Advances in evaluating and altering the rumen microbial ecosystem

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Summary

The manipulation of the rumen microbial ecosystem to improve animal productivity and lessen environmental impacts has been a research goal for many decades. Impediments to a targeted manipulation of the rumen have included a lack of knowledge of the diversity of the rumen microbial population as well as a lack of methods to thoroughly evaluate microbial changes. Molecular based studies of the rumen microbial population have shown it to be one of the most diverse environments studied. New molecular ecology based assays are now enabling a more precise evaluation of the establishment and effects of novel microorganisms in the rumen as well as a more accurate description of microbial changes induced by feeds and feed additives. This knowledge will help informed development of new strategies for the improvement of ruminant production.

Keywords: rumen, probiotic, 16S rRNA, real–time PCR, microbial populations

Introduction

The rumen is a dynamic ecosystem that converts feedstuffs into nutrients utilizable by the host animal. Rumen microbial populations include representatives from many domains of life including a large and diverse group of bacteria, eukaryotes (protozoa and fungi) and archaea (methanogenic bacteria) (Stewart *et al.* 1997). Lytic and temperate bacteriophages are also found in the rumen (Paynter *et al.* 1969; Klieve and Bauchop 1988). Although facultative anaerobic bacteria establish on the rumen wall and others are transient in the rumen (Cheng and Wallace 1979), the majority of rumen microorganisms are obligate anaerobes. Traditionally, anaerobic culture techniques have been used to characterize and enumerate microbial populations in the rumen (Hungate 1966) but these studies have been limited by the fastidious growth requirements of many rumen bacteria, as well as the inability to differentiate many phylogenetically diverse bacteria.

It has been the desire of many researchers to modify rumen microbial populations to increase production efficiency, alter fermentation products, express a novel trait, or alter the adaptability of the rumen to new feed sources. Although production and fermentation responses are monitored on a regular basis, the underlying changes in the rumen microbial populations have been much more difficult to elucidate. Recent advances in molecular ecology have permitted the introduction of new techniques that may be able to resolve the complexity of the rumen ecosystem. The measurement of quantitative changes in rumen microbial populations will be fundamental in evaluating the efficacy and the targeting of new strategies for rumen manipulation.

Rumen bacterial diversity

The estimation of changes in rumen bacterial populations has generally relied upon the culture and identification of individual bacteria or bacterial phenotypes. Examples are roll tube counting and Most Probable Number estimates of cellulolytic, amylolytic and proteolytic bacteria. These methods rely upon the ability to culture rumen microbes in synthetic laboratory media. However it has been estimated that only 10% of bacteria found in the environment can be cultured (Amann *et al.* 1995). A comparison of rumen bacterial numbers observed by direct microscopy and the numbers of colonies appearing on agar plates (Leedle *et al.* 1982), suggests that rumen bacterial populations also contain many uncultivable bacteria. Common rumen bacterial species obtained from culture based studies are shown in Table 1.

The isolation of pure cultures of rumen bacteria from rumen contents can be subject to numerous biases. For example, rumen bacteria have been found to produce bacteriocins that would inhibit the growth of some bacteria in roll tubes or on culture plates (Kalmokoff *et al.* 1996). Subjective selection of organisms for further study can also occur when bacteria

form minute colonies that are overlooked in favour of faster growing isolates that form large colonies. Bacteria that grow under the surface of the agar may not be sampled, and bacteria that do not transfer well or do not survive standard freezer storage conditions are excluded from further characterization. The high cost of media and the need to pick hundreds of colonies to define microbial changes have resulted in many researchers looking for alternative ways of enumerating bacteria and describing microbial diversity in the rumen.

Recently, studies utilizing molecular cloning technologies have been applied to the rumen ecosystem (Whitford *et al.* 1997; Wood *et al.* 1998; Tajima *et al.* 1999; Ramsak *et al*. 2000; Whitford *et al*. 2001). Molecular methods are independent of the need to culture or isolate bacteria and thus present an advantage over traditional techniques. The preferred target for studies of molecular phylogeny has been the 16S rRNA gene (Woese *et al*. 1983). The small subunit ribosomal RNA gene has highly variable as well as highly conserved regions that allow phylogenetically relevant information to be readily analysed. 16S rRNA genes present in samples of rumen contents have been amplified using the polymerase chain reaction (PCR), cloned into plasmid vectors, sequenced and characterized using phylogenetic analysis. These studies have found that up to 90% of the gene sequences isolated from the rumen were not closely related to cultured rumen bacteria that had 16S rRNA gene sequences available for comparison (Whitford *et al.* 1997; Tajima *et al.* 1999; Whitford *et al.* 2001). This figure correlates well with the estimate of 10% culturability of rumen contents. Many of the sequences found in these studies represented clusters of new genera of bacteria that have not been previously isolated. These include significant numbers of clones related to cellulolytic bacteria (Whitford *et al*. 1997) as well as novel rumen methanogens (Whitford *et al.* 2001). The importance and role of these novel rumen bacteria awaits evaluation using techniques that can quantify their presence directly in the rumen.

As with any technique, biases may be incorporated into the methods and PCR based assays are no exception. Recent reports have noted that artifacts are introduced into PCR amplified sequences and that artificial sequences may be cloned (Kopczynski *et al.* 1994). However these problems can be minimized by using conservative estimates of species similarity, keeping the number of PCR amplification steps to a minimum and by using computer programs that can detect chimeric sequences (Robison–Cox *et al.* 1995).

Molecular quantification of rumen bacteria

Oligonucleotide probes, targeting specific 16S rRNA gene sequences, have been used to identify, quantify and visualize ruminal bacterial populations (Stahl *et al.* 1988; McSweeney *et al.* 1993; Forster *et al.* 1997; Krause *et al.* 1999; McSweeney *et al.* 2001). 16S rRNA probes can be designed to hybridize to general classes of bacteria, such as the Gram–positive bacteria (MacGregor *et al.* 2001), or they can be progressively

Table 1 Common rumen bacterial isolates and availability of probes and assays for group (Gr), genus (G) or species (S).

Gram–negative		**Gram–positive**	**Gr**
Prevotella sp.	G, S	*Ruminococcus albus*	G
Ruminobacter amylophilus		*Ruminococcus flavefaciens*	G
Fibrobacter succinogenes	G, S	*Streptococcus bovis*	S
*Megasphaera elsdenii**	S	*Butyrivibrio fibrisolvens*	
*Selenomonas ruminantium**	S	*Pseudobutyrivibrio ruminis*	S
Anaerovibrio lipolytica	S	*Eubacterium ruminantium*	S
Fusobacterium sp.		*Eubacterium cellulosolvens*	
Succinomonas amylolytica		*Lactobacillus* sp.	G, S
Succinivibrio dextrinosolvens	S	*Lachnospira multipara*	
Wolinella succinogenes		*Succiniclasticum ruminis*	
Syntrophococcus sucromutans		*Bifidobacterium* sp.	G, S
Veillonella parvula		*Treponema* sp.	S

Archaebacteria	**Gr**
Methanobrevibacter ruminantium	G
Methanobrevibacter smithii	G
Methanosarcina barkeri	G
Methanomicrobium mobile	G

* phylogenetically related to gram positive bacteria

narrowed to cover specific genera or species. The probes generally work well when the defined set of bacterial targets is known and the signal response can be evaluated using controlled laboratory conditions. Probe sequences developed to date have been based on 16S rRNA gene sequences determined from organisms sampled from existing culture collections. The available set of probes does not cover the diversity present in the rumen and additivity problems have been evident when general probes were used to quantify major rumen bacterial populations (Forster *et al.* 1999).

A recent technique that may be used to quantify rumen microbial populations is the competitive PCR assay. In this method the primers in the PCR reaction are made to be specific to the target of interest as well as co–amplifying a DNA fragment that can be distinguished from the target, based on size or restriction site. The co–amplified DNA or competitive template acts as an internal control for the PCR reaction and competes for primers (Piatak *et al.* 1993). This technique has been used to quantify strains of proteolytic rumen bacteria (Reilly and Attwood 1998). The method is quite accurate and is equal in sensitivity to hybridization probes, however it is time consuming to design the assays and requires substantial post PCR signal processing.

An alternative method that shows great promise for the quantification of bacterial populations is the 5' fluorogenic exonuclease (TaqMan) PCR assay (Heid *et al.* 1996). This assay incorporates a set of primers for PCR as well as a probe that is 5' end–labeled with a fluorogenic reporter and 3' end–labeled with a quenching molecule. The release of fluorescent reporter as the PCR reaction progresses is directly related to the amount of original target template in the reaction. The PCR cycle at which the specific target is detected with confidence, noted as the threshold cycle (C_T), is compared to a standard curve and the unknown quantity is determined. The combination of primers and probe allows the assay to be very specific and the small amplicon size and real time detection reduces the bias inherent in other PCR based techniques (Becker *et al.* 2000). The assay requires a single tube reaction and the results can be read directly from the PCR thermocycler. Although the equipment required to perform these assays is expensive, 5' nuclease based PCR assays will become more popular in the future due to the speed of the assay, accuracy of results and increased sample throughput. The first TaqMan PCR assay applied to studies of rumen ecology was used to detect *Megasphaera elsdenii* in beef cattle (Ouwerkerk *et al.* 2000).

Probiotic applications of rumen bacteria

With the development of PCR based enumeration systems for bacteria it is now possible to determine whether introduced bacteria can survive and establish within the rumen. One of the first applications of novel bacteria to ruminant systems was the introduction of *Synergistes jonesii* to Northern Australian cattle to control toxicity caused by *Leucaena* (Jones and Megarrity 1986). The establishment and distribution of *S. jonesii* within inoculated herds has been demonstrated using PCR detection (A. Klieve, pers. comm.). The success of this application prompted further research to detoxify other compounds to which foraging cattle are exposed. Cattle in northern Australia consume *Acacia georgina* that contains the toxin fluoroacetate; this poison is responsible for significant animal losses in Australia. Gregg *et al.* (1994) developed a genetically modified rumen bacteria containing a dehalogenase gene that was capable of degrading fluoroacetate. PCR methods were used to confirm the establishment of the modified bacteria in inoculated sheep (Gregg *et al.* 1998). The widespread use of this technology is subject to approval by the Australian Genetic Manipulation Advisory Committee and acceptance of the use of genetically modified organisms (for a discussion see http://wwwwscience.murdoch.edu.au/centres/rumen/page.html).

Rumen bacteria are thought to be the main producers of an anticarcinogenic fatty acid, conjugated linoleic acid (CLA), which is found in milk fat and the fat of beef animals. CLA has been shown to have inhibitory effects on human malignant melanoma, colorectal, breast and lung cell lines as well as having anti-carcinogenic activity in animal studies. CLA may also play a role in reducing human body fat levels (Blankson *et al.* 2000) and has been shown to increase lean yield in pigs (Dunshea and Ostrowska 1999). Of the predominant rumen bacteria tested for CLA production, certain strains of *Butyrivibrio fibrisolvens* have been shown to be the most active. It is generally believed that these bacteria are responsible for the accumulation of CLA in meat and dairy products. When linoleic acid (the CLA precurser) rich oils have been fed to dairy cattle there is a general increase in the CLA content of milk, however the response is highly variable between individual cows (Kelly *et al.* 1998a). Animals that have been fed on pasture or forage based diets also have increased levels of CLA in their fat when compared to grain fed cattle (Jiang *et al.* 1996; Kelly *et al.* 1998b). This may be due to differences in rumen microbial composition between animals. In the future, probiotics containing active CLA producing rumen bacteria, may be used to boost levels of CLA in milk and meat. This could have an important effect on human health.

The strategic use of rumen bacteria to increase fibre digestion in the rumen has been of interest for many years and was one of the first proposed uses for genetically modified rumen bacteria (Hespell 1978). Krause *et al.* (1999) reported an experiment in which lambs were dosed with wild–type strains of the highly cellulolytic rumen bacteria *Ruminococcus albus* and *R. flavefaciens*. One strain was tracked by PCR whilst total ruminococci were quantified with a general

Ruminococcus 16S rRNA hybridization probe. The strain tracked by PCR decreased to the assay detection limit in 4 weeks whilst the general *Ruminococcus* probe indicated that the overall population of *Ruminococcus* was not different from the control in the post–dosing period. There did however seem to be a positive influence of dosing on other fibrolytic populations. More recently Krause *et al.* (2001) introduced into the rumen of cattle and sheep a *Butyrivibrio fibrisolvens* strain modified to contain a fungal xylanase. It was found that the modified strain not survive in the rumen beyond 28 days post–inoculation. This may have been due to a number of factors including the lack of fitness of the laboratory strain in the rumen environment as well as the production of antagonistic bacteriocins by the resident population of *Butyrivibrio* (Kalmokoff and Teather 1997).

It has been suggested that *Megasphaera elsdenii* could be a useful probiotic organism to reduce the incidence of acidosis and improve the efficiency of starch utilization in the rumen of grain fed cattle (Kung and Hession 1995; Wiryawan and Brooker 1995; Owens *et al.* 1998). When cattle are initially introduced to diets containing a high proportion of cereal grain, lactic acid, a by–product of anaerobic fermentation of starch produced by some ruminal bacteria, can accumulate in the rumen. This can result in the reduction of the pH of the rumen contents and a decrease in the efficiency with which feed is converted to volatile fatty acids and microbial protein for animal production (Strobel and Russell 1986). If the drop in pH is severe a condition known as acute lactic acidosis may result. The rumen bacterium *M. elsdenii* is thought to play a major role in preventing or controlling acidosis by catabolizing lactic acid (Stewart *et al.* 1997). Recent studies (Klieve *et al.* unpublished), using the technique of Real–Time PCR to track the establishment of an introduced population of *M. elsdenii* (Ouwerkerk *et al.* 2000), have shown that probiotically introduced *M. elsdenii* rapidly colonizes the rumen of beef cattle. In this work, numbers of *M. elsdenii* were found to increase more quickly with inoculation than occurs naturally. Inoculation provided a 5–7 day advantage in the establishment of a dense and stable population compared with the control animals.

In summary, the use of microbes as ruminal probiotics to enhance fermentation characteristics or remove antinutrient or toxic materials is expanding. It is likely that in the future these microbes will be utilized in place of chemical treatments of either the feed or of the animal. The development of DNA based technologies to determine the establishment and longevity of these microbes in the rumen ecosystem is necessary for the success of these strategies. A thorough understanding of the complexities and diversity of the rumen microbial ecosystem will also help target new systems for the manipulation of rumen fermentation.

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