METAGENOMIC ANALYSIS OF THE EFFECT OF NUTRITION ON MICROBIAL POPULATIONS IN THE RUMEN

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INTRODUCTION

Since the first observation of a four-chambered stomach in a ruminant by Aristotle (Russell, 2002) and the first discovery of the role of microbes within the rumen by Tappeiner in 1882 (Thaysen, 1946), the rumen has been investigated for its role in nutrient digestion, animal performance and efficiency. The rumen, which occupies 60-70% of a ruminant's digestive tract and 9-13% of the total body volume, is often compared to an anaerobic fermentation vat filled with microbes (Church, 1993; Flint, 1997). The symbiotic relationship between the rumen microbiota and the host animal has evolved into a highly efficient and complicated system where the host provides the microbes with nutrients, an ambient temperature, and a stable and buffered environment while the host animal is provided with the ability to utilize low-quality complex plant polymers that it cannot otherwise utilize, volatile fatty acids (VFAs) for growth, and microbial cell proteins (MCPs) (Church, 1993; Russell, 2002). Various studies using traditional culture and microscopic techniques have suggested that rumen microbiome is composed of bacteria, fungi, protozoa, Archaea and viruses (Church, 1993; Hobson, 1988; Lee et al., 2000).

Bacteria are the predominant organism within the rumen with an estimated concentration of 10^9 – 10^{11} cells per gram of ruminal content (Hungate, 1966; Lee et al., 2000). Vast majority of these bacteria are obligatory anaerobes while some facultative anaerobes also have been reported from the rumen. Rumen bacterial populations are known to fluctuate heavily in response to changes in diet and ruminal environment (Goad et al., 1998; Tajima et al., 1999; Tajima et al., 2000). Although several rumen microbial species have been isolated and biologically characterized (Kamra, 2005), only 10-50% of all rumen bacteria are considered to be cultivable (Kobayashi, 2006). However the high content turnover rate in the rumen suggest that the ratio between cultivable and total bacteria within the rumen might not be as drastically different as in most other environments (Amann et al., 1995). Over the years, many bacteria have been isolated from the rumen and have been characterized on their ability to utilize different substrates like cellulose, hemicelluloses, starch, sugars/dextrins, pectin, protein, urea etc. (Kamra, 2005). However, recent applications of molecular phylogeny has shown that the diversity within the rumen bacterial communities are much larger than ever assumed with culture-based technologies with many divergent strains of the same bacterial genera coexisting within the same host (Edwards et al., 2004; Fernando et al., 2008; Tajima et al., 1999; Tajima et al., 2000).

Archaea that are known to inhabit the rumen are strictly anaerobic methanogens (Janssen and Kirs, 2008). So far, seven species of methanogens belonging to five genera have been identified in the rumen. These methanogens are believed to play an important role in the rumen by scavenging molecular hydrogen generated during rumen fermentation and thereby making rumen fermentation a continual process (Kamra, 2005). Some of these organisms were observed

to have an interesting association with rumen protozoal organisms by attaching to the ciliate protozoa and thus getting access to a constant supply of hydrogen (Stumm et al., 1982).

The existence of a significant population of ciliated anaerobic protozoa in the rumen was known for over hundred years. They are thought to represent up to 40% of rumen microbial biomass with up to 10^6 cells pre mL of rumen content (Flint, 1997; Veira, 1986), and could be responsible for over 30% of fiber digestion in the rumen (Demeyer, 1981). They also have been associated with sequestration and subsequent metabolism of toxic compounds in the rumen (Veira, 1986). Hungate (1966) characterizes ciliate protozoa from the rumen into two groups, holotrich and entodiniomorphid protozoa, based on their morphology. Others have characterized them based on their metabolic properties (Kamra, 2005).

Rumen fungi are an integral part of the rumen ecosystem. Although these flagellated organisms were identified in the rumen a century ago, they were mistakenly classified as flagellated protozoa until Orpin (1975) correctly identified them as fungi. They are believed to have evolved from free-living chytridiomycetes to survive the anaerobic conditions of the gastrointestinal tracts of herbivores. Anaerobic gut fungi lack mitochondria and energy is generated in an organelle called hydrogenosome (Paul et al., 1990). Although anaerobic fungi have now been identified from a wide range of herbivore GI tracts, they have not been identified in any other anaerobic terrestrial or aquatic habitat (Hibbett et al., 2007). These organisms play a key role in fiber digestion in the rumen. They have the ability to penetrate the cuticle, the rigid outer layer of plant epidermis, have a synergistic association with rumen bacteria and help digest complex plant material (Dehority and Tirabasso, 2000). They are especially known to be capable of digesting lignified plant material (McSweeney et al., 1994). Removal of fungi from the rumen has resulted in a significant decrease in *in vitro* gas production and fiber degradation (Kamra, 2005). Rumen fungi are known to possess a range of highly active enzymes and they are the only known microorganism capable of producing an exo-acting cellulase (Forsberg et al., 1997).

Bacteriophages are viruses that inhabit bacteria. They are obligatory parasitic pathogens for specific bacteria. Like many other natural environments, rumen has its share of resident bacteriophages. One study estimates the rumen bacteriophage count to be between $3X10^6$ to $1X10^{10}$ units per mL of rumen content (Klieve and Swain, 1993). Bacteriophages are natural predators of bacteria and have an impact on bacterial population dynamics, horizontal gene transfer, antimicrobial resistance and bacterial virulence (Casas and Rohwer, 2007). Lysis of crucial bacteria involved in metabolic pathways inside the rumen could reduce the feed conversion efficiency and total animal performance while the lysis causes bacterial proteins be easily available to the animal as amino acids. Klieve and Bauchop (1991) while investigating lytic phages within the rumen were able to isolate a bacteriophage that infect *Streptococcus bovis*. *S. bovis* is a key bacterium that is involved in rumen acidosis and the introduction of these bacteriophages were later shown to significantly reduce *S. bovis* attached to the rumen epithelium (Styriak et al., 1991). Phage therapy has also been attempted for biological control of rumen methanogens (Klieve and Hegarty, 1999).

All early work on establishing the structure and complexity of the rumen microbiome was done using culture-based techniques and/or microscopy. However, it is estimated that 99% of

organisms found in any environment is resistant to *in-vitro* culture (Pace, 1997). (It is also suggested that this ratio would be a little lower for the rumen due to its high turnover rate (Amann et al., 1995)). The lack of a clear definition of a microbial species with culture-based techniques and the considerable effect of horizontal gene transfer among microbes severely limits the applicability of culture-based taxonomic identification to any environment (Hugenholtz et al., 1998; Hugenholtz and Pace, 1996). These challenges and the rapid advances in molecular technologies have prompted scientists to search for alternative methods in studying the rumen microbiome. Most of the inspiration for modernization of rumen microbiology has come from excellent advances made in various groups working on environmental microbiome projects (Venter et al., 2004) and from the National Institutes of Health -sponsored Human Microbiome Project (http://nihroadmap.nih.gov/hmp/) (Turnbaugh et al., 2007). One technological advance that made a profound effect on environmental microbiology is the use of natural polymorphisms in the 16S rRNA gene to study the diversity of microorganisms in any given eco system (Pace, 1997), and the subsequent rise of the new field of metagenomics - the culture-independent cloning and analysis of microbial DNA extracted directly from an environmental sample (Handelsman et al., 1998; Schloss and Handelsman, 2005). T-RFLP (Terminal Restriction Fragment Length Polymorphism) analysis has provided us with a reliable, low-cost option to study multiple metagenomic samples (Kent et al., 2003; Marsh et al., 2000; Osborn et al., 2000). The last, but one of the most important developments that helped transform the field of rumen microbiology is the rapid advances in DNA sequencing technology that was accompanied by an astonishing drop in sequencing cost. Pyrosequencing, developed by 454 LifeSciences (http://www.454.com) is one of those technologies. Major advantages of this system is the lack of a need to clone DNA prior to sequencing which eliminates the well-known bias of certain sequences in cloning and the ability to produce very large amounts of high-quality sequences produced in a short time. The latest version of this instrument is capable of producing 1.25 million reads at an average read-length of 400 bases resulting in the production of nearly 0.5Gb (half billion nucleotides) of sequence information per run (Margulies et al., 2005; Ronaghi et al., 1996; Ronaghi et al., 1998). Over the last several years many rumen microorganisms have been sequence analyzed using both Pyrosequencing and the more traditional Sanger sequencing technology (Brulc et al., 2010). Following is a discussion of our experiences in using metagenomics tools in an effort to better understand the dynamics of rumen microbial ecosystem as an animal adapts to a high concentrate diet and contracts sub acute ruminal acidosis.

RUMEN MICROBIAL POPULATION DYNAMICS DURING ADAPTATION TO A HIGH GRAIN DIET

High grain adaptation programs are widely used in the US feedlot cattle industry to balance enhanced growth performance against the risk of acidosis. During this adaptation process, significant changes in the ruminal environment and rumen bacterial population structure has been reported (Goad et al., 1998; Tajima et al., 2000). However, microbial changes during this transition were poorly understood and studies performed up to this point had utilized a few indicator bacteria to evaluate bacterial population changes. We utilized culture-independent approaches of Terminal Restriction Fragment Length Polymorphisms (T-RFLP) and sequence analysis of 16S rDNA libraries to compare rumen bacterial population structure in animals on prairie hay against animals adapting to a high grain diet. Four ruminally canulated beef steers

were adapted to high grain diet using a step-up diet regime containing 20:80, 40:60, 60:40, and 80:20 grain:hay ratios. Samples were collected at each stage after one week period of adaptation and total DNA was extracted. Using PCR primers designed against a highly-conserved region of bacterial 16S rRNA gene, a DNA fragment of ~800 bp was amplified and T-RFLP analyses performed. The same primers were used to amplify another set of the same DNA sequences to construct two 16S rDNA libraries for sequence analysis. We detected significant change in population structure during adaptation to high concentrate diet. Interestingly, no significant change in population structure was observed during the first two diets. By diet 3 and 4 changes in microbial population structure was clearly apparent. This could be attributable to the increased fermentable substrate present in the diet favoring amylolytic and starch digesting bacterial species. The phylogenetic assignment of T-RFLP data using RDP 16S database (Cole et al., 2003) was only able to assign phylogeny to 30% - 50% of the fragments generated. This suggests the presence of large number of uncharacterized bacteria within the rumen. Firmicutes were abundant in the 16S libraries and also in the T-RFLP profiles in both diets. Firmicutes are mainly comprised of gram positive, low G+C bacteria (Boone et al., 2001). Thus, the presence of Firmicutes in high numbers in both ruminal environments suggests that Firmicutes represent a core bacterial component within the rumen. The ratios between Firmicutes and Bacteroidetes in the T-RFLP analysis displayed a significant difference by diet 3, where the ratio was larger in hay-fed animals compared to grain-fed animals. Interestingly this is contrary to ratios observed in human gut where an increase in weight gain was reflected by a higher Firmicutes:Bacteroidetes ratio (Turnbaugh et al., 2006). However, the gastro-intestinal tract of humans and ruminants are completely different where humans are hind-gut fermenters and cattle are fore-gut fermenters (Church, 1993). As such, it is possible that in fore-gut fermenters the Firmicutes:Bacteroidetes ratio is lower during weight gain. T-RFLP analysis identified over 350 bacterial species belonging to 115 different genera. Bacterial genera identified included many previously described genera. These include *Bifidobacterium sp., Butyrivibrio sp., Eubacterium sp. Lactobacillus sp., Prevotella sp., Ruminococcus sp., Selenomonas sp., Streptococcus sp., Fusobacterium sp., and Peptostreptococcus sp.* T-RFLP analyses, although reliable and highly reproducible, only allows identification of previously characterized bacterial species. In addition, multiple bacterial species may share the same terminal restriction fragment. Thus, additional independent molecular approaches are needed to verify terminal restriction fragment results. For definitive identification of bacterial species in the samples analyzed, we constructed four 16S rDNA libraries and sequenced 384 clones from each library (4X384). Sequence analysis of the reads from animals fed prairie hay displayed a significantly larger number of bacteria belonging to phylum Bacteriodetes. This Bacteriodetes population accounted for a majority of the clones sequenced and was composed of bacteria belonging to genera *Prevotella, Anaerophaga*, and *Tannerella*. However, 82% of the bacteria belonging to phylum Bacteriodetes were unclassified Bacteriodes sp., suggesting that a greater number of bacterial species within the rumen are yet to be characterized. The 16S libraries demonstrated two distinct rumen microbial populations in hay fed and high-grain fed animals and detected only 24 common OTUs out of 398 and 315 respectively. The 16S libraries of hay-fed animals were significantly high in Fibrobacteres whereas, the 16S libraries of grain-fed animals were high in Bacteroidetes. Diversity estimates performed on this data predicts the rumen to contain >1700 bacterial species.

MICROBIAL POPULATION DYNAMICS DURING SUBACUTE RUMINAL ACIDOSIS

Ruminal acidosis is considered to be one of the most important nutritional disorders in the feedlot and dairy industries today. The economic loss of subacute acidosis is estimated to be around billion dollars in the dairy industry alone. Yet, only a few studies have investigated the role of microbial population dynamics in subacute ruminal acidosis (SARA). Four ruminally canulated steers were fed prairie hay *ad libitum* for two weeks, then they were put on a step-up diet containing increasing amounts of metabolic energy. After adaptation to the high concentrate diet, 2 animals were randomly selected and they were ruminally dosed with 1.2g/Kg body weight of ground corn to experimentally induce subacute ruminal acidosis. Rumen content was collected from all four steers before dosing and at 30 min intervals during the first six hours of induction. After the first 6 hours, rumen content was collected on an hourly basis for the next six hours and every two hours for 12 more hours. DNA was extracted from all samples and T-RFLP and 16S rDNA library sequencing was performed as described above. The T-RFLP analysis displayed a consistent increase *of Proteobacteria* and *Actinobacteria* during acidosis and a decrease in *Bacteroidetes* populations during the first few hours of induction. The number of different bacterial genera detected increased 2 hrs post induction in animals induced with acidosis. T-RFLP analysis identified 116 different bacterial genera among animals on high concentrate (control) and acidosis. We also performed quantitative real-time PCR analysis for some known and characterized bacteria to assess their individual fluctuations during subacute acidosis. qRT-PCR analysis displayed a 3-5 fold increase in *Megaspaera elsdenii* population during acidosis, and remained 4-fold higher 48 hrs later. *Streptococcus bovis* population increased 25-fold during acidosis. Forty eight hours post induction *S. bovis* population decreased to 15 fold. *Bifidobacterium ruminantium* displayed a similar trend *to Megaspaera elsdenii*, where the population increased 2-5 fold during acidosis. The *Bifidobacterium ruminantium* population increased 2-fold in the control animal 48 hrs post induction and displayed a similar population size to the animals in acidosis. *Selenomonas ruminantium and Mitsuokella jalaludinii* displayed similar population changes, where the population decreased 3-4 fold and 25-35 fold respectively, but recovered 48 hrs post induction. *Lactobacillus acidophilus* population decreased 2-3 fold in both control animals and animals in acidosis, but, recovered and increased by 3-5 fold 48 hrs post induction in the induced animals.

TAKE HOME MESSAGES

Rumen is a complex ecosystem consisting of bacteria, fungi, protozoa and viruses. The host animal and the rumen microbiota enjoy a unique relationship where the microbes play a role in nutrient utilization, feed efficiency, animal well-being and food safety. Although rumen microbes have been studied for a long time, our knowledge of their diversity and function is still in its infancy. Part of the reason for this lack of understanding is the inability to culture analyze a vast number of organisms that live within the rumen. The advent of metagenomics is rapidly changing our understanding of the rumen. Techniques like 16S rDNA based phylogeny, T-RFLP analyses and high-throughput Pyrosequencing is allowing insights into the rumen that was not available before. We have used some of those techniques to study how the rumen microbiota responds to some classic nutritional challenges like adaptation to a high concentrate diet or contracting subacute ruminal acidosis.

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