Asian-Aust. J. Anim. Sci.
Vol. 20, No. 2 : 283 - 294
February 2007

www.ajas.info

Application of Recent DNA/RNA-based Techniques in Rumen Ecology*

C. S. McSweeney[1, **], S. E. Denman[1], A.-D. G. Wright[1] and Z. Yu[2]

[1] CSIRO Livestock Industries, Queensland Bioscience Precinct, St Lucia, Qld, 4067, Australia

[2] Department of Animal Sciences, The Ohio State University, Columbus, Ohio, USA

ABSTRACT : Conventional culture-based methods of enumerating rumen microorganisms (bacteria, archaea, protozoa, and fungi) are being rapidly replaced by nucleic acid-based techniques which can be used to characterise complex microbial communities without incubation. The foundation of these techniques is 16S/18S rDNA sequence analysis which has provided a phylogenetically based classification scheme for enumeration and identification of microbial community members. While these analyses are very informative for determining the composition of the microbial community and monitoring changes in population size, they can only infer function based on these observations. The next step in functional analysis of the ecosystem is to measure how specific and, or, predominant members of the ecosystem are operating and interacting with other groups. It is also apparent that techniques which optimise the analysis of complex microbial communities rather than the detection of single organisms will need to address the issues of high throughput analysis using many primers/probes in a single sample. Nearly all the molecular ecological techniques are dependant upon the efficient extraction of high quality DNA/RNA representing the diversity of ruminal microbial communities. Recent reviews and technical manuals written on the subject of molecular microbial ecology of animals provide a broad perspective of the variety of techniques available and their potential application in the field of animal science which is beyond the scope of this treatise. This paper will focus on nucleic acid based molecular methods which have recently been developed for studying major functional groups (cellulolytic bacteria, protozoa, fungi and methanogens) of microorganisms that are important in nutritional studies, as well as, novel methods for studying microbial diversity and function from a genomics perspective. **Key Words :** Rumen, Microbial, Ecology, Ribosomal, Molecular, Functional Analysis

INTRODUCTION

Until recently, knowledge of rumen microbiology was primarily obtained using classical culture-based techniques, such as isolation, enumeration and nutritional characterization, which probably only account for 10 to 20% of the rumen microbial population. These traditional methods are time consuming and cumbersome, but have identified more than 200 species of bacteria and at least 100 species of protozoa and fungi inhabiting the rumen (Orpin and Joblin 1997; Stewart et al., 1998; Williams and Coleman 1998; White et al., 1999). New DNA-based technologies can now be employed to examine microbial diversity primarily through the use of small subunit (SSU) rDNA analysis (eg 16S and 18S rDNA) and to understand the function of complex microbial ecosystems in the rumen. Recent reviews and technical manuals written on the subject of molecular microbial ecology of animals provide a broad perspective of the variety of techniques available and their potential application in the field of animal science which is beyond the scope of this paper (see Zoetendal et al., 2003, 2004; Makkar and McSweeney, 2005). This paper will focus on DNA based molecular methods which have recently been developed for studying major functional groups of microorganisms that are important in nutritional studies, as well as, novel methods for studying microbial diversity and function from a genomics perspective.

EXTRACTION OF DNA FROM RUMEN DIGESTA FOR MOLECULAR ECOLOGY STUDIES

Nearly all the molecular ecological techniques analyze genomic community DNA directly extracted from samples collected from the rumen. As such, extraction of community

* This paper was presented at the 4th International Symposium on Recent Advances in Animal Nutrition during the 12th Animal Sciences Congress, Asian-Australasian Association of Animal Production Societies held in Busan, Korea (September 18-22th, 2006).

** Corresponding Author: C. S. McSweeney

[1] CSIRO Livestock Industries, Queensland Bioscience Precinct, St Lucia, Qld, 4067, Australia.

DNA representing the complete diversity of rumen microbial communities is required. Several DNA extraction methods, including commercial kits, have been tried on rumen samples (Forster et al., 1997; Whitford et al., 1998; Kocherginskaya et al., 2001; Krause et al., 2001; Sharma et al., 2003), but recently a repeated bead beating plus column (RBB+C) method was found to be superior to other methods because it improved DNA yields more than 5-fold (Yu and Morrison, 2004a). Conceivably, such improvement in DNA yields should translate into a better representation of community DNA. In future ecological studies of rumen microbial communities, representative DNA extraction by effective methods, such as the RBB+C method, should be employed so that the complete microbial diversity can be subsequently revealed by molecular ecology techniques.

MOLECULAR ECOLOGY OF RUMEN PROTOZOA

Rumen protozoa are implicated in fiber digestion positively (Machmuller et al., 2003) or negatively (Lee et al., 2000), in microbial protein turnover (Jouany 1996; Williams and Coleman, 1998), modulation of bacterial populations (Ronn et al., 2002; Schonhusen et al., 2003; Ozutsumi et al., 2005), and association with methanogens (Finlay et al., 1994; Kišidayová et al., 2000; Schonhusen et al., 2003). Traditionally, the roles that protozoa play in the rumen, beneficial or detrimental, were inferred from comparisons between faunated and defaunated ruminant animals. It has been documented that defaunation results in dramatic changes in the overall bacterial and fungal populations. Microscopy has been the method of choice in identifying and enumerating protozoal populations in rumen samples (Dehority, 2003); however, it has inherent limitations such as, misidentification and low sensitivity. Therefore, molecular techniques have recently been used in ecological studies of ruminal protozoa (Karnati et al., 2003; Regensbogenova et al., 2004a, b; Shin et al., 2004; Sylvester et al., 2004, 2005; Skillman et al., 2006) to better support analysis of ruminal protozoa.

The PCR-cloning-sequencing approach was first published by Karnati et al. (2003) in direct examination of the protozoal diversity in the rumen. They designed protozoa-specific primers, which primed PCR amplification of only protozoal 18S rDNA from community DNA derived from rumen contents. Besides *Entodinium*, which is dominant in the rumen, considerable phylogenetic diversity was found from a limited number of sequenced clones. However, using the same approach, Shin et al. (2004) reported primarily *Entodinium*-like 18S rDNA sequences. Such discrepancy may be attributed to diet, or differences in the primer used and samples analysed. Collectively, these two studies demonstrated that molecular approaches can be adapted to characterize protozoal populations in the rumen and ultimately should facilitate reliable and objective analyses of rumen protozoa independent from morphological examinations.

Alternatively, restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 18S rDNA sequences was shown to identify different rumen protozoa species (Regensbogenova et al., 2004b). However, because the 18S rDNA sequences among different protozoa are very similar, the phylogenetic resolution of such RFLP analysis remains to be determined. Because of the numerical dominance of *Entodinium* species, which can account for as much as 95-99% of rumen protozoa in animals fed high grain diets, genus-specific primers, such as those recently published by Skillman et al. (2006), may be required to effectively examine the complete protozoal diversity in the rumen. Moreover, given the high sequence similarity of 18S rDNA among different rumen protozoal species, other phylogenetic markers of higher sequence divergence, such as 28S rDNA or internal transcribed sequences (ITS), should be exploited for molecular ecological studies of rumen protozoa.

Community profiling by PCR-denaturing gradient gel electrophoresis (DGGE) has been widely used in comparing multiple bacterial communities (Kocherginskaya et al., 2001; Yu and Morrison, 2004b). Recently, Sylvester et al. (2005) and Regensbogenova et al. (2004) independently demonstrated the utility of PCR-DGGE in profiling protozoal communities in the rumen and duodenum by employing different protozoa-specific primers. They were able to show the impact of diets on protozoal diversity, and identified the major protozoal species (*Epidinium caudatum*, *Entodinium caudatum*, and *Isotrica prostoma*) by sequencing rDNA fragments recovered from predominant DGGE bands (Sylvester et al., 2004, 2005). However, both studies detected only a few species, probably due to the dominance of *Entodinium* species and low sequence divergence among different protozoal species. Again, phylogenetic markers with greater discriminating power, such as the 28S rDNA and/or ITS, probably better serve PCR-DGGE analysis of rumen protozoa. Owing to its high throughput capability, this approach will probably find numerous applications in ecological analyses of rumen protozoa to better support future studies on ruminant nutrition.

With respect to ruminant nutrition, it is important to accurately quantify protozoan biomass in the rumen and its passage to the duodenum (Firkins and Yu, 2006). Microscopic enumeration of protozoal populations is traditionally used in the rumen (Dehority, 2003), but it does not allow for measurement of protozoal passage to the duodenum, because all protozoal cells are lysed before reaching the duodenum (Sylvester et al., 2004, 2005).

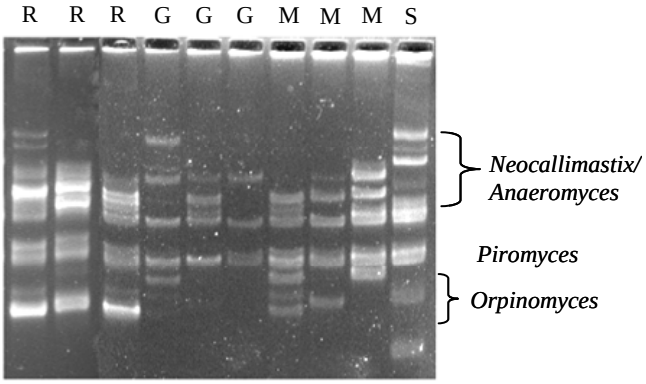

Figure 1. ITS-1 anaerobic fungal-specific amplicons separated on a spreadex EL 600 gel for 4.5 h at 55°C. Samples were collected from three animals for each dietary treatment R, roughage; G, grain; M, roughage and molasses; S, anaerobic fungal marker with genus mobility positions indicated.

Protozoal rRNA has been quantified by probe hybridization in rumen samples in earlier reports based on the difference in fungal and eukaryal 18S rRNA signals (Faichney et al., 1997; Krause et al., 1999b). Such analysis of rRNA should be considered semi-quantitative (Firkins and Yu, 2006). Additionally, RNA levels may vary dramatically while the protozoal populations remain unchanged. Therefore, rDNA was explored as a target and appeared to be a better marker that can be accurately and precisely quantified by real-time PCR assays (Sylvester et al., 2004, 2005; Skillman et al., 2006). Carefully prepared real-time PCR standards permit quantification of protozoan biomass in the rumen and duodenum both in terms of rDNA copy numbers and in N mass (Sylvester et al., 2005). However, it should be noted, that unlike bacteria which generally have a constant copy number of rDNA per cell (though the copy number vary among species), rumen protozoa probably vary their number of rDNA copies per cell over a life cycle, just as other closely related non-rumen protozoa do (Prescott, 1994). Further, cell sizes vary considerably among, and even within, protozoal species (Dehority, 2003). As such, more studies are needed to establish the accurate correlations between rDNA copies and the protozoal biomass.

Recently, Skillman et al. (2006) developed genus-specific primers to enumerate the most abundant protozoa in the rumen, *Entodinium spp.*, and demonstrated a real-time PCR method that was reproducible and correlated (r^2 = 0.8) with the conventional cell-count technique. However, there are advantages and disadvantages of each of these methods. Although microscopic methods are faster and more cost-effective, lysed or ingested protozoa are not counted. It is also likely that the temperature changes (39°C to ambient) associated with sampling can cause lysis of the fragile protozoal cells and may therefore underestimate protozoal counts. By contrast, the rapid freezing of samples for DNA extraction is likely to preserve DNA even from lysed protozoal cells. Although real-time PCR is more expensive than microscopic counts, it is more sensitive, detecting 1-10^6 protozoa.

ANAEROBIC FUNGI CLASSIFICATION

Anaerobic rumen fungi have been isolated from digesta and faeces of numerous herbivores including ruminant and monogastric animals. Currently, there are 18 species described based on morphology of thallus and zoospore ultra-structure. The recent isolation of a new genus, *Cyllamyces* from the faeces of a cow (Ozkose et al., 2001) takes the total validly described genera to six. *Cyllamyces* in its morphology is similar to that of the *Caecomyces* producing a bulbous holdfast rather than the rhizoidal system produced by the four other genera. Both *Neocallimastix* and *Piromyces* genera form monocentric thallus structures, but can be differentiated by examining their zoospore structures with the former producing polyflagellate zoospores and the latter producing uniflagellate. The genera *Orpinomyces* and *Anaeromyces* are seen as polycentric thallus forming fungi that produce polyflagellate and uniflagellate zoospores respectively. This in itself is an oversimplification of the characteristics of the fungi as even the monoflagellated species can be found to produce bi- or tetraflagellated zoospores (Orpin and Joblin, 1997) The ability to accurately distinguish and classify these fungi in an *in vivo* environment is difficult due to their pleomorphic tendencies, and this is compounded for environmental samples where the fungi are intimately entwined with plant material. Visual identification also relies on the presence of both the mature and zoospore stages of the fungi.

DNA-based molecular methods do not depend on the culturability of micro-organisms, and therefore offer an attractive alternative for the study of complex fungal community structures. Ribosomal RNA (rRNA) genes are commonly targeted by such DNA-based molecular methods. Use of 18S rRNA sequences for comparing rumen fungi is of limited use due to the highly conserved nature of 18S rRNA sequences in these organisms (97-100% sequence identity). Alternatively, the internal transcribed spacer region 1 (ITS-1) of the rRNA genes can be used for studying genetic diversity. Initially used by Li and Heath (1992) the ITS-1 region was found to be capable of discriminating between gut fungi to a moderate level of resolution, resulting in the formation of two clusters with *Orpinomyces*, *Neocallimastix* and *Piromyces* in one cluster and *Anaeromyces* and *Caecomyces* in the other. Further studies performed by Brookman et al. (2000) improved the resolution of ITS-1 discrimination when they were able to clearly separate the monocentric gut fungi.

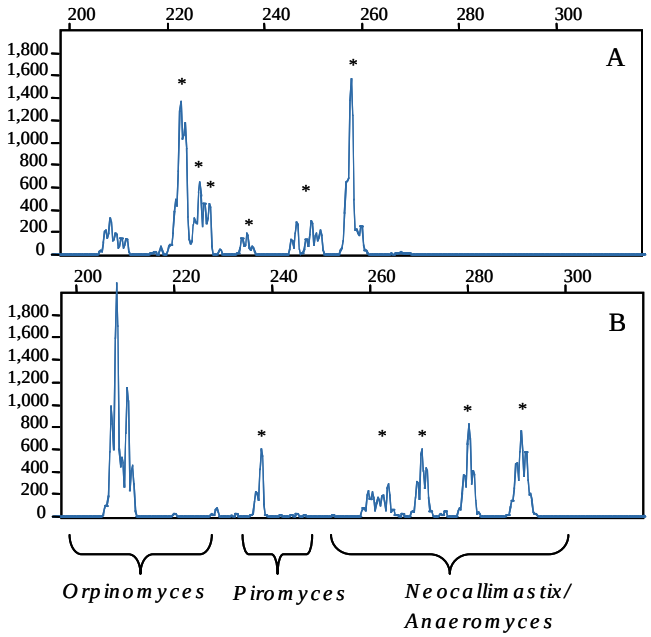

Figure 2. ARISA of rumen samples from animals on different diets Angelton grass (A) and oaten hay (B). Numbers along the X-axis represent base pairs, while the Y-axis indicates strength of fluorescence signal. Peaks marked with an * are seen to differ. The expected positions for specific genus are indicated.

The presence of a length-polymorphic region within the ITS-1 region of gut fungi ranging from approximately 220 bp for *Orpinomyces sp.* and up to ~280 bp for *Neocallimastix sp.* was first exploited by Nicholson et al. (2002). PCR amplification of the length polymorphic region using anaerobic fungal-specific primers, followed by separation of the PCR products on high-resolution gels was shown to be a useful tool for quickly identifying fungal members from cattle faecal samples (Denman et al., 2003). As members of the same genera tended to have a similar length polymorphic region, it was possible to discriminate fungal genera based on gel mobilities in high-resolution gels (Figure 1).

Further development of this method into an automated ribosomal intergenic spacer region analysis (ARISA) assay, through PCR using a carboxyfluorescein (FAM) labelled primer and then separation of the products using an automated sequencer (Figure 2) has led to increased throughput capabilities (Fisher and Triplett, 1999).

MONITORING THE PRE-DOMINANT CELLULOLYTIC MICROORGANISMS OF THE RUMEN

Most research in this field has centred on the role of the three predominant fibre degrading bacteria *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. In addition to the major cellulolytic bacterial populations, the rumen also possesses highly fibrolytic anaerobic rumen fungi.

The first quantitative molecular methods to appear for monitoring specific populations within the rumen were performed employing 16S rRNA probing techniques and then later competitive PCR (cPCR) (Stahl et al., 1988; Briescaher et al., 1992; Krause et al., 1999a; Lin et al., 1994; Michalet-Doreau et al., 2001; Weimer et al., 1999; Koike and Koybashi, 2001; Ziemer et al., 2002; Koike et al., 2003a). These studies enumerated bacterial populations from animals fed on various diets and at various times per day which indicated *F. succinogenes* as the predominant cellulolytic bacteria.

Anaerobic filamentous fugal populations have proven to be more difficult to enumerate than bacteria mainly due to their dual life stages: a motile free swimming zoospore and then a fibre attached mature thallus (Orpin, 1994). Initially, enumeration of anaerobic fungi focused on zoospore counts (Orpin, 1975). Zoospore counts could not be completely extrapolated to thallus forming units, particularly when considering polycentric species, which are capable of forming new rhizobium from fragments of old rhizomycelium in addition to zoospores (Hepsell et al., 1997). A most probable number (MPN) method of serially diluted fungal samples was developed to calculate thallus forming units (TFU's) (Theodorou et al., 1990; Obispo and Dehority, 1992). This method revealed that zoospore counts, although much more rapid, would underestimate the fungal populations of the rumen.

With the advancement of molecular enumeration methods, in particular 16S/18S rRNA probing techniques, researchers were able to monitor bacterial and fungal species within the rumen (Stahl et al., 1988; Dore et al., 1993). Due to the high level of conservation within the fungal 18S rDNA sequence (Bowman et al., 1992), the ITS-1 region is a more appropriate target for identification. Located between the 18S rDNA and 5.8S rDNA this region was identified as containing high level of sequence variations and was used for the phylogenetic identification of anaerobic rumen fungi (Li and Heath, 1992; Brookman et al., 2000).

Real-time PCR is a powerful tool that allows for the rapid quantification of a target DNA sequence (Freeman et al., 1999). Design of specific primer sets targeted against the 16S rDNA and the use of standard curves generated from known cell numbers allow for absolute quantification. Researchers have shown that this technique can be used successfully on nucleic acids extracted from rumen contents to monitor microbial populations in the rumen (Tajima et al., 2001; Ouwerkerk et al., 2002; Klieve et al., 2003; Skillman et al., 2006). However, care must be taken when designing primers, as Tajima and colleagues (2001) were able to demonstrate that the 16S rDNA gene from different rumen

bacteria exhibited varying rates of amplification. Standard curves generated from pure cultures of the target species must also be carefully investigated. Ouwerkerk et al. (2002) were able to demonstrate that when the target cells were added to fresh rumen fluid prior to DNA extraction they were able to obtain more accurate values. Target cells that were diluted in buffer without the addition of rumen fluid resulted in a standard curve that would have under-estimated the true population within the rumen by 10 fold (Ouwerkerk et al., 2002). This is most likely due to potential PCR inhibitory compounds that were present within the rumen fluid.

The design and use of a SYBR Green real-time PCR assay to monitor two fibrolytic bacterial species, *F. succinogenes* and *R. flavefaciens* and the total anaerobic fungal population has also recently been described (Denman and McSweeney, 2005, 2006). Standard curve generation for anaerobic rumen filamentous fungi cannot be accurately performed through serial dilutions, as the fungi grow as a non-homogenous culture in liquid media. In particular, some polycentric fungal isolates form a thick pellicle structure when grown *in vitro* which is difficult to disrupt. To overcome this, standard curves were generated based on fungal biomass or crude protein ml^{-1} with respect to cycle threshold values from fungal DNA content.

EXAMINATION OF MICROBIAL DIVERSITY IN THE RUMEN BY SERIAL ANALYSIS OF V1 RIBOSOMAL SEQUENCE TAGS (SARST-V1)

The ruminal microbial community is characterized by high cell density (up to 10^{11} microbial cells per gram or ml of rumen sample) complex interactions (White et al., 1999), as well as high diversity at low taxonomic levels (species and subspecies) and low diversity at phylum level. As previously mentioned, a number of studies have examined rumen microbial diversity using molecular techniques to overcome the limitation of cultivation-based methodologies. Exclusively, all recent studies involved cloning and sequencing of rRNA (*rrs*) genes originate from directly amplified PCR products from community DNA samples (Whitford et al., 1998; Tajima et al., 1999; Tajima et al., 2000; Koike et al., 2003b; Larue et al., 2004). Although these studies have substantially furthered our knowledge of bacterial diversity in the rumen, the current one-sequence-per-clone approach does not provide large enough datasets to support cost-effective, comprehensive analyses of microbial communities (Schloss et al., 2004). This is largely attributed to the substantial diversity and complexity of these microbial communities. Statistically, if a microbial community contains 200 species with the most abundant and least abundant having 10^9 and 10^4 bacterial cells per gram of sample respectively, then at least 50,000 random *rrs* clones need to be sequenced in order to have a 50% probability of one *rrs* sequence representing the least abundant species (St.-Pierre, personal communication). However, almost all studies reported so far involve the sequencing of several hundred *rrs* gene clones from a single sample, thus unveiling only a small portion of the complete diversity present in the rumen. Despite the recent advances in high-throughput DNA sequencing technologies, the time, labour and cost limitations associated with the conventional one-sequence-per-clone approach prevent any researcher from sequencing adequate numbers of clones. This may explain why not even a single microbial community, including ruminal microbial community, has ever been thoroughly characterized even after two decades of extensive studies using this approach. More efficient methodologies are required to generate adequate *rrs* sequence datasets so that the true bacterial diversity and microbiota composition can be determined.

To this end, we recently developed an innovative approach (referred to as Serial Analysis of V1 Ribosomal Sequence Tags, SARST-V1), which allows for sequencing multiple (up to 19) *rrs* genes per sequencing reaction (Yu et al., 2005). The SARST-V1 is an improved version of SARST (Neufeld et al., 2004b), which permits efficient sequencing of thousands or more 16S rDNA sequences in a single experiment. SARST-V1 uses a series of enzymatic reactions to amplify and ligate ribosomal sequence tags (RSTs) from the V1-regions, which is the most hyper-variable region of *rrs* (Yu and Morrison, 2004b), into concatemers that are subsequently cloned and sequenced. This approach offers a significant increase in throughput capacity over the traditional one-sequence-per-clone approach; up to 19 RSTs can be obtained from each sequencing reaction. A lot more bacterial genera, both previously described and novel, were also identified in this study than in all the previously reported studies combined (Yu et al., 2005). As demonstrated previously (Neufeld et al., 2004), full-length *rrs* can also be effectively recovered using specific primers designed from the RSTs. Thus, comprehensive analysis of microbial community composition made possible by new techniques (such as SARST-V1) should considerably further our knowledge on the functional implications of microbial diversity and ecology in the rumen.

RUMEN METHANOGENS

Methanogen 16S-RFLP/riboprinting

Methanogens play a significant part in the biological breakdown of organic matter in the digestive tracts of many vertebrates and invertebrates, and like eubacteria, methanogens have few morphological traits thus making them difficult to identify. Prior to the development of

molecular-based methods, classical microbiological techniques were also used to presumptively identify methanogens from the digestive tracts of animals (Miller and Wolin, 1986). Now, with the advent of molecular technology, a number of methanogen-specific fingerprinting assays have been developed with the aim of being more simple and rapid than conventional phenotypic characterizations.

Hiraishi and his colleagues (1995) were the first to use the 16S riboprinting technique for differentiating methanogens when they screened several methanogen species with eight restriction endonucleases. Their studies showed that *Hae*III and *Hha*I differentiated 10 methanogen species. Later, Wright and Pimm (2003) improved this strategy by screening with 55 additional restriction enzymes. They demonstrated that an initial digestion of DNA from 26 different methanogens with the restriction endonuclease *Hae*III generated 15 different riboprint sets. Six of the 15 riboprint patterns representing more than one strain could be further differentiated using additional restriction endonucleases. Now this simple and rapid method can be used to presumptively identify 22 of the 26 diverse strains of methanogens belonging to 13 different genera from a range of environments. This method has also been used to confirm the identity of methanogens isolated in culture, and to test the quality assurance of methanogen cultures received from overseas suppliers. Moreover, this 16S riboprint strategy has been an important first step to presumptively identify methanogens from 16S clone libraries constructed from rumen contents (Wright et al., 2004, 2006).

Gene markers for methanogens

As an alternative to the 16S gene, the methyl-coenzyme M reductase (*mcr*) gene has been analysed by phylogenetic analysis (Lueders et al., 2001; Luton et al., 2002; Hallam et al., 2003), DGGE and RFLP to study and identify the methanogen populations in peat bogs in the United Kingdom and Finland (Nercessian et al., 1999; Galand et al., 2002), and more recently in rumen contents (Denman et al., 2005). Methyl coenzyme-M reductase is ubiquitous to methanogens and is crucial to the terminal step of methanogenesis where it is involved in the reduction of the methyl group bound to coenzyme-M. Denman et al. (2005) analysed the methanogenic diversity within the rumen of cattle using the *mcrA* gene as the phylogenetic marker for both control and methanogen-inhibited animals and from this gathered information to design quantitative PCR (qPCR) primers for monitoring methanogenic populations. The qPCR was then used to monitor the effects of the anti-methanogenic compound bromochloromethane on the methanogen populations within the rumen (Denman et al., 2005).

Methanogen 16S rDNA clone libraries

With the growing use of molecular techniques to investigate complex microbial systems, the application of these methods has proven to be very effective for the characterization of methanogen diversity in the rumen (Lin et al., 1997; Tokura et al., 1999; Yanagita et al., 2000; Tajima et al., 2001; Whitford et al., 2001; Irbis and Ushida, 2004; Regensbogenova et al., 2004; Skillman et al., 2004; Wright et al., 2004, 2006), especially by the generation of 16S rDNA clone libraries. These libraries have been extensively used to examine methanogen diversity in domestic livestock from Australia (Wright et al., 2004, 2006), Canada (Whitford et al., 2001), Japan (Tajima et al., 2001) and New Zealand (Skillman et al., 2004). The main steps of 16S rDNA library generation and analysis have been discussed in detail elsewhere (Wright et al., 2005). Briefly, 16S or 18S rDNA sequences are PCR amplified from genomic community DNA with primers that are specific for either bacteria, archaea, fungi, or protozoa. The number of cycles needs to be low (i.e. 10 to 12 cycles) to avoid preferential amplification of certain templates and/or chimeric molecule formation (Wintzingerode et al., 1997; Bonnet et al., 2002).

In the most extensive methanogen 16S clone library study undertaken thus far, Wright et al. (2004) examined 733 clones from two year old Merino sheep in Western Australia and found that three *Methanobrevibacter* strains, SM9, M6 and NT7, accounted for more than 85% of the clones. In contrast, the same three strains accounted for only 9% of the 78 clones from five year old mature Merino sheep in Queensland. Such marked differences in ovine rumen methanogen diversity between these two studies from opposite sides of Australia are likely to be explained, in part, by diet. However, other factors such as the environment, fitness, genotype, and age of animal could also have an effect on the microbial diversity.

16S clone libraries have also uncovered a myriad of sequences representing uncultured archaea which were previously thought to be atypical for the rumen environment (Tajima et al., 2001; Irbis and Ushida, 2004; Shin et al., 2004; Wright et al., 2004). A recently published study by Wright et al (2006), revealed the largest assortment of uncharacterised archaeal sequences ever identified from the ovine rumen. Eighteen unique phylotypes (63 clones) clustered within a strongly supported phylogenetic group along with a novel sequence from pig manure storage pits (Snell-Castro et al., 2005), two novel sequences from sheep in Western Australia (Wright et al., 2004), three novel sequences from Holstein cattle in Japan (Tajima et al., 2001), and several novel sequences from rumen protozoa that had been isolated from a goat (Irbis and Ushida, 2004). The sister group to this distinct clade is a branch containing the thermophilic archaea, *Thermoplasma acidophilum* and

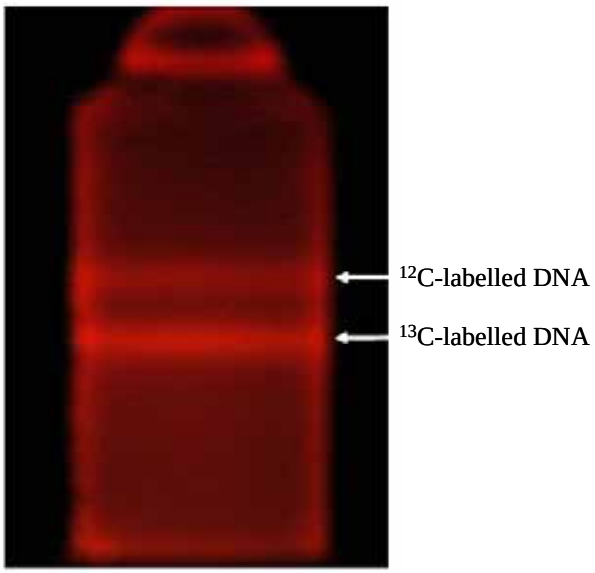

Figure 3. Cesium chloride gradient of DNA extracted from a mixed culture grown on a ^{13}C-labelled substrate.

Thermoplasma volcanium (order Thermoplasmatales).

Attempts to cultivate archaea belonging to this newly discovered group are needed, as well as further studies to examine the effect of environment, genotype, and age of animal on methanogen diversity in ruminant livestock.

FUNCTIONAL ANALYSIS OF THE RUMEN ECOSYSTEM

Over the last decade, molecular ecology studies of the rumen microbiota have focussed almost entirely on identifying and enumerating populations. These studies have relied primarily on DNA-based analysis of the SSU rDNA. While these analyses are very informative for determining the composition of the microbial community and monitoring changes in population size, they can only infer function based on these observations. The next step in functional analysis of the ecosystem is to measure how specific and/or predominant members of the ecosystem are operating and interacting with other groups. Progress in this area has been slow compared with the structural analysis of the ecosystem. However, recent developments in the use of stable isotopes to target microorganisms which utilise specific substrates *in-situ* have the potential to revolutionise our understanding of the key members involved in important metabolic pathways of the ecosystem (Radajewski et al., 2000).

In some cases the identity of important functional genes is already known and direct measurement of the expression of these genes can be used to monitor ecosystem-function. Gene expression can be sensitively analyzed by amplification of messenger RNA (mRNA) using reverse transcription-PCR (RT-PCR). Currently there are few published studies of gene expression in rumen microorganisms under either *in-vitro* or *in-vivo* conditions (Bera-Maillet et al., 2004; Krause et al., 2005). Gene expression studies are less developed than DNA-based analysis of community structure due to the inherent problems involved in extracting high quality RNA from digesta, and priming cDNA synthesis from bacterial mRNA that lacks a polyadenylated tail. RT-PCR requires the activities of reverse transcriptase (RTase) in making the complementary DNA (cDNA) from the template mRNA. A detailed description of the techniques involved in performing gene expression analysis for ruminant applications are described by Yu and Forster (2005). These techniques can be applied to studies of single genes or many genes in samples from pure cultures or complex microbial communities. The abundance of particular mRNA can be quantified by real-time RT-PCR or by using DNA microarrays. Quantitative real-time RT-PCR for gene expression analysis is similar in principle to real-time PCR for enumeration of microbial populations once cDNA template is synthesised from mRNA transcript (Yu and Forster, 2005). Until recently, DNA microarrays have been used mainly in biomedical research to study gene expression in eukaryotic cells of animals and yeasts because the techniques for mRNA extraction, purification and cDNA synthesis are well developed for eukaryotes. DNA expression arrays for prokaryotes have been used successfully for studying the genome of a single organism and these techniques have now been applied to complex microbial ecosystems (Dennis et al., 2003).

The limitation of many of the current methods in molecular microbial ecology is that they are dependant upon DNA sequence information from isolated microorganisms or from 16S rDNA clone libraries. This information represents only a small fraction of the microorganisms present in the gut ecosystem and also it is difficult to apply in terms of functional analysis. This limitation is currently being addressed by the application of the technique called “stable isotope probing” (SIP) which uses stable isotope labelled growth substrates (e.g. ^{13}C-labelled) to reveal the identity and function of predominant members of a mixed community that are metabolically active in utilising a particular growth substrate (Radajewski et al., 2000, 2004). The principle behind this technique is that the nucleic acids (DNA and RNA) from an organism which is actively utilising the labelled substrate become enriched with the label from the substrate. The label enriched nucleic acid (eg highly labelled ‘heavy’ DNA) can then be separated from the unlabelled ‘light’ DNA following density-gradient centrifugation (Figure 3).

The technique has also been applied to RNA labelling and recovery (Lueders et al., 2004; Manefield et al., 2002). The recovered ‘heavy’ DNA or RNA which represents the

Table 1. Commonly used DNA-based techniques that can be used to describe changes in the rumen microbial ecosystem

Method	Application	Advantages	Disadvantages
Restriction Fragment Length Polymorphisms (RFLP's)	Fingerprinting of isolates and microbial communities	Rapid screen for identifying and grouping similar organisms	Broad based screen which does not provide specific identity
Oligonucleotide probe hybridization	Enumeration of microbial populations at varying levels including domain, phylum, species	Quantitative and specific Probes based on phylogenetic sequence database	Laborious Quantitation expressed as % of total population and not absolute numbers
Fluorescence *in situ* hybridization	Enumeration of microrganisms *in situ* within their environment	Visualise culturable and unculturable microrganisms spatially in relation to substrate and other community members	Laborious and quantitation is difficult
16S/18S rDNA clone libraries	Identify the predominant microrganisms in a microbial community	Unculturable and culturable organisms can be identified from sequence analysis of cloned small sub unit ribosomal genes	Laborious and not quantitative. Sometimes, only predominant organisms are identified
Denaturing Gradient Gel Electrophoresis (DGGE) and Single Stranded Conformational Polymorphisms	Fingerprint pattern analysis of changes mixed microbial community composition	Culturable and unculturable organisms identified Microbial community composition can be determined by pattern analysis at domain, phylum and species level	Laborious Sometimes, only predominant organisms identified
Quantitative real time Polymeriase Chain Reaction (qPCR)	Quantitative estimates of microbial populations at the domain, phylum, species and sub-species level	Rapid quantitative method for estimating discrete population in a mixed environmental sample	Technique is based on small sub unit ribosomal sequence identity of previously sequenced cultured organisms and clone libraries
Quantitative real time reverse transcriptase Polymeriase Chain Reaction (qRT-PCR)	Quantitative estimates of microbial gene expression in complex microbial populations	Rapid quantitative method for estimating expression levels of single or select number of important genes in a mixed environmental sample	Global analysis of gene expression in complex ecosystem not possible
DNA phylogenetic microarray	Semi-quantitative estimates of microbial populations at the domain, phylum, species and sub-species level	Rapid method for monitoring gross changes in major populations in a complex ecosystem	Not quantitative Lower sensitivity and specificity than qPCR
DNA expression array	Semi-quantitative estimates of microbial gene expression in complex microbial populations	Rapid method for monitoring gross changes in gene expression of large numbers of genes from multiple organisms in a complex ecosystem	Not quantitative Lower sensitivity and specificity than qPCR
Stable isotope probing	Identification of a metabolically active microbial group	Culturable and unculturable organisms that are involved in a particular metabolic process can be distinguished from the general microbial community	Not quantitative. Culture conditions may bias for growth of organisms that are not the main group responsible for the metabolism under normal conditions in the ecosystem

nucleic acids from the populations that are involved in utilisation of the labelled substrate can then be analysed for identity, gene architecture and expression using standard molecular ecology tools. A significant advantage of SIP is that the heavy isotope enriched nucleic acid will contain the entire genome of functionally active members of the community which can be cloned into fosmid vectors and analysed for specific functional genes or amplified with 16S rDNA primers to determine identity of the organisms (Gray and Head, 2001). One limitation of the technique is that it is often necessary to provide large amounts of the labelled substrate combined with long incubation times (e.g. several days) to ensure sufficient uptake and labelling of the DNA to enable separation of recoverable amounts of labelled nucleic acid.

The SIP method therefore depends on the incorporation

of significant amounts of heavy isotope into the nucleic acids which means that these experiments are often performed as *in-vitro* fermentations. These conditions impose some limitations on the technique since *in-vitro* growth and excessive substrate may introduce biases and enrich for a population that is not representative of the functionally active organisms in the rumen.

CONCLUSION

As we embrace DNA-based technology, it is apparent that techniques which optimise the analysis of complex microbial communities rather than the detection of single organisms will need to address the issues of high throughput analysis of many primers/probes in a single sample. These technologies have the potential to revolutionize the understanding of rumen function and will overcome the limitations of conventional techniques, including isolation and taxonomic identification of strains important to efficient rumen function. The future of rumen microbiology research is dependant upon the adoption of these molecular research technologies to answer different questions about rumen ecology (Table 1). However, the challenge is in how these technologies can be used to improve ruminant production through a better understanding of microbial function and ecology.

REFERENCES

Bera-Maillet, C., A. Kwasiborski, P. Mosoni and E. Forano. 2004. Quantification of *Fibrobacter succinogenes* cellulase and xylanase gene expression in the rumen of a gnotobiotic lamb by real-time RT-PCR. INRA-RRI 2004 Gut Microbiology 4th Joint Symposium, Clermont-Ferrand, France. Rep. Nutr. Develop. 44(Suppl. 1):S57.

Bonnet, R., A. Suau, J. Dore, G. R. Gibson and M. D. Collins. 2002. Differences in rDNA libraries of faecal bacteria derived from 10- and 25-cycle PCRs. Int. J. Syst. Evol. Microbiol. 52:757-763.

Bowman, B. H., J. W. Taylor, A. G. Brownlee, J. Lee, S. D. Lu, and T. J. White. 1992. Molecular evolution of the fungi: relationship of the Basidiomycetes, Ascomycetes, and Chytridiomycetes. Mol. Biol. Evol. 9:285-296.

Briesacher, S. L., T. May, K. N. Grigsby, M. S. Kerley, R. V. Anthony and J. A. Paterson. 1992. Use of DNA probes to monitor nutritional effects on ruminal prokaryotes and *Fibrobacter succinogenes* S85. J. Anim. Sci. 70:289-295.

Brookman, J. L., G. Mennim, A. P. Trinci, M. K. Theodorou and D. S. Tuckwell. 2000. Identification and characterization of anaerobic gut fungi using molecular methodologies based on ribosomal ITS1 and 18S rRNA. Microbiol. 146:393-403.

Dehority, B. A. 2003. Rumen Microbiology. Nottingham, UK., Nottingham University Press.

Denman, S. E., M. J. Nicholson, M. K. Theodorou, J. L. Brookman and C. S. McSweeney. 2003. Tracking cellulolytic anaerobic rumen fungal populations along the digestive tract of cattle. In: MIE Bioforum 2003 'Biotechnology of Lignocellulose Degradation and Biomass Utilisation' Ise-Shima, Japan.

Denman, S. E. and C. S. McSweeney. 2005. PCR-based methods for analysis of populations and gene expression. 3.2 quantitative (Real time) PCR. In 'Methods in Gut Microbial Ecology for Ruminants'. (Ed. H. Makkar and C. S. McSweeney), Springer, The Netherlands.

Denman, S. E. and C. S. McSweeney. 2006. The use of quantitative PCR for monitoring diurnal fluxes of anaerobic fungal and bacterial populations within the rumen. FEMS Microb. Ecol. (in press).

Denman, S. E., N. Tomkins and C. S. McSweeney. 2005. Monitoring the effect of bromochloromethane on methanogen populations within the rumen using qPCR. In '2nd International Symposium on Greenhouse Gases and Animal Agriculture' (Ed. C. R. Soliva, J. Takahashi and M. Kreuzer). ETH Zurich, Switzerland, p. 112.

Dennis, P., E. A. Edwards, S. N. Liss and R. Fulthorpe. 2003. Monitoring gene expression in mixed microbial communities by using DNA microarrays. Appl. Environ. Microbiol. 69:769-778.

Dore, J., A. G. Brownlee, L. Millet, I. Virlogeux, M. Saigne, G. Fonty and P. Gouet. 1993. Ribosomal DNA-targeted hybridisation probes for the detection, identification and quantitation of anaerobic rumen fungi. Proc. Nutr. Soc. 52:176A.

Faichney, G. J., C. Poncet, B. Lassalas, J. P. Jouany, L. Millet, J. Doré and A. G. Brownlee. 1997. Effect of concentrates in a hay diet on the contribution of anaerobic fungi, protozoa, and bacteria to nitrogen in rumen and duodenal digesta in sheep. Anim. Feed Sci. Technol. 64:193-213.

Finlay, B. J., G. Esteban, K. J. Clarke, A. G. Williams, T. M. Embley and R. P. Hirt. 1994. Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiol. Lett. 117:157-161.

Firkins, J. L. and Z. Yu. 2006. Characterisation and quantification of the microbial populations of the rumen. Ruminant physiology: Digestion, metabolism and impact of nutrition on gene expression, immunology and stress (Ed. K. Sejrsen, T. Hvelplund and M. O. Nielsen). pp. 19-54. Wageningen Academic Publisher, The Netherlands.

Fisher, M. M. and E. W. Triplett. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Appl. Environ. Microbiol. 65:4630-4636.

Forster, R., J. Gong and R. J. Teather. 1997. Group-specific 16S rRNA hybridization probes for determinative and community structure studies of Butyrivibrio fibrisolvens in the rumen. Appl. Environ. Microbiol. 63:1256-1260.

Freeman, W. M., S. J. Walker and K. E. Vrana. 1999. Quantitative RT-PCR: pitfalls and potential. Biotech. 26:112-125.

Galand, P. E., S. Saarnio, H. Fritze and K. Yrjala. 2002. Depth related diversity of methanogen Archaea in Finnish oligotrophic fen. FEMS Microbiol. Ecol. 42:441-449.

Gray, N. D. and I. M. Head. 2001. Linking genetic identity and function in communities of uncultured bacteria. Environ. Microbiol. 3:481-492.

Hallam, S. J., P. R. Girguis, C. M. Preston, P. M. Richardson and E.

F. DeLong. 2003. Identification of methyl coenzyme M reductase A (*mcrA*) genes associated with methane-oxidizing archaea. Appl. Environ. Microbiol. 69:5483-5491.

Hespell, R. B., D. E. Akin and B. A. Dehority. 1997. Bacteria, fungi, and protozoa of the rumen. In: Gastrointestinal Microbiology, vol. 2, (Ed. R. I. Mackie, B. A. White and R. E. Isaacson). pp. 59-141. Chapman and Hall, New York.

Hiraishi, A., Y. Kamagata and K. Nakamura. 1995. Polymerase chain reaction amplification and restriction fragment length polymorphism analysis of 16S rRNA genes from methanogens. J. Ferment. Bioeng. 79:523-529.

Irbis, C. and K. Ushida. 2004. Detection of methanogens and proteobacteria from a single cell of rumen ci,liate protozoa. J. Gen. Appl. Microbiol. 50:203-212.

Jouany, J. P. 1996. Effect of rumen protozoa on nitrogen utilization by ruminants. J. Nutr. 126:1335S-46S.

Karnati, S. K., Z. Yu, J. T. Sylvester, B. A. Dehority, M. Morrison and J. L. Firkins. 2003. Specific PCR amplification of protozoal 18S rDNA sequences from DNA extracted from ruminal samples of cows. J. Anim. Sci. 81:812-815.

Kišidayová, S., Z. Varadyova, I. Zelenak and P. Siroka. 2000. Methanogenesis in rumen ciliate cultures of *Entodinium caudatum* and *Epidinium ecaudatum* after long-term cultivation in a chemically defined medium. Folia Microbiol. (Praha) 45:269-274.

Klieve, A. V., D. Hennessy, D. Ouwerkerk, R. J. Forster, R. I. Mackie and G. T. Attwood. 2003. Establishing populations of *Megasphaera elsdenii* YE 34 and *Butyrivibrio fibrisolvens* YE 44 in the rumen of cattle fed high grain diets. J. Appl. Microbiol. 95:621-630.

Kocherginskaya, S. A., R. I. Aminov and B. A. White. 2001. Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing, and statistical ecology approaches. Anaerobe 7:119-134.

Koike, S. and Y. Kobayashi. 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. FEMS Microbiol. Lett. 204:361-366.

Koike, S., J. Pan, Y. Kobayashi and K. Tanaka. 2003a. Kinetics of in sacco fiber-attachment of representative ruminal cellulolytic bacteria monitored by competitive PCR. J. Dairy. Sci. 86:1429-1435.

Koike, S., S. Yoshitanib, Y. Kobayashi and K. Tanaka. 2003b. Phylogenetic analysis of fiber-associated rumen bacterial community and PCR detection of uncultured bacteria. FEMS Microbiol. Lett. 229:23-30.

Krause, D. O., B. P. Dalrymple, W. J. Smith, R. I. Mackie and C. S. McSweeney. 1999a. 16S rDNA sequencing of Ruminococcus albus and Ruminococcus flavefaciens: design of a signature probe and its application in adult sheep. Microbiol. 145:1797-1807.

Krause, D. O., W. J. M. Smith, F. M. E. Ryan, R. I. Mackie and C. S. McSweeney. 1999b. Use of 16S-rRNA based techniques to investigate the ecological succession of microbial populations in the immature lamb rumen: Tracking of a specific strain of inoculated Ruminococcus and interactions with other microbial populations *in vivo*. Microb. Ecol. 38:365-376.

Krause, D. O., W. J. Smith and C. S. McSweeney. 2001. Extraction of microbial DNA from rumen contents containing plant tannins. Bio. Techn. 31:294-298.

Krause, D. O., W. J. M. Smith, J. D. Brooker and C. S. McSweeney. 2005. Tolerance mechanisms of streptococci to hydrolysable and condensed tannins. Anim. Feed Sci. Technol. 121:59-75.

Larue, R., Z. Yu, V. A. Parisi, A. R. Egan and M. Morrison. 2004. Novel microbial diversity adherent to plant biomass in the herbivore gastrointestinal tract, as revealed by ribosomal intergenic spacer analysis and rrs gene sequencing. Environ. Microbiol. 7:530-543.

Lee, S. S., J. K. Ha and K.-J. Cheng. 2000. Relative contributions of bacteria, protozoa, and fungi to *in vitro* degradation of orchard grass cell walls and their Interactions. Appl. Environ. Microbiol. 66:3807-3813.

Lueders, T., B. Pommerenke and M. W. Friedrich. 2004. Stable-isotope probing of microorganisms thriving at thermodynamic limits: syntrophic propionate oxidation in flooded soil. Appl. Environ. Microbiol. 70:5778-5786.

Li, J. L. and I. B. Heath. 1992. The phylogenetic-relationships of the anaerobic chytridiomycetous gut fungi (Neocallimasticaceae) and the chytridiomycota. 1. cladistic-analysis of ribosomal-RNA sequences. Can. J. Bot. 70:1738-1746.

Lin, C. Z., B. Flesher, W. C. Capman, R. I. Amann and D. A. Stahl. 1994. Taxon specific hybridization probes for fiber-digesting bacteria suggest novel gut-associated *Fibrobacter*. Syst. Appl. Microbiol. 17:418-424.

Lin, C., L. Raskin and D. A. Stahl. 1997. Microbial community structure in gastrointestinal tracts of domestic animals: comparative analysis using rRNA-targeted oligonucleotide probes. FEMS Microbiol. Ecol. 22:281-294.

Lueders, T., K. J. Chin, R. Conrad and M. Friedrich. 2001. Molecular analyses of methyl-coenzyme M reductase alpha-subunit (mcrA) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. Environ. Microbiol. 3:194-204.

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

Machmuller, A., C. R. Soliva and M. Kreuzer. 2003. Effect of coconut oil and defaunation treatment on methanogenesis in sheep. Reprod. Nutr. Dev. 43:41-55.

Makkar, H. and C. S. McSweeney. 2005. Methods in Gut Microbial Ecology for Ruminants. Springer, The Netherlands. p. 225.

Manefield, M., A. S. Whiteley, N. Ostle, P. Ineson and M. J. Bailey. 2002. Technical considerations for RNA based stable isotope probing: an approach to associating microbial diversity with microbial community function. Rapid Commun. Mass Spectrom. 16:2179-2183.

Michalet-Doreau, B., I. Fernandez, C. Peyron, L. Millet and G. Fonty. 2001. Fibrolytic activities and cellulolytic bacterial community structure in the solid and liquid phases of rumen contents. Reprod. Nutr. Dev. 41:187-194.

Miller, T. L. and M. J. Wolin. 1986. Methanogens in human and animal digestive tracts. Syst. Appl. Microbiol. 7:223-229.

Nercessian, D., M. Upton, D. Loyd and C. Edwards. 1999.

Phylogenetic analysis of peat bog methanogen populations. FEMS Microbiol. Lett. 173:425-429.

Neufeld, J. D., Z. Yu, W. Lam and W. W. Mohn. 2004a. SARST, Serial Analysis of Ribosomal Sequence Tags. pp. 543-568. In. 'Molecular Microbial Ecology Manual'. 2nd Edition. (Ed. G. A. Kowalchuk, F. J. de Bruijin, I. M. Head, A. D. L. Akkermans, J. D. van Elsaa), Kluwer Academic Publishers, The Netherlands.

Neufeld, J. D., Z. Yu, W. Lam and W. W. Mohn. 2004b. Serial analysis of ribosomal sequence tags (SARST): a high-throughput method for profiling complex microbial communities. Environ. Microbiol. 6:131-144.

Nicholson, M. J., E. J. Kim, M. K. Theodorou and J. L. Brookman 2002. Understanding the molecular ecology of rumen fungi-A DDGE approach. In 'Beyond Antimicrobials- the Future of Gut Microbiology' (Ed. R. J Wallace). Proceedings of the 3rd RRI-INRA, Aberdeen.

Obispo, N. E. and B. A. Dehority. 1992. A most probable number method for enumeration of rumen fungi with studies on factors affecting their concentration in the rumen. J. Microbiol. Methods. 16:259-270.

Orpin, C. G. 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. J. Gen. Microbiol. 91:249-262.

Orpin, C. G. 1994. Anaerobic fungi: taxonaomy, biology, and distribution in nature. In 'Anaerobic Fungi: Biology, Ecology and Function' (Ed. C. G. Orpin and D. O. Mountfort). Mycology series v. 12, Marcel Dekker, New York, pp. 1-45.

Orpin, C. G. and K. N. Joblin. 1997. The rumen anaerobic fungi. In 'The Rumen Microbial Ecosystem'. 2nd edn, (Ed. P. N. Hobson and C. S. Stewart), pp. 140-195. Blackie, London.

Ouwerkerk, D., A. V. Klieve and R. J. Forster. 2002. Enumeration of Megasphaera elsdenii in rumen contents by real-time Taq nuclease assay. J. Appl. Microbiol. 92:753-758.

Ozkose, E., B. J. Thomas, D. R. Davies, G. W. Griffith and M. K. Theodorou. 2001. *Cyllamyces aberensis* gen. nov sp. nov., A new anaerobic gut fungus with branched sporangiophores isolated from cattle. Can. J. Bot. 79:666-673.

Ozutsumi, Y., K. Tajima, A. Takenaka and H. Itabashi. 2005. The Effect of Protozoa on the Composition of Rumen Bacteria in Cattle Using 16S rRNA Gene Clone Libraries. Biosci. Biotechnol. Biochem. 69:499-506.

Prescott, D. M. 1994. "The DNA of ciliated protozoa". Microbiol. Rev. 58(2):233-67.

Radajewski, S., P. Ineson, N. R. Parekh and J. C. Murrell. 2000. Stable-isotope probing as a tool in microbial ecology. Nature 403:646-649.

Radajewski, S., I. R. McDonald and J. C. Murrell. 2004. Stable isotope probing of nucleic acids to identify active microbial populations. In 'Molecular Microbial Ecology Manual', 2 nd edn, pp. 1661-1672. Kluwer Academic Publishers, Netherlands.

Regensbogenova, M., P. Pristas, P. Javorsky, S. Y. Moon-van der Staay, G. W. Moon-van der Staay, J. H. Hackstein, N. R. McEwan and C. J. Newbold. 2004a. Assessment of ciliates in the sheep rumen by DGGE. Lett. Appl. Microbiol. 39:144-147.

Regensbogenova, M., S. Kisidayova, T. Michalowski, P. Javorsky, S. Y. Moon-van-der-Staay, G. W. Moon-an-der-Staay, J. H. Hackstein, N. R. McEwan, J.-P. Jouany, C. J. Newbold and P. Pristas. 2004b. Rapid identification of rumen protozoa by restriction analysis of amplified 18S rRNA genes. Acta Protozool. 43:219-224.

Ronn, R., A. E McCaig, B. S. Griffiths and J. I. Prosser. 2002. Impact of protozoan grazing on bacterial community structure in soil microcosms. Appl. Environ. Microbiol. 68:6094-6105.

Schloss, P. D., B. R. Larget and J. Handelsman. 2004. Integration of microbial ecology and statistics: a test to compare gene libraries. Appl. Environ. Microbiol. 70:5485-5492.

Schonhusen, U., R. Zitnan, S. Kuhla, W. Jentsch, M. Derno and J. Voigt. 2003. Effects of protozoa on methane production in rumen and hindgut of calves around time of weaning. Arch. Tierernahr 57:279-295.

Sharma, R., S. J. John, D. M. Damgaard and T. A. McAllister. 2003. Extraction of PCR-quality plant and microbial DNA from total rumen contents. Biotechniq. 34:92-97.

Shin, E. C., K. M. Cho, W. J. Lim, S. Y. Hong, C. L. An, E. J. Kim, Y. K. Kim, B. R. Choi, J. M. An, J. M. Kang, H. Kim and H. D. Yun. 2004. Phylogenetic analysis of protozoa in the rumen contents of cow based on the 18S rDNA sequences. J. Appl. Microbiol. 97:378-383.

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

Skillman, L. C., A. F. Toovey, A. J. Williams and A.-D. G. Wright. 2006. Development and validation of a real-time PCR method to quantify rumen protozoa and examination of variability between protozoal populations in sheep fed a hay based diet. Appl. Environ. Microbiol. 72:200-206.

Snell-Castro, R., J. J. Godon, J. P. Delgenes and P. Dabert. 2005. Characterisation of the microbial diversity in a pig manure storage pit using small subunit rDNA sequence analysis. FEMS Microbiol. Ecol. 52:229-242.

Stahl, D. A., B. Flesher, H. R. Mansfield and L. Montgomery. 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. 54:1079-1084.

Stewart, C. S., H. J. Flint and M. P. Bryant. 1998. The rumen bacteria, In 'The Rumen MIcrobial Ecosystem', 2nd ed., (Ed. P. N. Hobson and C. S. Stewart). pp. 10-72. Blackie Academic and Professionals, New York, NY.

Sylvester, J. T., S. K. R. Karnati, Z. Yu, M. Morrison and J. L. Firkins. 2004. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. J. Nutrition. 134:3378-3384.

Sylvester, J. T., S. K. Karnati, Z. Yu, C. J. Newbold and J. L. Firkins. 2005. Evaluation of a real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal nitrogen. J. Dairy Sci. 88:2083-2095.

Tajima, K., R. I. Aminov, T. Nagamine, K. Ogata, M. Nakamura, H. Matsui and Y. Benno. 1999. Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. FEMS Microbiol. Ecol. 29:159-169.

Tajima, K., S. Arai, K. Ogata, T. Nagamine, H. Matsui, M. Nakamura, R. I. Aminov and Y. Benno. 2000. Rumen bacterial community transition during adaptation to high-grain diet. Anaerobe 6:273-284.

Tajima, K., R. I. Aminov, T. Nagamine, H. Matsui, M. Nakamura and Y. Benno. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl. Environ. Microbiol. 67:2766-2774.

Theodorou, M. K., M. Gill, C. Kingspooner and D. E. Beever. 1990. Enumeration of Anaerobic Chytridiomycetes as Thallus-

Forming Units- Novel Method for Quantification of Fibrolytic Fungal Populations From the Digestive-Tract Ecosystem. Appl. Environ. Microbiol. 56:1073-1078.

Tokura, M., I. Chagan, K. Ushida and Y. Kojima. 1999. Phylogenetic study of methanogens associated with rumen ciliates. Curr. Microbiol. 39:123-128.

Weimer, P. J., G. C. Waghorn, C. L. Odt and D. R. Mertens. 1999. Effect of diet on populations of three species of ruminal cellulolytic bacteria in lactating dairy cows. J. Dairy Sci. 82:122-134.

White, B. A., I. K. O. Cann, S. A. Kocherginskaya, R. I. Aminov, L. A. Thill, R. I. Mackie and R. Onodera. 1999. Molecular analysis of archaea, bacteria and eucarya communities in the rumen: Review. Asian-Aust. J. Anim. Sci. 12:129-138.

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

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

Williams, A. G., and G. S. Coleman. 1998. The rumen protozoa, In (Ed. P. N. Hobson and C. S. Stewart), The Rumen Microbial Ecosystem, Blackie Acad. Profess. New York. pp. 73-139.

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

Wright, A.-D. G. and C. L. Pimm. 2003. Improved strategy for presumptive identification of methanogens using 16S riboprinting. J. Microbiol. Mthds. 55:337-349.

Wright, A. D. G., K. Tajima and R. I. Aminov. 2005. 16S/18S rDNA clone library construction and analysis. In Methods in Gut Microbial Ecology for ruminants (Ed. H. makkar and C. S. McSweeney). Springer, The Netherlands.

Wright, A-D. G., A. F. Toovey and C. L. Pimm. 2006. Molecular identification of methanogenic archaea from sheep in Queensland, Australia reveal more uncultured novel archaea. Anaerobe. In press.

Wright, A.-D. G., A. J. Williams, B. Winder, C. Christophersen, S. Rodgers and K. Smith. 2004. Molecular diversity of rumen methanogens from sheep in Western Australia. Appl. Environ. Microbiol. 70:1263-1270.

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

Yu, C.-P., R. Ahuja, G. Sayler and K.-H. Chu. 2005. Quantitative molecular assay for fingerprinting microbial communities of wastewater and estrogen-degrading consortia. Appl. Environ. Microbiol. 71:1433-1444.

Yu, Z. and M. Morrison. 2004a. Improved extraction of PCR-quality community DNA from digesta and fecal samples. BioTech. 36:808-812.

Yu, Z. and M. Morrison. 2004b. "Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis." Appl. Environ. Microbiol. 70:4800-4806.

Yu, Z. and R. J. Forster. 2005. PCR-based methods for analysis of populations and gene expression. 3.1 Nucleic acid extraction, oligonucleotide probes and PCR methods. In 'Methods in Gut Microbial Ecology for Ruminants' (Ed. H. Makkar and C. S. McSweeney). Springer, The Netherlands.

Ziemer, C. J., R. Sharp, M. D. Stern, M. A. Cotta, T. R. Whitehead and D. A. Stahl. 2000. Comparison of microbial populations in model and natural rumens using 16S ribosomal RNA-targeted probes. Environ. Microbiol. 2:632-643.

Zoetendal, E. G., S. Koike and R. I. Mackie. 2003. A critical view on molecular ecology of the gastrointestinal tract. In 'Matching Herbivore Nutrition to Ecosystem Biodiversity'. (Ed.: L. tMannetje). Proceedings of the sixth international symposium on the nutrition of herbivores,. pp. 59-78. Merida, Mexico.

Zoetendal, E. G., C. T. Collier, S. Koike, R. I. Mackie and H. R. Gaskins. 2004. Molecular ecological analysis of the gastrointestinal microbiota: A review. J. Nutr. 134:465-472.