27: Similarity Matrix of 16S rDNA Sequences Including Isolate 48 (% Identity)

		1	2	3	4	5	6	7	8	9	10	11
1	*Methanosarcina barkeri* Sar (AF028692)	100.0	96.4	99.1	98.3	87.9	92.2	92.6	98.6	92.6	98.7	99.2
2	*Methanosarcina lacustera* ZS (AF432127)	96.4	100.0	96.4	96.8	87.6	92.3	92.1	96.7	92.2	96.7	96.6
3	*Methanosarcina barkeri* CM1 (AJ002476)	99.1	96.4	100.0	98.2	88.5	92.5	93.0	98.5	93.0	98.6	99.1
4	*Methanosarcina mazei* C16 (AJ012095)	98.3	96.8	98.2	100.0	88.9	92.7	93.3	98.7	92.7	98.8	98.1
5	*Methanimicrococcus blatticola* PA (AJ238002)	87.9	87.6	88.5	88.9	100.0	87.7	87.6	88.0	87.5	88.8	88.4
6	*Methanococcoides methylutens* TMA-10 (M59127)	92.2	92.3	92.5	92.7	87.7	100.0	95.1	92.3	94.8	92.7	92.3
7	*Methanohalophilus mahii* SLP (M59133)	92.6	92.1	93.0	93.3	87.6	95.1	100.0	92.9	95.3	92.7	93.0
8	*Methanosarcina acetivorans* C2A (M59137)	98.6	96.7	98.5	98.7	88.0	92.3	92.9	100.0	92.9	99.1	98.8
9	*Methanolobus vulcani* PL-12/M (U20155)	92.6	92.2	93.0	92.7	87.5	94.8	95.3	92.9	100.0	93.1	92.8
10	*Methanosarcina siciliae* C2J (U89773)	98.7	96.7	98.6	98.8	88.8	92.7	92.7	99.1	93.1	100.0	98.5
11	**Isolate 48**	99.2	96.6	99.1	98.1	88.4	92.3	93.0	98.8	92.8	98.5	100.0

Table 28: Similarity Matrix of 16S rDNA Sequences Including Isolate 110 (% Identity)

		1	2	3	4	5	6	7	8	9	10	11	12
1	*Methanobacterium formicicum* MF (AF169245)	100.0	96.1	91.1	92.4	93.8	90.9	91.1	90.6	89.7	90.0	89.4	90.7
2	*Methanobacterium bryantii* RiH2 (AF028688)	96.1	100.0	90.7	92.0	92.0	90.9	90.4	89.8	89.4	89.4	89.7	90.2
3	*Methanobrevibacter smithii* SM9 (AJ009958)	91.1	90.7	100.0	93.5	93.4	98.2	98.5	96.2	97.8	97.9	91.0	98.6
4	*Methanobrevibacter ruminantium* NT7 (AJ009959)	92.4	92.0	93.5	100.0	93.8	94.1	93.6	94.1	92.7	92.7	93.0	93.2
5	*Methanobrevibacter cuticularis* RFM-1 (U41095)	93.8	92.0	93.4	93.8	100.0	93.6	92.9	93.0	92.1	92.4	91.1	92.9
6	*Methanobrevibacter smithii* PS (U55233)	90.9	90.9	98.2	94.1	93.6	100.0	97.4	96.8	97.0	97.0	92.4	97.8
7	*Methanobrevibacter thaueri* CW (U55236)	91.1	90.4	98.5	93.6	92.9	97.4	100.0	95.9	97.7	97.9	91.4	99.1
8	*Methanobrevibacter woesei* GS (U55237)	90.6	89.8	96.2	94.1	93.0	96.8	95.9	100.0	95.0	94.8	91.4	95.8
9	*Methanobrevibacter gottschalkii* HO (U55238)	89.7	89.4	97.8	92.7	92.1	97.0	97.7	95.0	100.0	99.3	90.9	98.1
10	*Methanobrevibacter gottschalkii* PG (U55239)	90.0	89.4	97.9	92.7	92.4	97.0	97.9	94.8	99.3	100.0	90.9	98.4
11	*Methanobrevibacter wolinii* SH (U55240)	89.4	89.7	91.0	93.0	91.1	92.4	91.4	91.4	90.9	90.9	100.0	91.1
12	**Isolate 110**	90.7	90.2	98.6	93.2	92.9	97.8	99.1	95.8	98.1	98.4	91.1	100.0

8 References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W. and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research. 25:3389-3402.

Annison, E.F. and Lewis, D. (1959). Introduction. In: *Metabolism in the Rumen*. Methuen and Co., London. pp 14-22.

Balch, W.E., Fox, G.E., Magrum, L.J., Woese, C.R. and Wolfe, R.S. (1979). Methanogens: re-evaluation of a unique biological group. Microbiological Reviews. 43:260-296.

Balch, W.E. and Wolfe, R.S. (1981). Validation list No. 6. International Journal of Systematic and Evolutionary Microbiology. 31:215-218.

Barker, H.A. (1940). Studies upon the methane fermentation. IV. The isolation and culture of *Methanobacterium omelianskii*. Antonie Van Leeuwenhoek. 6:201–220.

Barns, S.M., Delwiche, C.F., Palmer, J.D. and Pace, N.R. (1996). Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proceedings of the National Academy of Sciences, USA. 93:9188-9193.

Beijer, W.H. (1952). Methane fermentation in the rumen of cattle. Nature. 170:576-577.

Benstead, J., Archer, D.B. and Lloyd, D. (1991). Formate utilization by members of the genus *Methanobacterium*. Archives of Microbiology. 156:34-37.

Biavati, B., Vasta, M. and Ferry, J.G. (1988). Isolation and characterization of *Methanosphaera cuniculi* sp. nov.. Applied and Environmental Microbiology. 54:768-771.

Boone, D.R., Whitman, W.B. and Rouviere, P. (1993). Diversity and taxonomy of methanogens. In: *Methanogens*. Ferry, J.G. (ed), Chapman and Hall Inc, New York. pp 35-80.

Brown, J.W., Daniels, C.J. and Reeve, J.N. (1989). Gene structure, organization, and expression in archaebacteria. Critical Reviews in Microbiology. 16:287-338.

Brown, L. and Petrie, S. (2003). Trends in greenhouse gas emissions. In: *New Zealand's Greenhouse Gas Inventory 1990-2003*. Ministry for the Environment, Wellington. pp 11-14.

Bryant, M.P., Small, N., Bouma, C. and Robinson, I. (1958). Studies on the composition of the ruminal flora and flora of young calves. Journal of Dairy Science. 41:1747-1767.

Bryant, M.P. and Small, N. (1960). Observations on the ruminal microorganisms of isolated and inoculated calves. Journal of Dairy Science. 36:205-217.

Bryant, M.P., Wolin, M.J., Wolin, E.A. and Wolfe, R.S. (1967). *Methanobacillus omelianskii*, a symbiotic relationship between two species of bacteria. Archives of Microbiology. 59:20-31.

Chaucheyras-Durand, F. and Fonty, G. (2001). Establishment of cellulolytic bacteria and development of fermentative activities in the rumen of gnotobiotically-reared lambs receiving the microbial additive *Saccharomyces cerevisiae* CNCM I-1077. Reproduction Nutrition Development. 41:57-68.

Chesson, A. and Forsberg, C.W. (1997). Microbe-microbe interactions. In: *The Rumen Microbial Ecosystem, 2nd edn*. Hobson, P.N. and Stewart, C.S. (eds). Chapman and Hall, London, United Kingdom. pp 329-381.

Cirne, D.G., Delgado, O., Marichamy, S. and Mattiasson, B. (2006). *Clostridium ludense* sp. nov., a novel lipolytic bacterium isolated from the bovine rumen. International Journal of Systematic and Evolutionary Microbiology. 65:625-628.

Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M. and Tiedje, J.M. (2003). The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Research. 31:442-443.

Cord-Ruwisch, R., Seitz, H. and Conrad, R. (1988). The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Archives of Microbiology. 149:350-357.

Daniel, S.L., Hsu, T., Dean, S.I. and Drake, H.L. (1990). Characterization of the H_2- and CO_2-dependant chemolithotrophic potentials of the acetogens *Clostridium thermoacticum* and *Acetogenium kivui*. Journal of Bacteriology. 172:4464-4471.

Deppenmeier, U., Lienard, T. and Gottschalk, G. (1999). Novel reactions involved in energy conservation by methanogenic archaea. FEBS Letters. 457:291-297.

Dijkstra, J. and Tamminga, S. (1995). Simulation of the effects of on the contribution of rumen protozoa to degradation of fibre in the rumen. British Journal of Nutrition. 74:617-634.

Eirich, L.D., Vogels, G.D. and Wolfe, R.S. (1978). Proposed structure for coenzyme-F-420 from *Methanobacterium* sp.. Biochemistry. 17:4583-4593.

Ellis, J.E., Hillman, K., Williams, A.G. and Lloyd, D. (1990). Hydrogen production by rumen ciliate protozoa. In: Microbiology and biochemistry of strict anaerobes involved in interspecies hydrogen transfer. Belaich, J.P., Bruschi, M., Garcia, J.L. (eds), Plenum Press, New York. pp 377-379.

Faichney, G.J., Graham, N.M. and Walker, D.M. (1999). Rumen characteristics, methane emissions, and digestion in weaned lambs reared in isolation. Australian Journal of Agricultural Research. 50:1083-1089.

Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package). 3.5c edition. Department of Genetics, University of Washington, Seattle.

Finlay, B.J., Esteban, G., Clarke, K.J., Williams, A.G., Embley, T.M. and Hirt, R.P. (1994). Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiology Letters. 117:157-162.

Fonty, G., Gouet, P., Jouany, J-P., Rieu-Lesme, F., Senaud, J., Citron, A. and Breton, A. (1986). Microbial colonisation of the rumen in young lambs. In: *Biology of anaerobic bacteria*. Dubourguier, H.C., Albagnac, G., Montreuil, M., Romond, C., Sautière, P. and Guillaume, J. (eds). Elsevier Science Publishers, Amsterdam. pp 40-46.

Fonty, G., Gouet, P., Jouany, J-P. and Senaud, J. (1987). Establishment of the microflora and anaerobic fungi in the rumen of lambs. Journal of General Microbiology. 133:1835-1843.

Fonty, G., Jouany, J-P., Chavarot, M., Bonnemoy, F. and Gouet, P. (1991). Development of the rumen digestive functions in lambs placed in a sterile isolator a few days after birth. Reproduction Nutrition Development. 31:521-528.

Fonty, G., Williams, A.G., Bonnemoy, F., Morvan, B., Withers, S.E. and Gouet, P. (1997). Effect of *Methanobrevibacter* sp. MF1 inoculation on glycoside hydrolase and polysaccharide depolymerase activities, wheat straw degradation and volatile fatty acid concentrations in the rumen of gnotobiotically-reared lambs. Anaerobe. 3:383-389.

Garcia, J-L., Patel, B.K.C. and Ollivier, B. (2000). Taxonomic, phylogenetic, and ecological diversity of methanogenic archaea. Anaerobe. 6:205-226.

Gibson, G.R. (1990). Physiology and ecology of the sulfate-reducing bacteria. Journal of Applied Bacteriology. 69:769-797.

Grant, W.D., Pinch, G., Harris, J.E., De Rosa, D. and Gambacorta, A. (1985). Polar lipids in methanogen taxonomy. Journal of General Microbiology. 131:3277-3286.

Grosskopf, R., Janssen, P.H. and Liesack, W. (1998). Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Applied and Environmental Microbiology. 64:960-969.

Hillman, K., Lloyd, D. and Williams, A. G. (1998). Interactions between the methanogen *Methanosarcina barkeri* and rumen holotrich ciliate protozoa. Letters in Applied Microbiology. 7:49-53.

Hobson, P.N. (1997). Introduction. In: *The Rumen Microbial Ecosystem, 2nd edn.*. Hobson, P.N. and Stewart, C.S. (eds). Chapman and Hall, London, United Kingdom. pp 1-9.

Hobson, P.N., Mann, S.O. and Oxford, A.E. (1958). Some studies on the occurrence and properties of a large Gram-negative coccus from the rumen. Journal of General Microbiology. 19:462-465.

Holdeman, L.V. and Moore, W.E.C. (1972). Roll tube techniques for anaerobic bacteria. The American Journal of Clinical Nutrition. 25:1314-1317.

Huber, H., Hohn, M.J., Rachel, R., Fuchs, T., Wimmer, V.C. and Stetter, K.O. (2002). A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. Nature. 417:63-67.

Hungate, R.E. (1950). The anaerobic and mesophilic cellulolytic bacteria. Journal of Bacteriological Reviews. 14:1.

Hungate, R.E. (1966). A roll tube method for cultivation of strict anaerobes. Journal of Bacteriology. 91:908-909.

Hungate, R.E., Smith, W., Bauchop, T., Yu, I. and Rabinowitz, J.C. (1970). Formate as an intermediate in the bovine rumen fermentation. Journal of Bacteriology. 102:389-397.

Jarvis, G.N., Strompl, C., Burgess, D.M., Skillman, L.C., Moore, E.R. and Joblin, K.N. (2000). Isolation and identification of ruminal methanogens from grazing cattle. Current Microbiology. 40:327-332.

Jayne-Williams, D.J. (1979). The bacterial flora of the rumen of healthy and bloating calves. Journal of Applied Bacteriology. 47:271-284.

Joblin, K.N., Naylor, G.E. and Williams, A.G. (1990). Effect of *Methanobrevibacter smithii* on xylanolytic activity of anaerobic ruminal fungi. Applied and Environmental Microbiology. 56:2287-2295.

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

Joblin, K.N. (2006). Climate change: New Zealand's agriculture green-house-gases and abatement strategies. In: *Global Perspective on Greenhouse Gas Emission from the Agricultural Sector, Japan. August 3 – 4*. Takahashi, J. and Kishimoto, T. (eds). Obibrio press, Japan.

Kandler, O. and Hippe, H. (1977). Lack of peptidoglycan in the cell walls of *Methanosarcina barkeri*. Archives of Microbiology. 113:57-60.

Karr, E.A., Ng, J.M., Belchik, S.M., Sattley, W.M., Madigan, M.T. and Achenbach, L.A. (2006). Biodiversity of methanogenic and other archaea in the permanently frozen lake Fryxell, Antarctica. Applied and Environmental Microbiology. 72: 1663–1666.

Kimura, M. (1980). A simple method of estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution. 16:111-120.

Lai, M-C. and Chen, S-C. (2001). *Methanofollis aquaemaris* sp. nov., a methanogen isolated from an aquaculture fish pond. International Journal of Systemic and Evolutionary Microbiology. 51:1873–1880.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In: *Nucleic acid techniques in bacterial systematics*. Stackebrandt, E. and Goodfellow, M. (eds). John Wiley and Sons Ltd., West Sussex, England.

Leadbetter, J.R. and Breznak, J.A. (1996). Physiological ecology of *Methanobrevibacter cuticularis* sp. nov. and *Methanobrevibacter curvatus* sp. nov., isolated from the hindgut of the termite *Reticulitermes flavipes*. Applied and Environmental Microbiology. 62:3620-3631.

Leadbetter, J.R., Crosby, L.D. and Breznak, J.A. (1998). *Methanobrevibacter filiformis* sp. nov., a filamentous methanogen from termite hindguts. Archives of Microbiology. 169:287-292.

Leedle, J.A.Z. and Greening, R.C. (1988). Postprandial changes in the methanogenic and acidogenic bacteria in steers fed high- or low-forage diets once daily. Applied and Environmental Microbiology. 54:502-506.

Lin, C.Z. and Miller, T.L. (1998). Phylogenetic analysis of *Methanobrevibacter* isolated from faeces of humans and other animals. Archives of Microbiology. 169:397-403.

Lovley, D.K., Greening, R.C. and Ferry, J.G. (1984). Rapidly growing rumen methanogenic organism that synthesizes co-enzyme M and has a high affinity for formate. Applied and Environmental Microbiology. 48:81-87.

Ludwig, W. and Klenk, H-.P. (2001). Overview: a phylogenetic backbone and taxonomic framework for prokaryotic systematics. In: *Bergey's manual of systematic bacteriology, 2nd edn.*. Vol. 1. Boone, D.R. and Castenholz, R.W. (eds). Springer-Verlag, New York. pp 49-65.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A. and Schleifer, K.H. (2004). ARB: a software environment for sequence data. Nucleic Acids Research. 32:1363-1371.

Lysons, R.J., Alexander, T.J.L., Wellstead, P.D. and Jennings, I.W. (1976). Observations on alimentary-tract of gnotobiotic lambs. Research in Veterinary Science. 20:70-76.

Maugh, T.H. (1977). Phylogeny: Are methanogens a third class of life? Science. 198:812.

Merck Index (1999) 12th edition. Merck, Whitehouse Station, NJ.

Miller, T.L., Wolin, M.J., Hongxue, Z. and Bryant, M.P. (1986). Characteristics of methanogens isolated from bovine rumen. Applied and Environmental Microbiology. 51:201-202.

Miller, T.L. and Wolin, M.J. (2001). Inhibition of growth of methane-producing bacteria of the ruminant forestomach by hydroxymethylglutaryl~SCoA reductase inhibitors. Journal of Dairy Science. 84:1445-1448.

Miller, T.L. (2001). *Methanobrevibacter* Genus. In: *Bergey's manual of systematic bacteriology, 2nd edn.*. Vol. 1. Boone, D.R. and Castenholz, R.W. (eds). Springer-Verlag, New York. pp 218-229.

Minato, H., Otsuka, M., Shirasaka, S., Itabashi, H. and Mitsumori, M. (1992). Colonization of microorganisms in the rumen of young cattle. Journal of General Applied Microbiology. 38:447-456.

Ministry for the Environment (2006). Trends in greenhouse gas emissions In: *New Zealand's greenhouse gas inventory 1990-2004*. Key Contributors: Petrie, S., Wear, S., Nottage, R., Gulliver, S., Wilson, T., Tsui, K. and Evans, D.. Ministry for the Environment, Wellington, New Zealand. pp 20-23.

Morvan, B., Dore, J., Rieu-Lesme, F., Foucat, L., Fonty, G. and Gouet, P. (1994). Establishment of hydrogen-utilizing bacteria in the rumen of the newborn lamb. FEMS Microbiology Letters. 117:249-256.

Morvan, B., Bonnemoy, F., Fonty, G. and Gouet, P. (1996). Quantitative determination of H_2-utilizing acetogenic and sulfate-reducing bacteria and methanogenic archaea from digestive tract of different mammals. Current Microbiology. 32:129-133.

Muyzer, G. (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. Current Opinion in Microbiology. 2:317-322.

Naylor, G.E. (1998). The isolation and characterisation of ruminal mycoplasmas and their interactions with cellulolytic bacteria. Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand. Masters Thesis. pp 101.

Nicholson, M.J., Evans, P.N. and Joblin, K.N. (2007). Analysis of methanogen diversity in the rumen using temporal temperature gradient gel electrophoresis: identification of a new lineage of methanogens. Microbial Ecology. In Press.

Oppermann, R.A., Nelson, W.O. and Brown, R.E. (1957). In vitro studies on methanogenic rumen bacteria. Journal of Dairy Science. 40:779-788.

Patterson, J.A. and Hespell, R.B. (1979). Tri-methylamine and methylamine as growth substrates for rumen bacteria and *Methanosarcina barkeri*. Current Microbiology. 3:79-83.

Paynter, M.J.B. and Hungate, R.E. (1968). Characterization of *Methanobacterium mobilis*, sp. nov., isolated from the bovine rumen. Journal of Bacteriology. 95:1943-1951.

Pounden, W.D. and Hibbs, J.W. (1948). The influence of the ration and rumen inoculation on the establishment of certain microorganisms in the rumens of young calves. Journal of Dairy Science. 31:1041-1050.

Ramaswamy, V., Boucher, O., Haigh, J., Hauglustaine, D., Haywood, J., Myhre, G., Nakajima, T., Shi, G.Y., Solomon, S., Betts, R., Charlson, R., Chuang, C., Daniel, J.S., Del Genio, A., van Dorland, R., Feichter, J., Fuglestvedt, J., de F. Forster, P.M., Ghan, S.J., Jones, A., Kiehl, J.T., Koch, D., Land, C., Lean, J., Lohmann, U., Minschwaner, K., Penner, J.E., Roberts, D.L., Rodhe, H., Roelofs, G.J., Rotstayn, L.D., Schneider, T.L., Schumann, U., Schwartz, S.E., Shine, K.P., Smith, S., Stevenson, D.S., Stordal, F., Tegen, I. and Zhang, Y. (2001). Radiative forcing of climate change. In: *Climate Change 2001: The Scientific Basis: Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change*. Houghton, J.T., Ding, Y., Griggs, D., Noguer, M., van der Linden, P.J., Dai, X., Maskell, K., Johnson, C.A. (eds). Cambridge University press, United Knigdom. pp 351-416.

Rieu-Lesme, F., Morvan, B., Collins., M.D., Fonty, G. and Willems, A. (1996). A new H_2/CO_2-using acetogenic bacterium from the rumen: a description of *Ruminococcus schinkii* sp. nov. FEMS microbiology Letters. 140:281-286.

Russell, J.B., Strobel, H.B. and Chen, G.J. (1988). Enrichment and isolation of a ruminal bacterium with a very high specific activity of ammonia production. Applied and Environmental Microbiology. 54:872–877.

Skillman, L.C., Jarvis, G.N., Strompl, C.N., Moore, E.R.B. and Joblin, K.N. (2001). Isolation, purification and identification of a new ruminal methanogen. Proceedings of the workshop on the science of atmospheric trace gases 2001. Compiler: Clarkson, T.S. NIWA Technical Report 103.

Skillman, L.C., Evans, P.N., Naylor, G.E., Morvan, B., Jarvis, G.N. and Joblin, K.N. (2004). 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. Anaerobe. 10:277-285.

Skillman, L.C., Evans, P.N., Strompl, C. and Joblin, K.N. (2006). 16S rDNA directed PCR primers and detection of methanogens in the bovine rumen. Letters in Applied Microbiology. 42:222-228.

Smith, P.H. and Hungate, R.E. (1958). Isolation and characterization of *Methanobacterium ruminantium* sp. nov. Journal of Bacteriology. 75:713-718.

Stahl, D.A., Flesher, B., Mansfield, H.R. and Montgomery, L. (1988). Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Applied and Environmental Microbiology. 54:1079-1084.

Statistics New Zealand (2004a). Agriculture. In: *New Zealand Official Yearbook 2004, 104th edn*. David Bateman Ltd., Wellington, New Zealand. pp 325-342.

Statistics New Zealand (2004b). Overseas trade. In: *New Zealand Official Yearbook 2004, 104th edn*. David Bateman Ltd., Wellington, New Zealand.

Stewart, C.S. and Bryant M.P. (1988). The rumen bacteria. In: *The rumen microbial ecosystem*. Hobson, P.N. (ed). Chapman and Hall, London, United Kingdom. pp 10-72.

Stewart, J.A., Chadwick, V.S., and Murray, A. (2006). Carriage, quantification, and predominance of methanogens and sulfate-reducing bacteria in faecal samples. Letters in Applied Microbiology. 43:58-63.

Tajima, K., Nagamine, T., Matsui, H., Nakamura, M. and Aminov, R.I. (2001). Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. FEMS Microbiology Letters. 200:67-72.

Ushida, K., Newbold, C.J. and Jouany, J.P. (1997). Interspeceis hydrogen transfer between the rumen ciliate *Polyplastron multiversiculatum* and *Methanosarcina barkeri*. Journal of General and Applied Microbiology. 43:129-131.

Verhulst, A., Semjen, G., Janssen, G., Parmentier, G., Asselberghs, S., Van Hespen, H., Eyssen, H. (1985). Biohydrogenation of linoleic acid by *Clostridium sporogenes*, *Clostridium bifermentans*, *Clostridium sordellii* and *Bacteroides* sp. FEMS Microbiology Ecology. 31:255-259.

Wallace, R.J., Onodera, R. and Cotta, M.A. (1997). Metabolism of nitrogen-containing compounds. In: *The rumen microbial ecosystem*. Hobson, P.N. and Stewart, C.S. (eds). Chapman and Hall, London, United Kingdom. pp 283-328.

Watanabe, K., Kodama, Y., Hamamura, N. and Kaku, N. (2002). Diversity, abundance, and activity of archaeal populations in oil-contaminated groundwater accumulated at the bottom of an underground crude oil storage cavity. Applied and Environmental Microbiology. 68:3899-3907.

Whitelaw, F.G., Eadie, J.M., Bruce, L.A. and Shand, W.J. (1984). Methane formation in faunated and ciliate-free cattle and its relationship with rumen volatile fatty acid proportions. British Journal of Nutrition. 52:261-275.

Whitford, M.F., Teather, R.M. and Forster, R.J. (2001). Phylogenetic analysis of methanogens from the bovine rumen. BMC Microbiology. 1:5.

von Wintzingerode, F.V., Gobel, U.B. and Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews. 21:213-229.

Wilson, I.G. (1997). Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology. 63:3741-3751.

Woese, C.R. (1977). A comment on methanogenic bacteria and the primitive ecology. Journal of Molecular Evolution. 9:369-371.

Woese, C.R., Kandler, O. and Whellis, J.L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eucarya. Proceedings of the National Academy of Sciences, USA. 87:4576-4579.

Wolfe, R.S. (1993). A historical overview of methanogenesis. In: *Methanogenesis*. Ferry, J.G. (ed). Chapman and Hall, London. pp 1-32.

Wolin, M.J. and Weinberg, G. (1960). A theorectical rumen fermentation balance. Journal of Dairy Science. 43:825-830.

Wolin, M.J. and Miller, T.L. (1997). Microbe-microbe interactions. In: *The rumen microbial ecosystem*. Hobson, P.N. and Stewart, C.S. (eds). Chapman and Hall, London, United Kingdom. pp 467-491.

Wright, A-D.G., Toovey, A.F. and Pimm, C.L. (2006). Molecular identification of methanogenic archaea from sheep in Queensland, Australia reveal more uncultured novel archaea. Anaerobe. 12:134-139.

Wu, S.Y., Chen, S-C. and Lai, M-C. (2005). *Methanofollis formosanus* sp. nov., isolated from a fish pond. International Journal of Systematic and Evolutionary Microbiology. 55:837-842.

Yanagita, K., Kamagata, Y., Kawaharasaki, M., Suzuki, T., Nakamura, Y. and Minato, H. (2000). Phylogenetic analysis of methanogens in sheep rumen ecosystem and detection of *Methanomicrobium mobile* by fluorescence *in situ* hybridization. Bioscience, Biotechnology and Biochemistry. 64:1737-1742.

Zabel, H.P., König, H. and Winter, J. (1984). Isolation and characterization of a new coccoid methanogen, *Methanogenium tationis* sp. nov. from a solfataric field on Mount Tatio. Archives of Microbiology. 137:308–315.

Zellner, G., Sleytr, U.B., Messner, P., Kneifel, H. and Winter, J. (1990). *Methanogenium liminatans* spec-nov, a new coccoid, mesophilic methanogen able to oxidize secondary alcohols. Archives of Microbiology. 153:287-293.

Zellner, G., Messner, P., Winter, J. and Stackebrandt, E. (1998). *Methanoculleus palmolei* sp. nov., an irregularly coccoid methanogen from an anaerobic digester treating wastewater of a palm oil plant in North-Sumatra, Indonesia. International Journal of Systematic Bacteriology. 48:1111-1117.

Zellner, G., Boone, D.R., Keswani, J., Whitman, W.B., Woese, C.R., Halgelstein, A., Tindall, B.J. and Stackebrandt, E. (1999). Reclassification of *Methanogenium tationis* and *Methanogenium liminatans* as *Methanofollis tationis* gen. nov., comb. nov and *Methanofollis liminatans* comb. nov. and description of a new strain of *Methanofollis liminatans*. International Journal of Systematic Bacteriology. 49:247-255.

Zellner, G. and Boone, D.R. (2001). *Methanobacterium* genus. In: *Bergey's manual of systematic bacteriology, 2nd edn.*. Vol. 1. Boone, D.R. and Castenholz, R.W. (eds). Springer-Verlag, New York. pp 215-218.

Ziolecki, A. and Briggs, C.A.E. (1961). The microflora of the rumen of the young calf: II. Source, nature and development. Journal of Applied Bacteriology. 24:148-163.