META-FUNCTIONAL GENOMICS OF THE BOVINE RUMEN

By

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 2008

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Dedication

I dedicate this thesis to my parents Leslie and Ajantha Fernando. Thank you for all your love and support.

Acknowledgements

The time I spent at Oklahoma State University has been a learning experience for me as a person and as a professional. I consider it a privilege to acknowledge many people who have encouraged, inspired, and guided me throughout my stay at the Oklahoma State University.

First, I would like to extend my sincere thanks to my adviser Dr. Udaya DeSilva for giving me this opportunity to further my education and to follow my dreams. Dr. Desilva you have been an inspiration to me and have always been there to guide and encourage me on all my endevours. You have taught me not only science, but also valueable lessons in life. Thank you for all the advice, guidance and encouragement. I would also like to thank my thesis committee members, Dr. Robert Burnap, Dr. Clint Krehbiel, and Dr. Hebbie Purvis for all the guidance, advice, and motivation given to me.

I am thankful to Dr. Bruce Roe and his lab for helping with the 454-pyro sequencing and 16S rDNA sequencing. I would also like to extend a special thanks to Xiaoping Guo for all her help, friendship and advice. Also I would like to thank Asitha Vasudevan pillai and Saumya Wickramasinghe for all their help, friendship and given to me during my day-to-day activities in the lab.

I also wish to thank the Faculty and Staff of the Department of Animal Science for making my learning experience more memorable.

Finally, I wish to thank the people who are most responsible for my achievements, my parents, Ajantha and Leslie Fernando for providing me with the basis from which to succeed, I extend my sincere thanks and love to you both. I also thank my brothers Sudham and Dhanushka for all the help and support given to me. Thanks guys.

Now, I thank the most wonderful person in my life, my wife Anju. Thank you for the love, support and encouragement; you have been a tower of strength to me. Thank you.

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INTRODUCTION

Introduction

The ruminant animal

The ruminant animal has played a pivotal role in our history and has been a valuable source of food for our early ancestors (Russell, 2002). Ruminants have been the major food source for the early hunter-gathers and have possibly been hunted for as long as 750,000 years (Church, 1993). In the past many “normadic American Indian tribes” have been dependent on elk, deer, bison, and other ruminants for food, clothing, weapons, shelter and other needs. Even, today the Masai tribes of Africa are dependent on their cattle for meat, blood, and milk (Church, 1993).

The domestication of these magnificent creatures have been estimated to have occurred at the time when our early ancestors started cultivating crops (Church, 1993). According to archeological findings this time frame falls 8,500-11,000 years ago, where sheep were the first to be domesticated about 11,000 years ago, followed by goats about 9,000 years ago, and cattle 8,500 year ago (Church, 1993).

In today’s world the ruminant animal plays an important role, and has a significant impact in the US food industry. Currently, according to the USDA cattle inventory as of January 1st 2008, the US cattle industry has 96.7 million head of cattle (see Table 1) (National Agricultural Statistics Service et al., 2008) and 6.06 head of sheep and goats (Economic Research Service, 2008).

The Rumen

Since the first observation of the four chambered stomach of ruminants by Aristotle (Russell, 2002), and the identification of the microbial role within the rumen by

Tappeiner in 1882 (Thaysen, 1946), the rumen has been investigated for its role in nutrient digestion and to manipulate its microbial ecosystem to increase animal performance and efficiency.

Class	2008 *1000 Head*
Cattle and Calves	*96,669*
Cows and Heifers that have calved	*41,777*
Beef cows	*32,553*
Milk cows	*9,224*
Heifers 500 pounds and over	*20,003*
For beef cow replacement[2]	*5,670*
Expected to calve	*3,420*
For milk cow replacement[2]	*4,457*
Expected to calve	*2,928*
Other Heifers	*9,876*
Steers 500 pounds and over	*17,305*
Bulls 500 pounds and over	*2,207*
Calves under 500 pounds	*15,378*
Cattle on feed	*14,317*
Calf crop	*37,361*

[1] Totals may not add due to rounding
[2] Replacement heifers expected to calve during this year

Table 1. Cattle and Calves: Number by Class and Calf Crop., United States January 1st, 2007-2008[1] (Reproduced from USDA Cattle inventory report released on February 1st 2008 by the National Agricultural Statistics Service (NASS), Agricultural Statistics Board, and U.S. Department of Agriculture)

The rumen which holds around 40-60 gallons of digesta is considered as an anaerobic fermentation chamber that is full of microbes (Church, 1993). The synergistic relationship between the host animal and the rumen microbial flora, provides the microbes with nutrients, ambient temperatures and a buffered

environment to enhance microbial growth (Russell, 2002), while the microbes provide the host animal with nutrients, volatile fatty acids (VFAs), and microbial cell proteins (MCPs) (Church, 1993; Russell, 2002) which help increase animal performance. Therefore, the microbial population that reside within the rumen plays an important role in nutrient digestion and fermentation within the host animal (Chalupa, 1980; Church, 1993; Edwards et al., 2004; Fernando et al., 2008a; Mrazek et al., 2006; Owens et al., 1998; Russell, 1987; Skillman et al., 2006; Tajima et al., 2001)). Using traditional culture dependent techniques and direct microscopic observations it is estimated that the rumen microbial population is composed of bacteria, fungi, protozoa, archaea, and viruses (Church, 1993; Hobson, 1988; Lee et al., 2000), where the bacterial population within the rumen predominates. These microbial populations provide the animal with energy and protein and helps the animal to survive on low nutrient diets (Church, 1993; Hobson, 1988). However, these microbial populations are not stable within the rumen and fluctuates with changes in the ruminal environment and diet (Biavati and Mattarelli, 1991; Counotte et al., 1981; Fernando et al., 2008d; Fernando et al., 2008c; Goad et al., 1998; Kotarski et al., 1992; Tajima et al., 1999; Tajima et al., 2000). Thus, the microbial populations that reside within the rumen are populations that have adapted to the ruminal environment (Church, 1993) which results in changes in volatile fatty acid composition and concentration within the rumen (Goad et al., 1998; Kotarski et al., 1992; Russell and Baldwin, 1978; Russell and Hino, 1985). Therefore, currently there is a great interest to investigate the rumen microbial population changes in

different ruminal environments and diets to identify the role of rumen microbes in nutrient digestion.

Within the rumen microbial ecosystem, the predominant microbial population is the bacterial population, which is estimated to be between 10^9-10^{11} cells per gram of ruminal contents based on direct microscopic counts (Church, 1993; Hungate, 1966; Lee et al., 2000). Among this large bacterial population a majority of the bacterial species are obligate anaerobes or facultative anaerobes (Church, 1993). As mentioned earlier, the rumen bacterial population is not static and fluctuates according to changes in the diet and ruminal environment (Biavati and Mattarelli, 1991; Counotte et al., 1981; Fernando et al., 2008d; Fernando et al., 2008c; Goad et al., 1998; Kotarski et al., 1992; Tajima et al., 1999; Tajima et al., 2000). Thus, the predominant rumen bacterial population depends on the diet and ruminal environment. Therefore, early studies of rumen bacterial populations have characterized bacteria based on substrate utilization patterns (Church, 1993).

Development of the rumen bacterial population

At birth the new born baby calf is naive and contains no microbial species within its rumen. But, as the animal develops, the rumen starts being colonized with bacterial populations from its environment, which mainly includes, dam's saliva and vagina, cud, manure, bedding and environmental flora, the udder, milk and from contact with other animals (Church, 1993). Among these sources of microbes, the animal to animal contact that occurs during grooming and feeding is the primary source of colonization. It is estimated that the majority of the colonization of the rumen

microbial flora occurs over the first 9-13 weeks and by the 13th week the rumen microbial population is comparatively similar to the adult animal.

Rumen bacteria

The rumen microbial ecosystem is a complex environment predominated by bacteria. But our understanding of this complex rumen microbial ecosystem is in its infancy, as we have only identified and characterized a few bacteria species from the rumen. These bacterial populations isolated include; cellulolytic bacteria, hemicellulose and pectinolytic bacteria, amylolytic bacteria, simple-sugar utilizing bacteria, Intermediate acid utilizing bacteria, proteolytic bacteria, ammonium producing bacteria, lipolytic bacteria, and methane-producing bacteria (Church, 1993). Among these bacterial populations, several bacterial populations have been isolated in pure culture and have been studied extensively. In the following section, a few such species is described.

Cellulolytic and hemicellulolytic bacteria

The cellulolytic bacteria identified within the rumen, plays an important role in cellulose digestion, and have been reported in high roughage diets (Church, 1993). These cellulolytic bacteria, include *Bacteroides succinogenes, Ruminococcus flavefaciens, Ruminococcus albus* and *Butyrivibrio fibriosolvens* and secrete the cellulase enzyme to breakdown the 1-4 beta glycosidisc bonds in cellulose (Bera-Maillet et al., 2004; Church, 1993; Tarakanov, V and Lavlinskii, 1998; Tarakanov and Lavlinskii, 1998; Weimer, 1996; Weimer et al., 1999; Yu and Hungate, 1979). The

hemicellulolytic bacteria identified within the rumen include *Butyrivibrio fibriosolvens, Prevotella ruminicola,* and *Ruminococcus sp.* These bacterial species detected are also efficient cellulose digesters and therefore predominate in high fiber diets together with the cellulose digesters (Church, 1993). The pectinolytic bacteria detected within the rumen include *Butyrivibrio fibriosolvens, Prevotella ruminicola, Lachnospira multiparus, Succinovibrio dextrinosolvens, treponema sp.* and *Streptococcus bovis*. These bacterial species secrete extracellular and intracellular pectinases that help pectin digestion (Dehority, 1969; Wojciechowicz et al., 1982; Wojciechowicz and Ziolecki, 1984).

Amlylolytic bacteria

The starch digesting amylolytic bacteria found within the rumen predominate in high starch diets. This increase of amylolytic bacteria have been detected when animals are adapted to high energy diets (Aurangzeb et al., 1992; Fernando et al., 2008c; Martin et al., 1999; Tajima et al., 2000). The amylolytic bacteria isolated from the rumen include *Bacteroides amylophilus, Bacteroides succinogenes, Succinovibrio dextrinosolvens, Streptococcus bovis, Succinimonas amylolytica and Prevotella ruminicola*, where they secrete α-amylase to breakdown long chain carbohydrates randomly along the carbohydrate chain (Aurangzeb et al., 1992; Church, 1993; Martin et al., 1999). Many of the microbial species involved in starch digestion also play an important role in utilization of simple sugars present within the rumen. In addition to the amylolytic bacteria, *Ruminococcus flavefaciens* secretes cellobiose

phosphorylase to degrade cellobiose, while *Lactobacillus sp.* within the rumen are known as efficient sugar fermentors within the rumen (Church, 1993).

Intermediate acid-utilizing bacteria

As the rumen is a complex microbial ecosystem, there is a large number of synergistic relationships within the rumen. One such relationship that occurs among bacterial species is the secondary metabolite utilization, where the by product or the end product of one bacterial fermentation process acts as a substrate for the fermentation by other organisms within the rumen. These types of organisms present within the rumen are characterized as intermediate acid-utilizing bacteria as these organisms utilize volatile fatty acid such as formate, lactate and succinate (Church, 1993). Bacterial species such as *Megasphaera elsdenii* and *Selenomonas ruminantium*, play an important role in intermediate acid-utilization and in controlling ruminal pH where they ferment lactate to produce acetate, propionate or larger chain fatty acids (Counotte et al., 1981; Russell, 2002; Russell and Dombrowski, 1980; Stewart et al., 1997). In addition to lactate utilizing bacteria such as *Megasphaera elsdenii* and *Selenomonas ruminantium; Veillonella alcalescens, Anaerovibrio lipolytica* and *Propionibacterium sp.* have been shown to utilize succinate to produce CO_2 and propionate (Church, 1993). The formate produced within the rumen is further utilized by *Methanobrevibacter ruminantium* as a precursor for methane production (Church, 1993; Russell, 2002).

Proteolytic bacteria

A majority of the bacteria found in the rumen are proteolytic in nature, where the proteolytic activity has been detected in 38% of the isolates identified from the rumen. This suggests that the proteolytic activity within the rumen is common to a majority of the bacteria (Church, 1993). Therefore, the predominant proteolytic bacterial species present within a given ruminal environment depends on the protein source available. *Bacteroides amylophilus, Prevotella ruminicola, Butyrivibrio fibrosolvens* and *Streptococcus bovis* are among the most widely studied bacterial species from the rumen (Nagaraja and Titgemeyer, 2007; Owens et al., 1998) and have increased proteolytic activity where they synthesize endo- and exo-peptidases (Brock et al., 1982; Church, 1993; Hazlewood and Edwards, 1981). Due to the high proteolytic activity within the rumen, the rumen microbes utilize low quality protein sources present in the animal's diet, and provide the host animal with higher quality microbial cell protein (Church, 1993). Therefore, the rumen microbial population meets the animal's protein requirement.

Ammonia-producing bacteria

Many bacteria within the rumen prefer ammonia as the nitrogen source than amino acids or peptides (Church, 1993). Therefore, the ammonia producing bacterial species such as *Bacteroides ruminicola, Megasphaera elsdenii, Selenomonas ruminantium* and *Butyrivibrio sp.* play a critical role within the rumen to provide the required ammonia for the growth of rumen microbial species, especially the bacteria that digest complex carbohydrates (Chen and Russell, 1989; Church, 1993; Rychlik

et al., 2002). These bacteria that synthesizes branched chain volatile fatty acids provide essential nutrients for other rumen bacteria (Church, 1993). Based on the diet, the ammonium requirement of rumen bacteria can also be met by the ureolytic bacteria that hydrolyses urea to produce ammonia (Church, 1993; Wozny et al., 1977). These ureolytic bacteria include, *Succinivibrio dextrinosolvens, Bacteroidetes ruminicola, Butyrivibrio sp., Treponema sp.,* and *Bifidobacterium sp.* (Church, 1993; Wozny et al., 1977).

Lipolytic bacteria

Within the rumen triglycerides and phospholipids are metabolized by lipolytic bacteria *Anaerovibrio lipolytica* to free fatty acids and glycerol (Church, 1993; Russell, 2002). The sulfolipids, galactolipids and phospholipids which are present in forages have been shown to be utilized by *Butyrivibrio sp.* (Church, 1993). As the rumen is anaerobic, no β-oxidation occurs within the rumen. However within the rumen, unsaturated long chain fatty acids are hydrogenated by the rumen bacteria (Church, 1993; Liolios et al., 2008). This hydrogenation of unsaturated fatty acids by the rumen bacteria helps maintain the body fat composition of the animal and also to have “high concentrations of unusual fatty acids in their milk” (Church, 1993). In addition to *Anaerovibrio lipolytica*, other bacterial species such as *Treponema bryantii, Fusocillus sp., Eubacterium sp.,* and *Micrococcus sp.* are capable of hydrogenation of unsaturated fatty acids (Church, 1993).

Methane-producing bacteria

Methane producing bacteria play an important role within the rumen and helps regulate the hydrogen gas concentration (Church, 1993). Within the rumen, carbohydrate fermentation by amylolytic and proteolytic bacteria produces glycolytic substrates and $NADH_2$. This $NADH_2$ produced needs to be converted back to NAD to maintain ruminal fermentation and to continue glycolysis. The conversion of $NADH_2$ back to NAD occurs through the enzyme NAD hydrogenase (Church, 1993; Russell, 2002). NAD hydrogenase is sensitive to the hydrogen ion concentration within the rumen and requires low hydrogen ion concentrations to be active. The methonogenic bacteria present within the rumen helps maintain this low hydrogen ion concentration by combining the H_2 released by NAD hydrogenase with CO_2 to produce CH_4 (Church, 1993; Russell, 2002). By doing so, the methonogenic bacteria helps glycolysis to continue within the rumen. This interspecies H_2 transfer is facilitated by methenogenic bacteria such as *Methanobrevibacter ruminantium, Methanomicrobium mobile,* and *Methanobacterium formicicum* (Church, 1993).

Why study the rumen?

The microbial population within the rumen plays an important role in nutrient digestion, where the microbes provide the animal with >60% of its protein requirement (Church, 1993). The synergistic association between the animal and the microbial flora provides the animal with nutrients that are not available to the monogastric animal (Church, 1993). Therefore, the interaction between the complex microbial population and the animal is an important relationship which directly

impacts the efficiency and performance of the animal. Hence, understanding this relationship between the rumen microbial community and the host animal with respect to microbial diversity and the functional role of bacteria within the rumen will provide an opportunity to make effective management decisions to enhance microbial and animal production.

Based on direct microscopic observations it is estimated that 99% of the bacteria found in any given environment is resistant to invitro culture (Pace, 1997). This observation is also common to the ruminal environment where, based on direct microscopic counts it is estimated that 10^9-10^{11} (Grubb and Dehority, 1975; Maki and Foster, 1957) bacterial cells are present per gram of ruminal contents, but we have only identified a handful of the species using culture dependent techniques from what we observe under a microscope. Thus, traditional culture dependent techniques have only helped us identify and evaluate the functional role of a few microbes that reside within the rumen (Goad et al., 1998; Hungate et al., 1952; Hungate, 1968). Traditional bacterial isolation techniques have been laborious and time consuming (Bryant and Robinson, 1961; Ferguson et al., 1984). Furthermore, these traditional techniques have been bias towards specific groups of organisms (Bryant and Robinson, 1961; Ferguson et al., 1984). Recently, researchers have begun to understand the importance of microbial community analysis, and have initiated many studies to investigate microbial diversity in different environments using T-RFLP (Terminal Restriction Fragment Length Polymorphism) analysis, 16S rDNA libraries and metagenomic approaches (Abulencia et al., 2006; Fernando et al., 2008d; Fernando et al., 2008c; Tyson et al., 2004; Venter et al., 2004; Woyke et

al., 2006). This meta-genome and 16S sequencing projects (Fernando et al., 2008a; Fernando et al., 2008c; Fernando et al., 2008d) of environmental samples have provided valuable information into the phylogenetic diversity of microbial species and their capabilities in different environments (Schloss and Handelsman, 2005; Tringe et al., 2005; Tyson et al., 2004; Venter et al., 2004). 16S rRNA based molecular approaches have also been used within the rumen (Edwards et al., 2004; Tajima et al., 1999; Tajima et al., 2000) which have provided a glimpse into the large phylogenetic diversity present within the rumen. This suggests that there is far greater diversity than anticipated and that there is an imperative need to identify and characterize the rumen microbiome.

Therefore, in an effort to better understand rumen microbial population diversity and function, we undertook a meta-functional genomics approach to analyze the rumen biome. As the first part of my dissertation, to study the changes in rumen microbial population structure and diversity, we evaluated microbial population changes in three different ruminal environments and have surveyed the microbial population diversity and abundance throughout the gastro intestinal tract in cattle. The three ruminal environments include;

1) During the adaptation of an animal to a high concentrate diet from a forage-based diet, which is widely used in feedlots.

2) During ionophore treatment, an important management practice used in cattle

3) During development of ruminal acidosis, an important metabolic disorder in cattle

Within the rumen, a majority (grater than 75%) of the bacteria are attached or embedded to feed particles and have hindered previous attempts to identify rumen

microbes. Therefore, one of the challenges in evaluating rumen microbial populations is to identify the microbes that are embedded within feed particles which may be playing an important role in nutrient digestion. During this dissertation work we established new protocols to successfully isolate intact DNA from total rumen microbial populations to identify microbial populations that are free living as well as attached to feed particles. The real time fluctuations of microbial population diversity and structure within each ruminal environment was evaluated using the extracted DNA by means of Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis, sequence analysis of 16S rDNA libraries and by real-time PCR analysis. Real-time microbial population fluctuations detected through T-RFLP analysis and sequence analysis using16S rDNA libraries revealed great diversity within the rumen microbial population, where we detected over 500 different bacterial species belonging to 112 different bacterial genera. Before our investigation of the rumen, it was believed that the rumen bacterial population diversity was ~250 bacterial species. But, this investigation adds over 250 previously unreported bacterial species to the rumen bacterial species list, more than doubling the amount of known bacteria within the rumen.

We also propose a new method of real time PCR analysis of microbial population fluctuations which gives a better estimate of microbial population changes than the traditional absolute quantification method. Thus, using molecular techniques, we have added a large amount of new information to the current understanding of the rumen, and also provide new analysis methods to study the rumen microbial population.

As the second part of my dissertation, to identify the functional role of the rumen microbial population, we performed a large-scale, community level transcriptome, analysis of the rumen microbes using the 454 pyro-sequencing strategy combined with several bioinfomatic approaches. In this study we have established new protocols to extract good quality RNA from environmental samples, and is the first to use community transcriptomics to study the functional role of complex microbial environments. Messenger RNA (mRNA) was isolated from the total RNA extracted and was sequenced using the 454 Pyro-sequencing strategies to identify the functional role of microbes within the rumen in samples collected from prairie hay and high concentrate animals. 60,000 reads were generated from the two populations and were analyzed using bioinformatic approaches to identify the functional role of the bacterial populations. This analysis has helped better understand the functional role of bacteria within communities and to identify potential microbial species involved in nutrient digestion. This approach has also helped to determine the rate of nutrient digestion within the rumen. Therefore, using community transcriptomics we propose a new method to study microbial populations that allows identification of function as well as identity of bacterial species within an environment.

The 16S rDNA analysis and the community transcriptomics approach identified many uncharacterized bacteria suggesting that there is a large amount of unknown bacterial species within the rumen. Therefore, as the final part of my thesis, I have attempted to culture new bacterial species from the rumen. Using rumen fluid as the culture media and a new 16S rDNA based screening strategy; we have isolated 38

new bacterial species that have not been present in pure culture. We have further confirmed 13 of these bacterial species using biochemical tests via API50 kits. This dissertation research project has provided great advances in the field of rumen microbial population diversity function and structure, and provides new methodologies to study microbial communities using a transcriptomics approach. Also, this study provides a new strategy for screening for uncultured bacteria which may enable more efficient screening for new microbes.

Reference List

Hobson,P.N., (1988) *The rumen microbial ecosystem*. New York: Elsevier Applied Science.

Abulencia,C.B., Wyborski,D.L., Garcia,J.A., Podar,M., Chen,W.Q., Chang,S.H. et al. (2006) Environmental whole-genome amplification to access microbial populations in contaminated sediments. *Applied and Environmental Microbiology* **72:** 3291-3301.

Aurangzeb,M., Qadeer,M., and Iqbal,J. (1992) Production of raw starch hydrolyzing amylolytic enzymes by Streptococcus bovis PCSIR-7B. *Pakistan Journal of Scientific and Industrial Research* **35:** 520-523.

Bera-Maillet,C., Ribot,Y., and Forano,E. (2004) Fiber-degrading systems of different strains of the genus Fibrobacter. *Applied and Environmental Microbiology* **70:** 2172-2179.

Biavati,B. and Mattarelli,P. (1991) Bifidobacterium-Ruminantium Sp-Nov and Bifidobacterium-Merycicum Sp-Nov from the Rumens of Cattle. *International Journal of Systematic Bacteriology* **41:** 163-168.

Brock,F.M., Forsberg,C.W., and Buchanansmith,J.G. (1982) Proteolytic Activity of Rumen Microorganisms and Effects of Proteinase-Inhibitors. *Applied and Environmental Microbiology* **44:** 561-569.

Bryant,M.P. and Robinson,I.M. (1961) An Improved Nonselective Culture Medium for Ruminal Bacteria and Its Use in Determining Diurnal Variation in Numbers of Bacteria in Rumen. *Journal of Dairy Science* **44:** 1446-&.

Chalupa,W. (1980) Chemical control of rumen microbial metabolism. In *Digestive Physiology and Metabolism in Ruminants*. Ruckebush,Y. and Thivend,P. (eds). Westport, CT: AVI Publishing Co., Inc..

Chen,G.J. and Russell,J.B. (1989) More Monensin-Sensitive, Ammonia-Producing Bacteria from the Rumen. *Applied and Environmental Microbiology* **55:** 1052-1057.

Church,D.C. (1993) *Ruminant Animal: Digestive Physiology and Nutrition*. New Jersey: Prentice Hall.

Counotte,G.H.M., Prins,R.A., Janssen,R.H.A.M., and Debie,M.J.A. (1981) Role of Megasphaera-Elsdenii in the Fermentation of Lactate-2-C-13 in the Rumen of Dairy-Cattle. *Applied and Environmental Microbiology* **42:** 649-655.

Dehority,B.A. (1969) Pectin-Fermenting Bacteria Isolated from Bovine Rumen. *Journal of Bacteriology* **99:** 189-&.

Economic Research Service . Livestock, Dairy, and Poultry Outlook. Harley, M. M. 2008. www.ers.usda.gov.
Ref Type: Report

Edwards,J.E., Mcewan,N.R., Travis,A.J., and Wallace,R.J. (2004) 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **86:** 263-281.

Ferguson,R.L., Buckley,E.N., and Palumbo,A.V. (1984) Response of Marine Bacterioplankton to Differential Filtration and Confinement. *Applied and Environmental Microbiology* **47:** 49-55.

Goad,D.W., Goad,C.L., and Nagaraja,T.G. (1998) Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. *Journal of Animal Science* **76:** 234-241.

Grubb,J.A. and Dehority,B.A. (1975) Effects of An Abrupt Change in Ration from All Roughage to High Concentrate Upon Rumen Microbial Numbers in Sheep. *Applied Microbiology* **30:** 404-412.

Hazlewood,G.P. and Edwards,R. (1981) Proteolytic Activities of A Rumen Bacterium, Bacteroides-Ruminicola R8-4. *Journal of General Microbiology* **125:** 11-15.

Hungate,R.E. (1966) *The rumen and its microbes*. New York: Academic press.

Hungate,R.E. (1968) Ruminal fermentation. In *Handbook of Physiology*. Code,C.F. (ed). Washington, D.C.: American Physiology Society, pp. 2725-2745.

Hungate,R.E., Dougherty,R.W., Bryant,M.P., and Cello,R.M. (1952) Microbiological and Physiological Changes Associated with Acute Indigestion in Sheep. *Cornell Veterinarian* **42:** 423-449.

Kotarski,S.F., Waniska,R.D., and Thurn,K.K. (1992) Starch Hydrolysis by the Ruminal Microflora. *Journal of Nutrition* **122:** 178-190.

Lee,S.S., Ha,J.K., and Cheng,K.J. (2000) Relative contributions of bacteria, protozoa, and fungi to in vitro degradation of orchard grass cell walls and their interactions. *Applied and Environmental Microbiology* **66:** 3807-3813.

Liolios,K., Mavromatis,K., Tavernarakis,N., and Kyrpides,N.C. (2008) The Genomes On Line Database (GOLD) in 2007: status of genomic and metagenomic projects and their associated metadata. *Nucleic Acids Research* **36:** D475-D479.

Maki,L.R. and Foster,E.M. (1957) Effect of Roughage in the Bovine Ration on Types of Bacteria in the Rumen. *Journal of Dairy Science* **40:** 905-913.

Martin,C., Devillard,E., and Michalet-Doreau,B. (1999) Influence of sampling site on concentrations and carbohydrate-degrading enzyme activities of protozoa and bacteria in the rumen. *Journal of Animal Science* **77:** 979-987.

Mrazek,J., Tepsic,K., Avgustin,G., and Kopecny,J. (2006) Diet-dependent shifts in ruminal butyrate-producing bacteria. *Folia Microbiologica* **51:** 294-298.

Nagaraja,T.G. and Titgemeyer,E.C. (2007) Ruminal acidosis in beef cattle: The current microbiological and nutritional outlook. *Journal of Dairy Science* **90:** E17-E38.

National Agricultural Statistics Service, Agricultural Statistics Board, and U.S.Department of Agriculture . Cattle inventry. 2008.
Ref Type: Report

Owens,F.N., Secrist,D.S., Hill,W.J., and Gill,D.R. (1998) Acidosis in cattle: A review. *Journal of Animal Science* **76:** 275-286.

Pace,N.R. (1997) A molecular view of microbial diversity and the biosphere. *Science* **276:** 734-740.

Russell,J.B. (1987) A Proposed Mechanism of Monensin Action in Inhibiting Ruminal Bacterial-Growth - Effects on Ion Flux and Protonmotive Force. *Journal of Animal Science* **64:** 1519-1525.

Russell,J.B. (2002) *Rumen microbiology and its role in ruminant nutrition*. Ithaca, NY: James B. Russell.

Russell,J.B. and Baldwin,R.L. (1978) Substrate Preferences in Rumen Bacteria - Evidence of Catabolite Regulatory Mechanisms. *Applied and Environmental Microbiology* **36:** 319-329.

Russell,J.B. and Dombrowski,D.B. (1980) Effect of Ph on the Efficiency of Growth by Pure Cultures of Rumen Bacteria in Continuous Culture. *Applied and Environmental Microbiology* **39:** 604-610.

Russell,J.B. and Hino,T. (1985) Regulation of Lactate Production in Streptococcus-Bovis - A Spiraling Effect That Contributes to Rumen Acidosis. *Journal of Dairy Science* **68:** 1712-1721.

Rychlik,J.L., LaVera,R., and Russell,J.B. (2002) Amino acid deamination by ruminal Megasphaera elsdenii strains. *Current Microbiology* **45:** 340-345.

Schloss,P.D. and Handelsman,J. (2005) Metagenomics for studying unculturable microorganisms: cutting the Gordian knot. *Genome Biology* **6**.

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

Stewart,C.S., Flynt,H.J., and Bryant,M.P. (1997) The Rumen Bacteria. In *The Rumen Microbial Ecosystem*. Hobson,P.N. and Stewart,C.S. (eds). Blackie Academic and Professional, NY, pp. 10-72.

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

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

Tajima,K., Arai,S., Ogata,K., Nagamine,T., Matsui,H., Nakamura,M. et al. (2000) Rumen bacterial community transition during adaptation to high-grain diet. *Anaerobe* **6:** 273-284.

Tarakanov,B., V and Lavlinskii,D. (1998) Cellulolytic bacteria of the genus Ruminococcus from bovine rumen. *Mikrobiologiya* **67:** 518-521.

Tarakanov,B.V. and Lavlinskii,D.Y. (1998) Cellulolytic bacteria of the genus Ruminococcus from bovine rumen. *Microbiology* **67:** 425-428.

Thaysen,A. (1946) The nutritional role of the microflora in the alimentary tract. The microbiological aspect of rumen digestion. *Proc Nutr Soc* **3:** 195-199.

Tringe,S.G., von Mering,C., Kobayashi,A., Salamov,A.A., Chen,K., Chang,H.W. et al. (2005) Comparative metagenomics of microbial communities. *Science* **308:** 554-557.

Tyson,G.W., Chapman,J., Hugenholtz,P., Allen,E.E., Ram,R.J., Richardson,P.M. et al. (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428:** 37-43.

Venter,J.C., Remington,K., Heidelberg,J.F., Halpern,A.L., Rusch,D., Eisen,J.A. et al. (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304:** 66-74.

Weimer,P.J. (1996) Why don't ruminal bacteria digest cellulose faster? *Journal of Dairy Science* **79:** 1496-1502.

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

Wojciechowicz,M., Heinrichova,K., and Ziolecki,A. (1982) An Exopectate Lyase of Butyrivibrio-Fibrisolvens from the Bovine Rumen. *Journal of General Microbiology* **128:** 2661-2665.

Wojciechowicz,M. and Ziolecki,A. (1984) A Note on the Pectinolytic Enzyme of Streptococcus-Bovis. *Journal of Applied Bacteriology* **56:** 515-518.

Woyke,T., Teeling,H., Ivanova,N.N., Huntemann,M., Richter,M., Gloeckner,F.O. et al. (2006) Symbiosis insights through metagenomic analysis of a microbial consortium. *Nature* **443:** 950-955.

Wozny,M.A., Bryant,M.P., Holdeman,L.V., and Moore,W.E.C. (1977) Urease Assay and Urease-Producing Species of Anaerobes in Bovine Rumen and Human Feces. *Applied and Environmental Microbiology* **33:** 1097-1104.

Yu,I. and Hungate,R.E. (1979) Extracellular Cellulases of Ruminococcus-Albus. *Annales de Recherches Veterinaires* **10:** 251-254.

CHAPTER II

RUMEN MICROBIAL POPULATION DYNAMICS DURING

SUB ACUTE ACIDOSIS

Introduction

Acidosis is a digestive disorder commonly found in feedlot and dairy cattle (Kleen et al., 2003; Osborne et al., 2004). It is characterized by episodes of ruminal pH fluctuations from the normal 6.5 - 7.5 to an acidic pH of 5.2 - 5.6 (subacute acidosis) and in extreme cases to values below pH 5 (acute acidosis) (Osborne et al., 2004; Owens et al., 1998). Acidosis is usually caused as a result of diets that are high in fermentable carbohydrates or forage that is low in effective fiber, or both (Nocek, 1997; Osborne et al., 2004). The decrease in the ruminal pH is one of the first indications of the onset of acidosis, where the ruminal environment is not adapted to ferment, tolerate or absorb the increasing short chain fatty acids synthesized within the rumen (Kleen et al., 2003). This change in the ruminal pH initiates several systemic changes within the rumen, such as increased organic acids (especially lactic acid), decreased ruminal mortality, stasis, ruminitis and hyperkeratosis (Nocek, 1997).

Nutritionists, researchers and veterinarians classify acidosis into two major categories. The first, acute acidosis, where ruminal pH is seen to fall below 5, is associated with significantly impaired physiological functions, an increase in volatile fatty acids and a significant decrease in the total number of rumen bacteria and protozoa (Nagel and Broderick, 1992; Nocek, 1997). The second milder form, subacute acidosis is somewhat harder to detect (Kleen et al., 2003). It is often misdiagnosed as a problem due to poor forage quality or bunk management (Nocek, 1997). Subacute ruminal acidosis (SARA) accounts for a tremendous

economic loss in both dairy and feedlot cattle industries by decreasing the potential productive efficiency and performance of animals.

Potentially, SARA can occur at any time, but it is most common during transition from a forage diet to a high concentrate diet where the ruminal environment is not adapted to tolerate or absorb the increasing short chain fatty acids (SCFA) synthesized within the rumen to keep the ruminal pH within physiological limits (Kleen et al., 2003). The microbial population which metabolizes the abundant carbohydrates and fermentable sugars to produce VFAs play a critical role in causing as well as controlling SARA (Goad et al., 1998). A study carried out by Goad and coworkers in 1998 evaluated ruminal microbial and fermentative changes associated with induced SARA. They reported a significant increase in total counts of viable anaerobic and amylolytic bacteria in grain adopted animals than in hay-adapted animals suggesting a change in rumen microbial population during acidosis. An increase in the number of lactate utilizing bacteria have also been observed in grain adapted sheep and cattle (Mackie and Heath, 1979; Olumeyan et al., 1986). These observations of changes in microbial population structure suggest that the rumen microbial population plays a crucial role in controlling SARA in the animal. Previous research conducted in the rumen during acidosis has evaluated pH, Volatile fatty acids (VFA), ammonia, passage rate, rumen volume and mass, and nutrient digestibility. However, microbial population dynamics in terms of total population numbers, diversity and relative contribution of each species in the rumen are rarely measured and therefore not much is known about the detailed alterations and systemic manifestations of ruminal bacteria during SARA (Brown et al., 2000).

Hence, evaluating the microbial fluctuations that occur during SARA may provide new insight into the role of microbes in controlling or causing SARA, help identify new microbial species for early detection or control of acidosis and provide a clearer explanation of the observed changes in cattle with SARA.

As a first step towards understanding the microbial fluctuations during acidosis, we have evaluated microbial population structure and diversity using Terminal restriction Fragment length Polymorphisms (T-RFLP). We have also constructed several 16S rDNA libraries and have compared microbial populations in animals with sub acute acidosis to animals on high concentrate diet. Finally, we have evaluated microbial population changes in previously reported starch digesters using quantitative realtime PCR (qRT-PCR) to evaluate the role of these organisms during acidosis.

Experimental Procedures

Animals and Diets

Four ruminally cannulated beef steers (837$\pm$ 60 lbs) were fed prairie hay *ad libitum*, to have relatively similar background rumen bacterial population. Following the adaptation phase on prairie hay, the animals were put on a step-up diet containing increasing amounts of metabolic energy. The step up diets were formulated to meet the animals requirements as described in NRC-1996, but was composed with 2.0, 2.4, 2.7 or 3.0 Mcal of ME/kg of dry matter with a fiber to concentrate ratio of 80:20, 60:40, 40:60 and 20:80 respectively. The animals were fed each diet for 7 days and were gradually changed over to the next diet in a “step-up” fashion.

After adaptation to high concentrate diet, the animals were utilized to evaluate the microbial population changes that occur during transition from high concentrate diet to sub acute ruminal acidosis and during recovery from acidosis. Out of the four animals, 2 animals (steer 72 and 63) were randomly selected and were maintained on a diet formulated with 3.0 Mcal of ME/kg of dry matter with a fiber to concentrate ratio of 20:80 (control animals). The other two animals (steer 62 and 65) were ruminally dosed with 1.2 g/Kg body weight of ground corn to experimentally induce sub-acute ruminal acidosis. After dosing all 4 animals were fed with the regular diet of 3.0 Mcal of ME/kg of dry matter with a fiber to concentrate ratio of 20:80.

Sampling

Rumen contents were collected from all 4 steers before dosing ("0" time point). Upon ruminally dosing two animals with corn, rumen samples were collected at 30 min intervals for the first six hours, at one hour intervals for the next 6 hours and at two hour intervals for the next 12 hours. A final sample was collected at 48 hrs later. At each time point, the rumen pH was recorded and rumen samples were collected and snap frozen in liquid nitrogen for microbial community analysis. During sampling steer 73 developed fever, became anorectic and was administered antibiotics. Therefore, steer 73 was removed from the analysis.

DNA isolation

Frozen rumen samples were homogenized using a coffee grinder, and 0.5 g of the homogenized sample was used to extract DNA. Total bacterial DNA was extracted

using the QIAamp® DNA mini stool kit (Qiagen, Valencia, CA, USA) according to manufacturers protocol with a few modifications. Briefly, 1.4 ml of buffer ASL was added to 0.5g of rumen sample and was vortexed for 15 sec. Following vortexing the sample was incubated at 95°C for 10 min. The sample was then vortexed again for 15 sec and was centrifuged at 20,000g for 1 min to pellet the cell debris and other suspended particles present in the sample. The supernatant was aspirated to a fresh 2 ml tube and an inhibitorEX tablet was added and vortexed immediately until the tablet was completely dissolved (~1 min). The sample was then incubated at room temperature for 1 min to bind all inhibitors, and was centrifuged at 20,000g for 3 min to remove the inhibitorEX bound inhibitors. The solution was aspirated and re-centrifuged for an additional 3 min to remove any residual inhibitor. To 400 µl of the supernatant, 20 µl of proteinase K and 400 µl of AL buffer was added and incubated at 70°C for 30 min. 400 µl of 100% ethanol was added to the proteinase digested sample. This sample was then added to the QIAamp spin column and centrifuged at 20,000g for 1 min to bind the DNA. The column was washed with 500 µl of buffer AW1 and AW2 respectively. The spin columns were centrifuged at 20,000g to remove residual buffer AW2 and the DNA was eluted using 20 µl of distilled water. The concentration of the extracted DNA was measured using a UV spectrophotometer, and the quality of the DNA was evaluated on a 1.2% agarose gel.

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis

100 ng of total bacterial genomic DNA isolated from the rumen samples were used for Terminal-Rescticton Fragment Length Polymorphisum (T-RFLP) analysis (Kent et al., 2003; Marsh et al., 2000; Osborn et al., 2000). 100 ng of total bacterial DNA was PCR amplified in a MJ dyad thermocycler (MJ research, Watertown, MA) using FAMBacT0008F 5'-AGAGTTTGATCCTGGCTCAG-3' and BacT0805R 5'-GGACTACCAGGGTATCTAATCCC-3' primers to amplify a 809 bp fragment (in *E. coli*) of the 16S rRNA gene in all Eubacteria. The FAMBacT008F primer is labeled at the 5' end with the fluorophore 6-FAM. A 50 µl PCR reaction contained, 100 ng of DNA, 1X PCR buffer (Promega, Madison, WI, USA) 1.5 mM $MgCl_2$, 400 nM of each primer, 200 µM dNTPs, 100 ng/µl BSA and 3 U Taq DNA Polymerase (Promega, Madison, WI, USA). Cycling conditions were: one cycle of 1 min at 95ºC, 30 sec at 52ºC, 1 min at 72ºC followed by 34 additional cycles of 30 sec at 95ºC, 30 sec at 52ºC, 1 min at 72ºC and a final extension of 3 min at 72ºC.

The amplified PCR products were ethanol precipitated (Sambrook and Russel, 2001) and resuspended in 5 µl of distilled water. The precipitated PCR product was digested separately with *Rsa I (*Invitrogen, Carlsbad, CA,USA), *Hae III (*Invitrogen, Carlsbad, CA,USA) and *Msp I* (Promega, Madison, WI, USA) according to the manufacturers protocols. The restriction enzymes used in T-RFLP analysis were identified using software tools available at the Microbial community analysis web site (MiCA3) (http://mica.ibest.uidaho.edu/default.php). A 10 µl reaction contained 1X buffer, 2.5 U Enzyme and 5 µl of the PCR product. 250 ng/µl of BSA was added to the MSP I digestion. The Digestion reaction was incubated at 37°C for 4 hours,

followed by 65°C for 20 min. 2 µl of the digested PCR product was mixed with 0.5 µl of GeneScan ROX 1000 size standard (Applied Biosystems, Foster City, CA) and 3.5 µl of loading dye (Applied Biosystems, Foster City, CA) and was electrophoresed for 8 hours in an ABI 377 sequence analyzer (Applied Biosystems, Foster City, CA). The data collected by the ABI 377 sequence analyzer was analyzed using GeneScan 3.1 analysis software (Applied Biosystems, Foster City, CA). The data set was subsequently normalized by dividing the cumulative peak height of each sample by the sample with the smallest cumulative peak height. The normalized T-RFLP profiles were further analyzed using Phylogenetic Assignment Tool (PAT) (Kent et al., 2003a) to assign species for each restriction fragment generated. The bacterial species assignment was based on the restriction digestion patterns obtained for all 3 enzymes of each sample.

The normalized peak area was used to construct Population fluctuation and microbial diversity plots. The normalized peak areas for the 3 different digests were averaged and were used as an estimate of bacterial population. The representation of each bacterial species within the total bacterial population was calculated as the percentage of peak area of a bacterial species relative to the total peak area of the sample. Therefore, the Population fluctuation and microbial diversity plots show the bacterial diversity and the relative abundance of each bacterial species identified within the population.

Construction and sequence analysis of 16S rRNA libraries

100 ng of total DNA from the initial stages of acidosis (samples collected during the first 1.5 hrs) and during the low pH stationary phase of acidosis (samples collected between the 3rd hour to the 5th hour), from steers 63 (control) and 65 (Induced) was used for 16S library construction. Total rumen DNA from the control and induced animals were used to PCR amplify an 809 bp fragment (in *E.coli*) using BacT0008F 5'-AGAGTTTGATCCTGGCTCAG-3' and BacT0805R 5'-GGACTACCAGGGTATCTAATCCC-3' primers using a high fidelity taq polymerase. The 50 µl PCR reaction contained, 100 ng of DNA, 1X PCR buffer (Invitrogen, Carlsbad, CA, USA) 1.5 mM $MgCl_2$, 400 nM of each primer, 200 µM dNTPs, and 2 U Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). Cycling conditions were: one cycle of 2 min at 95ºC, 30 sec at 52ºC, 1 min at 68ºC followed by 34 additional cycles of 20 sec at 95ºC, 30 sec at 52ºC, 1 min at 68ºC and a final extension of 3 min at 68ºC. The blunt ended PCR products generated were ligated in to PCR-Blunt® (Invitrogen, Carlsbad, CA) plasmid vector and transformed to DH5α max efficiency competent *E.coli* cells (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol to construct four different 16S libraries. The transformed cells were grown overnight at 37°C on 22X22 cm Nunc® bioassay plates (Nalge Nunc International, Rochester, NY, USA) containing 2X YT with 100 µg/ml of kanamycin. Random libraries of approximately 1920 colonies were picked from each library, grown in Terrific Broth (TB) medium (Sambrook and Russel, 2001) supplemented with 100 micrograms of kanamycin for 14 hours at 37°C with shaking

at 250 rpm, and the sequencing templates were isolated by a cleared lysate-based protocol (Bodenteich et al., 1993; Roe, 2004).

Sequencing reactions were performed as previously described (Chissoe et al., 1995) using Taq DNA polymerase, and either the Amersham ET fluorescent-labeled terminator or the Perkin-Elmer Cetus fluorescent-labeled Big Dye Taq terminator sequencing kits at a 1:16 dilution. The reactions were incubated for 60 cycles in a Perkin-Elmer Cetus DNA Thermocycler 9600 and after removal of unincorporated dye terminators by ethanol precipitation followed by a 70% ethanol wash, the fluorescent-labeled nested fragment sets were resolved by electrophoresis on an ABI 3700 Capillary DNA Sequencers. After base calling with Phred (Ewing et al., 1998; Ewing and Green, 1998), the analyzed data was transferred to a Sun Workstation Cluster, and assembled using Phrap (Ewing and Green, 1998) Overlapping sequences (contigs) were analyzed using Consed (Gordon et al., 1998). Each clone was sequenced from both directions to have two fold coverage of each base. The resulting data was cleaned of vector sequences, using the software "Crossmatch" and was analyzed using analysis tools available at the Ribosomal Database Project (http://rdp.cme.msu.edu). The assembled data was blasted against the 16S RDP database (Cole et al., 2005; Cole et al., 2003) and the NCBI database to identify the bacterial populations present.

Phylogenetic and Comparative Analyses

The data was aligned using the RDP pipline available at *myRDP* space (Cole et al., 2007). The alignment generated was imported to the ARB software package

(Ludwig et al., 2004) to generate phylogenetic trees with hierarchical classification of the sequences. Phylogenetic trees were constructed using the neighbor-joining method. Furthermore, “Classifier” (Wang et al., 2007) and “Library Compare” (Cole et al., 2005) software tools available at ribosomal database project (http://rdp.cme.msu.edu) were used to further analyze the sequences and to classify the clones to taxanomic hierarchy. The “Library Compare” software tool was used to compare the libraries with each other and to identify statistically, significant changes in bacterial populations present among the libraries (Cole et al., 2003; Cole et al., 2005).

Quantitative Real-Time PCR analysis

Quantitative analysis of population changes of selected bacterial species were conducted using real-time polymerase chain reaction (qPCR) analysis. Samples collected a) before induction of acidosis, b) during acidosis (pH<5.2), and c) 48 hrs after acidosis was used for realtime PCR analysis. The population changes were evaluated using SYBR green I reporter assay kit available from Roche Diagnosticis (Indianapolis, IN, USA). The primers used for analyzing *Prevotella ruminicola, Prevotella bryantii, Prevotella albensis, Megaspaera elsdenii and Selenomonas ruminantium* bacterial populations were, primers previously reported by Tajima et. al (2001), Ouwerkerk et. al (2002) and Ozutsumi et. al (2006). Primers for *Streptococcus bovis (*AB002482*), Bifidobacterium ruminantium (*D86197*), Butyrivibrio fibrosolvens (*AM039822*), Mitsuokella jalaludinii (*AF479674*), Lactobacillus acidophilus (*X61138), and *Propionibacterium acnes (*AY642054) were

developed using PRIMROSE 16S primer and probe design software (Ashelford et al., 2002) using sequences available at the RDP database and National Center for Biotechnology Information (NCBI) (Cole et al., 2003; Cole et al., 2005). For all assays the PCR products generated were sequenced to confirm the amplification of the correct bacterial species.

A 15 µl reaction contained 400 nM forward primer, 400 nM reverse primer, 1X Master mix (Roche Diagnosticis, Indianapolis, IN, USA), and 30 ng of total rumen DNA. Thermal cycling conditions were 95°C for 10 min, followed by 50 additional cycles of 95°C for 30 sec 50-62°C for 30 sec, depending on the annealing temperature of each primer set (see Table 1) and 72°C for 40 sec or 1 min based on the size of the amplicon followed by the melting curve. The single copy universal gene *rpoB* was used for normalization (Dahllof et al., 2000; Santos and Ochman, 2004). As the rpoB primers will amplify the gene from all eubacteria, the length of the product will be a few bases different in certain bacterial species, therefore we observed a single broadened peak in the melting curve. The qRT-PCR reactions were performed in an ABI PRISM 7500® sequence detection system (Applied Biosystems, Foster City, CA).

Relative quantification of bacterial population changes was performed using the comparative C_T method as previously described (Hettinger et al., 2001). The ΔC_T value was determined by subtracting the 16S C_T value of each sample from the corresponding sample *rpoB* gene C_T value. Calculation of $\Delta\Delta C_T$ was carried out using the zero time point (before induction of sub-acute ruminal acidosis) mean ΔC_T value as an arbitrary constant to subtract from all other ΔC_T mean values. Fold

changes in bacterial populations were calculated from the ΔΔCT values using the formula $2^{\pm\Delta\Delta Ct}$.

Results

pH measurement

The decrease in ruminal pH, one of the first indications of the onset of acidosis, (Kleen et al., 2003; Nocek, 1997) was used as a measurement to detect the induction of sub-acute ruminal acidosis. Ruminal pH was measured at regular intervals in animals experimentally induced with sub-acute ruminal acidosis as well as in control animals maintained on 3.0 Mcal of ME/kg of dry matter. Figure 1 depicts the ruminal pH fluctuation in control and experimentally induced animals over a period of 24 hrs. During the course of the study, Steer 73 developed fever, was anorectic and had to be administered antibiotics. Intake of steer 73 was uneven and pH fluctuations were irregular. Therefore, steer 73 was removed from the analysis. Ruminal pH reached an acidotic pH of 5.2> within a period of 1.5 hrs post dosing in animals infused with corn, whereas the control animals maintained a ruminal pH of 6.0-7.0 during the 24 hr collection period. The animals induced with sub-acute ruminal acidosis started increasing ruminal pH to normal physiological pH levels 20 hrs post induction (Figure 1).

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis

Population fluctuation and microbial diversity plots generated based on T-RFLP analysis is shown in Figure 2. Each identified bacterial genus is represented by a

unique color where width of each colored band represents the abundance of that genus within the population. The number of genera identified within each sample is shown in parentheses. T-RFLP analysis shows significant changes in the microbial population structure and diversity between the control and induced animals within the first 5 hrs of induction. This change in microbial population structure and diversity is apparent in the different colored banding pattern and the increasing number of bacterial genera detected during acidosis (Figure 2). Animals with sub-acute acidosis display increased microbial population diversity, as more genera can be identified by 2 hrs post induction of acidosis. The bacterial diversity in the control animals tend to stay constant with a slight increase in diversity by the 3rd hour which tends to decrease by the 5th hour. T-RFLP analysis identified 116 different bacterial genera among animals on high concentrate and acidosis. The increasing bacterial populations during sub-acute ruminal acidosis included, *Tissierella sp., Thermobacillus sp., Thermobiospora sp., Telluria sp., Stenotrophomonas sp., spirilum sp., Rubrivivax sp., Rikenellasp., Rhodococcus sp., Moorella sp., Micrococcus sp., Methylobacillus, Halobacteriodes sp., Herbaspirilum sp.,Glaciecola sp., Francisella sp., Frankia sp., Fibrobacter sp., Clavibacter sp., Acidosphaera sp., Actinobacillus sp., Amycolatopsis sp., Anaerofilum sp., Anaeroplasma sp., Aneurinibacillus sp., Prevotella sp., and Actinobacillus sp..* T-RFLP analysis also revealed a decrease in several bacterial populations, which included *Buchnera sp., Capnocytophaga sp., Chrysiogenes sp., Clostridium sp., Colwellia sp., Flectobacillus sp., Fusibacter sp., Fusobacterium sp., Kingella sp., Leptospira sp., Leuconostoc sp., Melittangium sp., Methylosinus sp., Microscilla sp., Myxococcus sp.,*

Nitrosomonas sp., Paracoccus sp., Pedobacter sp., Persicobacter sp., Psychroserpens sp., Rhizobium sp., Rhodospirillum sp., Ruminococcus sp., Sphingobacterium sp., Spirochaeta sp., Streptobacillus sp., Thauera sp., and Treponema sp. We further analyzed the microbial populations using tools available at the ribosomal database project. Classification of organisms at phylum level is shown in figure 3. This Classification displayed an increase in *Proteobacteria* and *Actinobacteria* populations and a decrease in *Bacteroidetes* populations during acidosis.

16S rRNA library and Phylogenetic analysis

We constructed four 16S rDNA libraries from control and induced animals. Samples collected during the initial pH drop (First 1.5 hrs post induction) and during the low pH stationary phase (4.5 - 6.5 hrs post induction) was used to construct the libraries (Figure 1). 384 clones from each library was sequenced analyzed. Resulting sequences were compared using "Library Compare" (Cole et al., 2005) to identify microbial population changes during acidosis. The results are presented as differences at phylum level. Library 1 (control) and Library 3 (induced) constructed from samples collected during the initial stages of induction detected very significant changes in rumen bacterial population diversity and structure within the first 1.5 hrs of induction (Table 2a & 3 and figure 4a), where the control animals displayed a significantly larger number of bacterial species belonging to *Bacteroidetes, Spirochaetes*, and *Firmicutes* when compared to animals in acidosis. However, the *Proteobacterial* population was significantly higher in animals with sub acute ruminal

acidosis (Table 2a and figure 4a). Similarly, Library 2 (control) and Library 4 (induced) constructed from samples collected during the Low pH stationary phase of acidosis detected a similar change in microbial population diversity and structure (Table 2b and figure 4b), where the control animal on high concentrate displayed significantly larger populations of *Bacteroidetes* and *firmicutes*. Libraries constructed from the animal in acidosis displayed decreased numbers in bacteria belonging to *Firmicutes* and *Bacteroidetes*, but displayed a significant increase in bacteria belonging to *Proteobacteria* and *Actinobacteria* (Table 2b and figure 4b). Comparison of libraries 3 and 4 constructed from the animal in acidosis displayed a significant increase in *Actinobacteria* with time (Table 2c and figure 4c), suggesting that during low ruminal pH the *Actinobacterial* populations increase. Comparison of Library 1 and 2, from the control animals did not display any significant change in bacterial population.

The taxonomic hierarchy of the clones sequenced is shown in table 3 and contains over 20% of unclassified bacteria. The hierarchical classification identified 362 (Lib 1), 326 (Lib 2), 343 (Lib 3) and 329 (Lib 4) 16S rRNA clones belonging to domain *Bacteria*. Among the 326 16S rRNA clones identified in Lib 1 constructed from samples collected from control animals during initial stages of induction, 150 clones belonged to phylum *Firmicutes*, 17 to *Spirochaetes,* and 171 phylum *Bacteroidetes*. But only 2 clones belonging to *Proteobacteria* and 1 clone belonging to *Actinobacteria* was detected. Similarly, Lib 2 constructed from samples collected from control animals during the low pH stationary phase contained 122 clones belonging to *Firmicutes,* 7 belonging to *Spirochaetes,* and 134 belonging to phylum

Bacteroidetes. But, only 4 clones belonging to *Proteobacteria* was detected. No clones from phylum Actenobacteria was present in Lib 2. The 16S rRNA clones sequenced from Lib 3 & 4 constructed during initial and low pH stationary phases of acidosis displayed significantly different composition of bacteria when compared to control animals on high concentrate diet, where 95 and 85 clones belonging to *Firmicutes*, 115 and 93 clones belonging to *Bacteroidetes*, 82 and 76 clones belonging to *Proteobacteria,* and 7 and 24 clones belonging to *Actinobacteria* were detected in Lib 3 & 4 respectively. No clones belonging to *Spirochaetes* was detected in Library 3 & 4.

Phylogenetic analysis of the 16S rRNA libraries sequenced was performed using ARB software environment (Ludwig et al., 2004). The sequences were characterized using the Bayesian classifier (Wang et al., 2007) which assigned the sequences into bacteria taxanomy. A non-redundant set of Operational Taxanomic Units (OTUs) were identified from each of the four libraries and was used to construct phylogenetic trees for each stage of acidosis. Phylogenetic trees were constructed using the neighbor joining method (Figures 5-8). Phylogenetic analysis demonstrated great diversity in all four libraries, but displayed a similar organization in libraries 1 & 2 constructed from control animals. Similarly, Lib 3 & 4 constructed from animals in acidosis also displayed similar organization, but contained more *Actinobacterial sp*. at later stages of acidosis. Libraries 1 & 2 constructed from control animals on high concentrate diet contained 137 and 100 different OTUs, and libraries 3 & 4 constructed from animals in acidosis displayed 113 and 70 OTUs respectively. A significantly large number of phylogenetically distant *Proteobacteria*

population was present in animals induced with acidosis. These OTUs accounted for more than 23% of the total sequences. The increasing *Proteobacteria* populations also displayed a higher redundancy than other bacterial species. An increasing *Actinobacterial* population was also detected during acidosis.

Quantitative Real-Time PCR analysis

Quantitative realtime PCR analysis was performed to validate T-RFLP analysis and to evaluate the fluctuations in some known starch digesters. Primers were developed to amplify specific regions of the 16S ribosomal subunit. Quantitative realtime PCR (qRT-PCR) results are summarized in Figure 9. qRT-PCR analysis displayed a 3-5 fold increase in *Megaspaera elsdenii* population during acidosis, and maintained at 4-fold during the recovery phase of acidosis. *Streptococcus bovis* population increased 25-fold in steer 65 but did not change significantly in steer 62 and the control animal during acidosis. During the recovery phase of acidosis *S. bovis* population decreased by 15-fold in steer 65, but was constantly low in steer 62 and control animal. *Bifidobacterium ruminantium* displayed a similar trend to *Megaspaera,* where the population increased 2-5 fold during acidosis and was 3-fold grated when compared before induction. The *Bifidobacterium ruminantium* population increased 2-fold in the control animal during the recovery phase and displayed a similar population size to the animals in acidosis. *Butyrivibrio fibrosolvens* population increased 3-fold in steer 65, but did not change in steer 62 and control animal during acidosis, but decreased during the recovery phase of acidosis in steers 62 and 65. The *Butyrivibrio fibrosolvens* population increased in

the control animal during the recovery phase. The *Propionibacterium acnes* populations remained constant in animals induced with acidosis but decreased 4 & 8 fold in the control animal during acidosis and recovery phase of acidosis. *Prevotella ruminicola, Prevotella bryantii,* and *Prevotella albensis,* populations decreased 140-fold, 1600-fold and 40-fold in steer 62 during acidosis, but did not change in steer 65 and the control animal. The decreased *Prevotella* populations recovered rapidly during the recovery phase of acidosis and displayed populations similar to the control animal and steer 65. *Selenomonas ruminantium* and *Mitsuokella jalaludinii* displayed a similar change in bacterial populations, where the population decreased 3-4 fold and 25-35 fold respectively, but recovered during the recovery phase of acidosis. *Lactobacillus acidophilus* population decrease 2-3 fold in both control animals and animals in acidosis, but, recovered and increased by 3-5 fold during the recovery phase. The *Lactobacillus acidophilus* population in the control animal remained decreased during the recovery phase.

Figure 1

Ruminal pH fluctuations during induction of sub acute ruminal acidosis. The ruminal pH was used as a measurement to detect ruminal acidosis. Steer 73 has irregular pH fluctuations and was running a fever. Steer 73 was subsequently removed from the analysis.

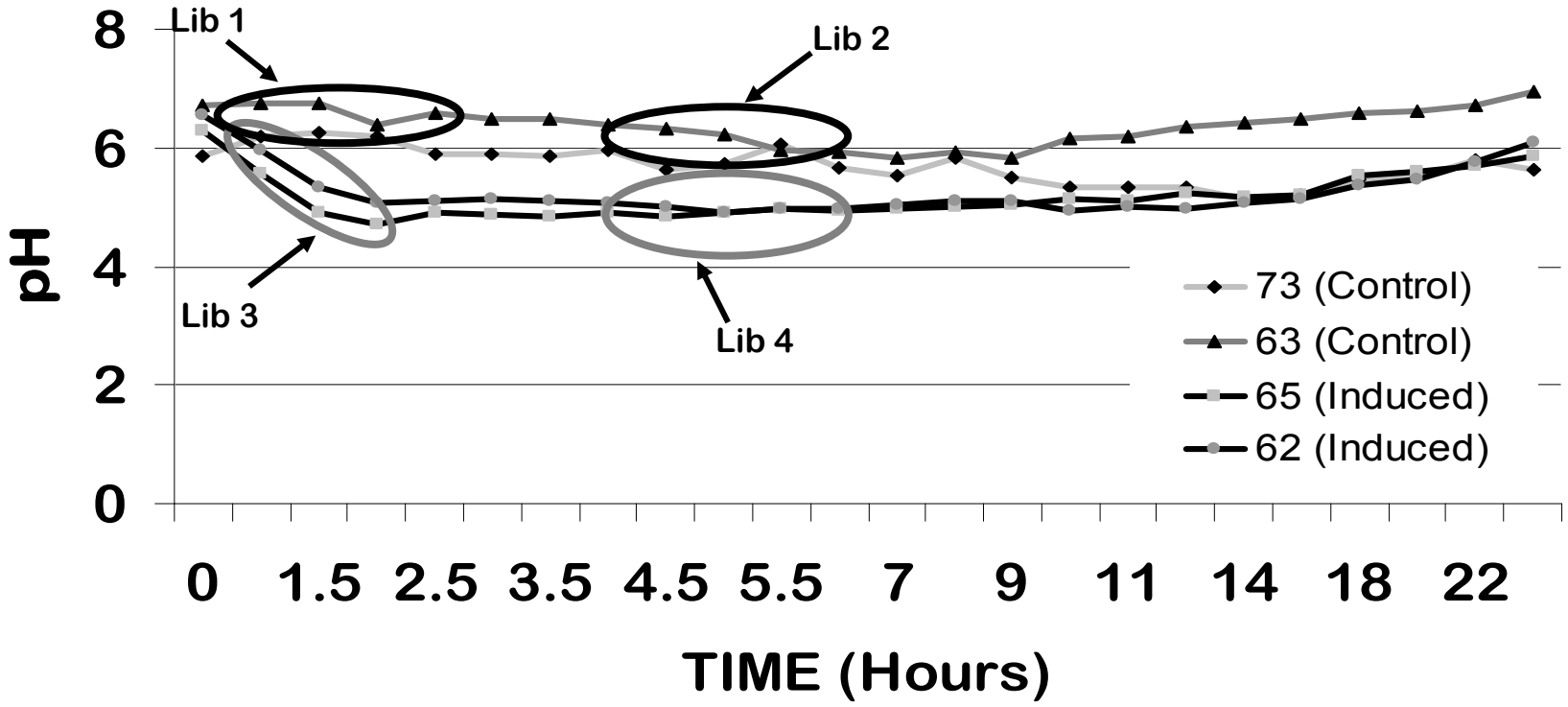
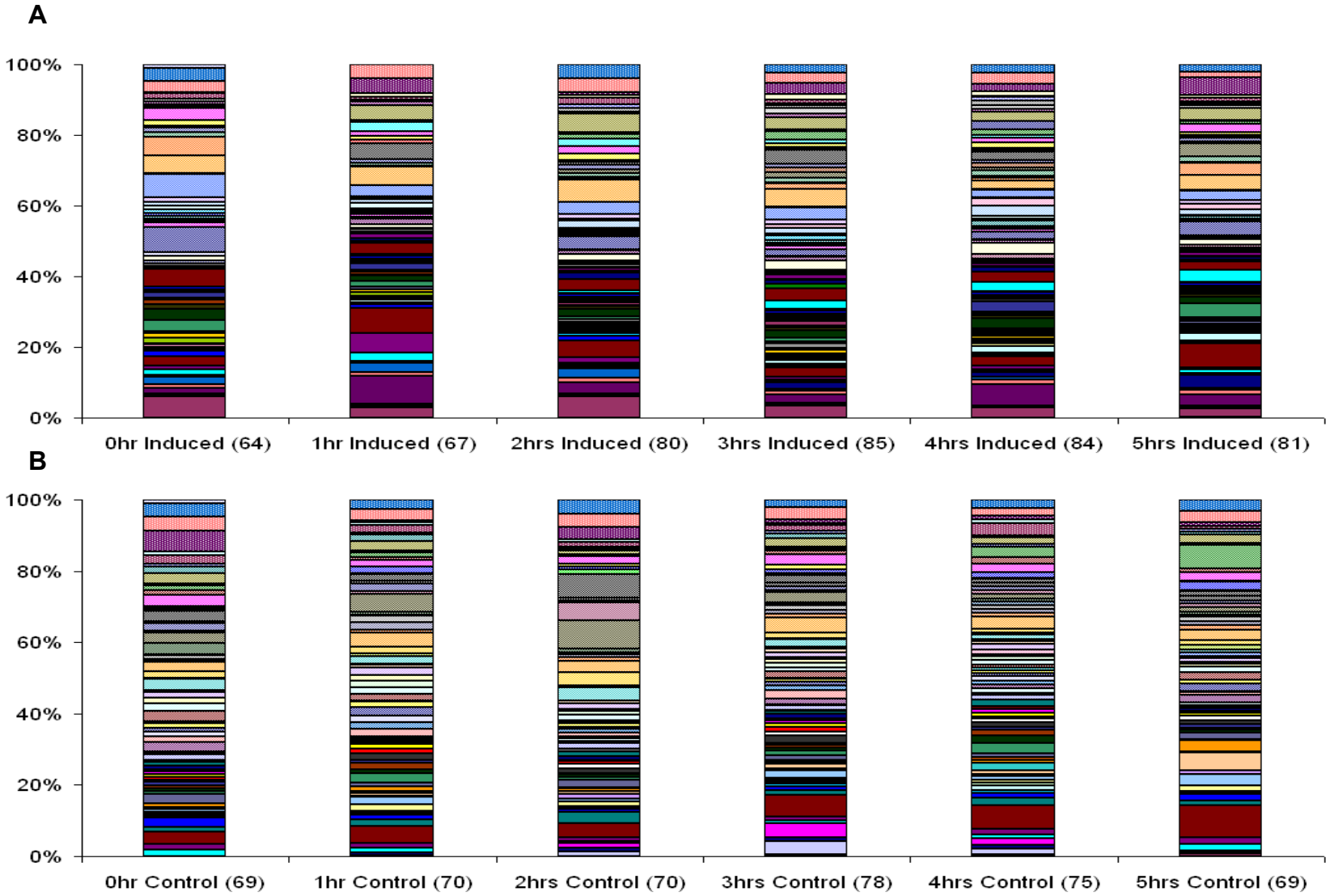

Lib 1
Lib 2
Lib 3
Lib 4
pH
8
6
4
2
0
0
1.5
2.5
3.5
4.5
5.5
7
9
11
14
18
22
TIME (Hours)
73 (Control)
63 (Control)
65 (Induced)
62 (Induced)

Figure 2

Population fluctuation and microbial diversity plots of animals induced with sub acute ruminal acidosis and control animals on high concentrate diet. The Population fluctuation and microbial diversity plots were constructed based on T-RFLP analyses. The different color banding pattern shows significant changes in microbial population diversity. The numbers within parentheses represent the number of different genera identified in each sample. (A) animals induced with sub acute ruminal acidosis, (B) control animals on high concentrate diet.


A
100%
80%
60%
40%
20%
0%
0hr Induced (64)
1hr Induced (67)
2hrs Induced (80)
3hrs Induced (85)
4hrs Induced (84)
5hrs Induced (81)
B
100%
80%
60%
40%
20%
0%
0hr Control (69)
1hr Control (70)
2hrs Control (70)
3hrs Control (78)
4hrs Control (75)
5hrs Control (69)


Acidosphaera sp.
Actinobacillus sp.
Amycolatopsis sp.
Anaerobranca sp.
Anaerofilum sp.
Anaeroplasma sp.
Aneurinibacillus sp.
Angiococcus sp.
Aranicola sp.
Archangium sp.
Arthrobactersp.
Azoarcus sp.
Bacillus sp.
Bacteroides sp.
Bifidobacterium sp.
Bordetella sp.
Brevibacillus sp.
Brevibacterium sp.
Buchnera sp.
Burkholderia sp.
Butyrivibrio sp.
Caldicellulosiruptor sp.
Capnocytophaga sp.
Chlorobium sp.
Chrysiogenes sp.
Clavibacter sp.
Clostridium sp.
Clostridium sp.
Colwellia sp.
Cytophaga sp.
Dechlorisoma sp.
Dermatophilus sp.
Desulfobacterium sp.
Desulfotomaculum sp.
Dictyoglomus sp.
Entomoplasma sp.
Erysipelothrix sp.
Eubacterium sp.
Exiguobacterium sp.
Fibrobacter sp.
Flectobacillus sp.
Flexibacter sp.
Francisella sp.
Frankia sp.
Fusibacter sp.
Fusobacterium sp.
Glaciecola sp.
Haemophilus sp.
Haliscomenobacter sp.
Halobacillus sp.
Halobacteroides sp.
Herbaspirillum sp.
Kingella sp.
Kitasatospora sp.
Kurthia sp.
Lactobacillus sp.
Lactococcus sp.
Lentzea sp.
Leptospira sp.
Leptothrix sp.
Leuconostoc sp.
Melittangium sp.
Mesoplasma sp.
Methylobacillus sp.
Methylophilus sp.
Methylosinus sp.
Microbacterium sp.
Micrococcus sp.
Microscilla sp.
Moorella sp.
Moraxella sp.
Mycobacterium sp.
Mycoplasma sp.
Myxococcus sp.
Nitrosomonas sp.
Nocardia sp.
Oerskovia sp.
Paenibacillus sp.
Paracoccus sp.
Pasteurella sp.
Pedobacter sp.
Peptostreptococcus sp.
Persicobacter sp.
Prevotella sp.
Pseudomonas sp.
Pseudoramibacter sp.
Psychroserpens sp.
Rhizobium sp.
Rhodococcus sp.
Rhodospirillum sp.
Rikenella sp.
Rubrivivax sp.
Ruminococcus sp.
Selenomonas sp.
Shewanella sp.
Sphingobacterium sp.
Spirillum sp.
Spirochaeta sp.
Spiroplasma sp.
Sporosarcina sp.
Staphylococcus sp.
Stenotrophomonas sp.
Streptobacillus sp.
Streptococcus sp.
Streptomyces sp.
Taylorella sp.
Telluria sp.
Thauera sp.
Thermobacillus sp.
Thermobispora sp.
Thermodesulfovibrio sp.
Thermomonospora sp.
Tissierella sp.
Treponema sp.
Vibrio sp.
Xanthomonas sp.
Xylella sp.

Figure 3

Summarized, hierarchical classification of Terminal Restriction Fragment length Polymorphism (T-RFLP) data. The data displayed is classified based on phylum. The predominant phyla are depicted. The remaining phyla are pooled and displayed as others.

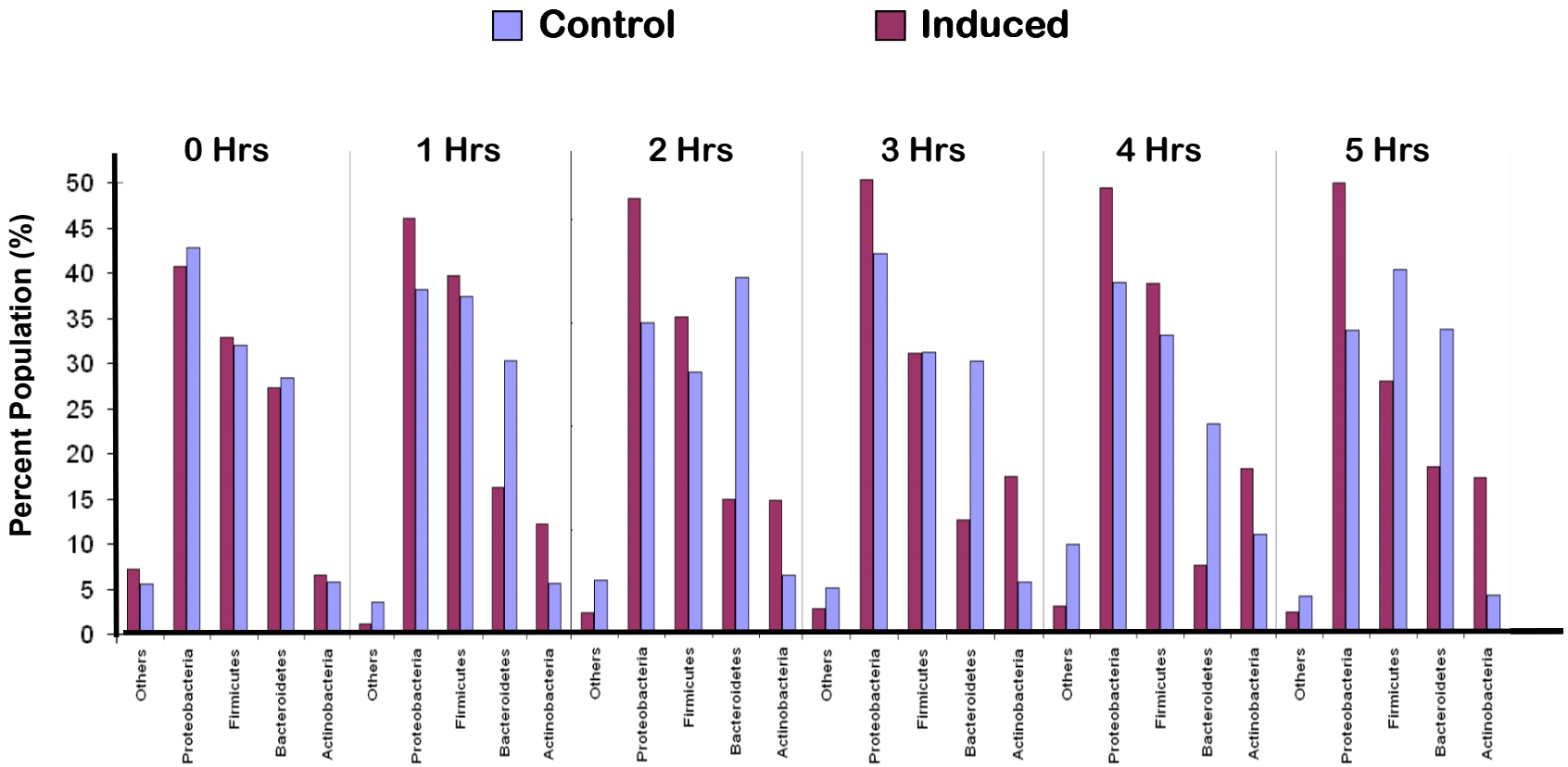

Control
Induced
0 Hrs
1 Hrs
2 Hrs
3 Hrs
4 Hrs
5 Hrs
Percent Population (%)
0
5
10
15
20
25
30
35
40
45
50
Others
Proteobacteria
Firmicutes
Bacteroidetes
Actinobacteria

Figure 4

Comparison of Libraries at the phylum level using “Library Compare” software available at the ribosomal database project. Populations that are significantly different at p> 0.01 are shown with an asterix “*”.

(A) Library 1 Vs Library 3

(B) Library 2 Vs Library 4

(C) Library 3 Vs Library 4

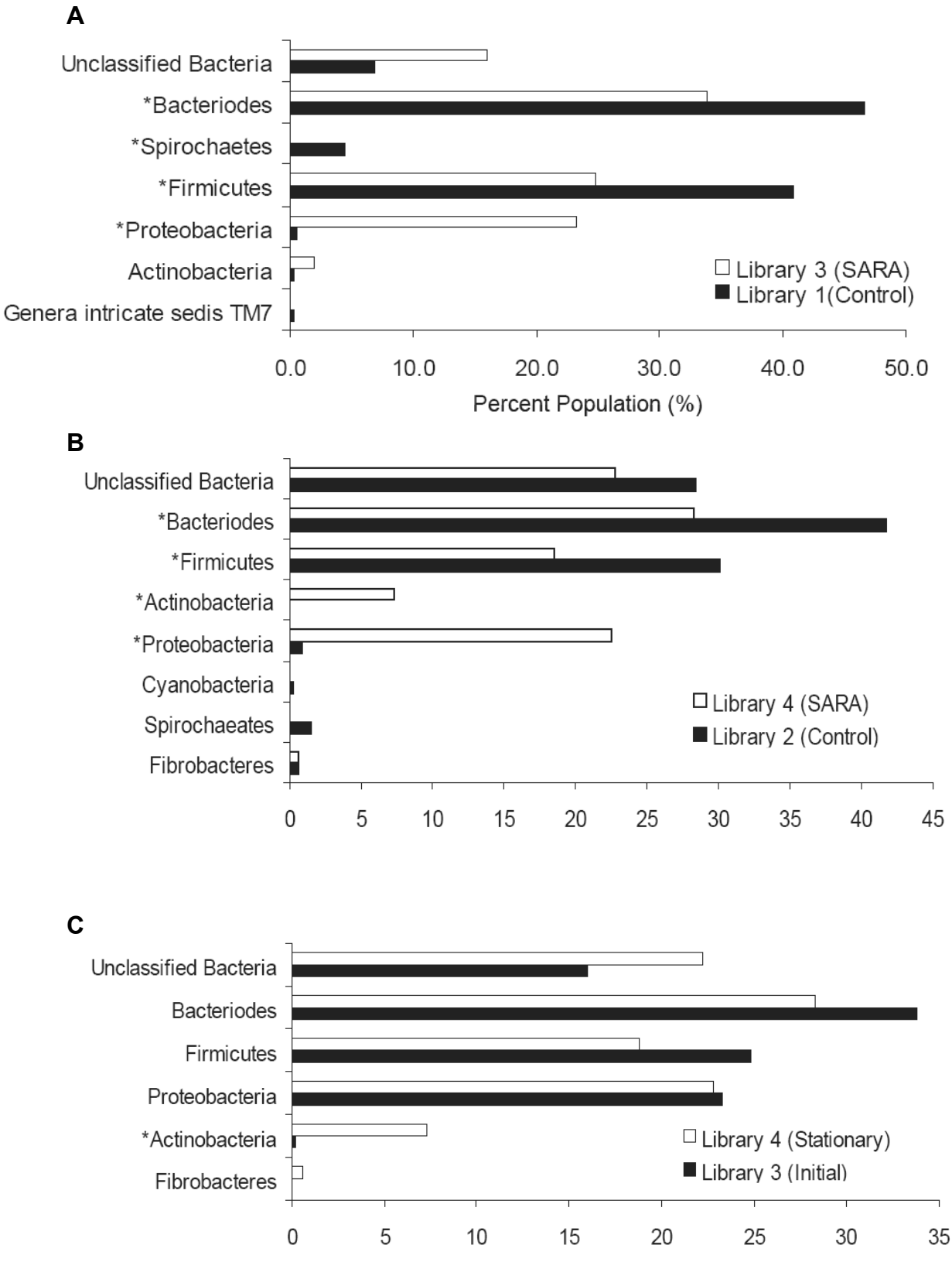

A
Unclassified Bacteria
*Bacteriodes
*Spirochaetes
*Firmicutes
*Proteobacteria
Actinobacteria
Genera intricate sedis TM7
Library 3 (SARA)
Library 1(Control)
0.0
10.0
20.0
30.0
40.0
50.0
Percent Population (%)
B
Unclassified Bacteria
*Bacteriodes
*Firmicutes
*Actinobacteria
*Proteobacteria
Cyanobacteria
Spirochaeates
Fibrobacteres
Library 4 (SARA)
Library 2 (Control)
0
5
10
15
20
25
30
35
40
45
C
Unclassified Bacteria
Bacteriodes
Firmicutes
Proteobacteria
*Actinobacteria
Fibrobacteres
Library 4 (Stationary)
Library 3 (Initial)
0
5
10
15
20
25
30
35

Figure 5

Phylogenetic analysis of library 1 constructed from steer 63 on high concentrate diet during the initial stages of acidosis. A consensus phylogenetic tree constructed using the Neighbour joining method is shown. The scale bars indicates the length of 10 substitutions per 100 residues. The numbers displayed represents the redundancy of each clone.

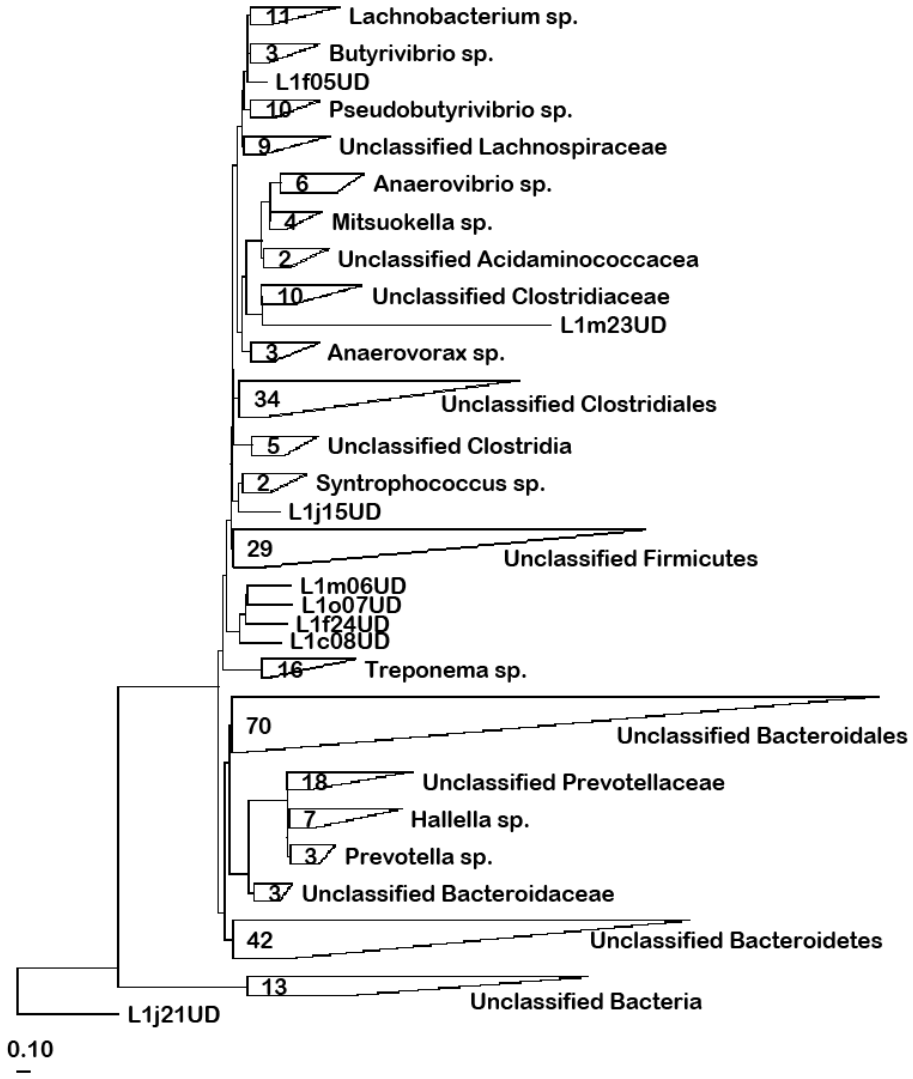

11
Lachnobacterium sp.
3
Butyrivibrio sp.
L1f05UD
10
Pseudobutyrivibrio sp.
9
Unclassified Lachnospiraceae
6
Anaerovibrio sp.
4
Mitsuokella sp.
2
Unclassified Acidaminococcacea
10
Unclassified Clostridiaceae
L1m23UD
3
Anaerovorax sp.
34
Unclassified Clostridiales
5
Unclassified Clostridia
2
Syntrophococcus sp.
L1j15UD
29
Unclassified Firmicutes
L1m06UD
L1o07UD
L1f24UD
L1c08UD
16
Treponema sp.
70
Unclassified Bacteroidales
18
Unclassified Prevotellaceae
7
Hallella sp.
3
Prevotella sp.
3
Unclassified Bacteroidaceae
42
Unclassified Bacteroidetes
13
Unclassified Bacteria
L1j21UD
0.10

Figure 6

Phylogenetic analysis of library 2 constructed from steer 63 on high concentrate diet during the low pH stationary phase of acidosis. A consensus phylogenetic tree constructed using the Neighbour joining method is shown. The scale bars indicates the length of 10 substitutions per 100 residues. The numbers displayed represents the redundancy of each clone.

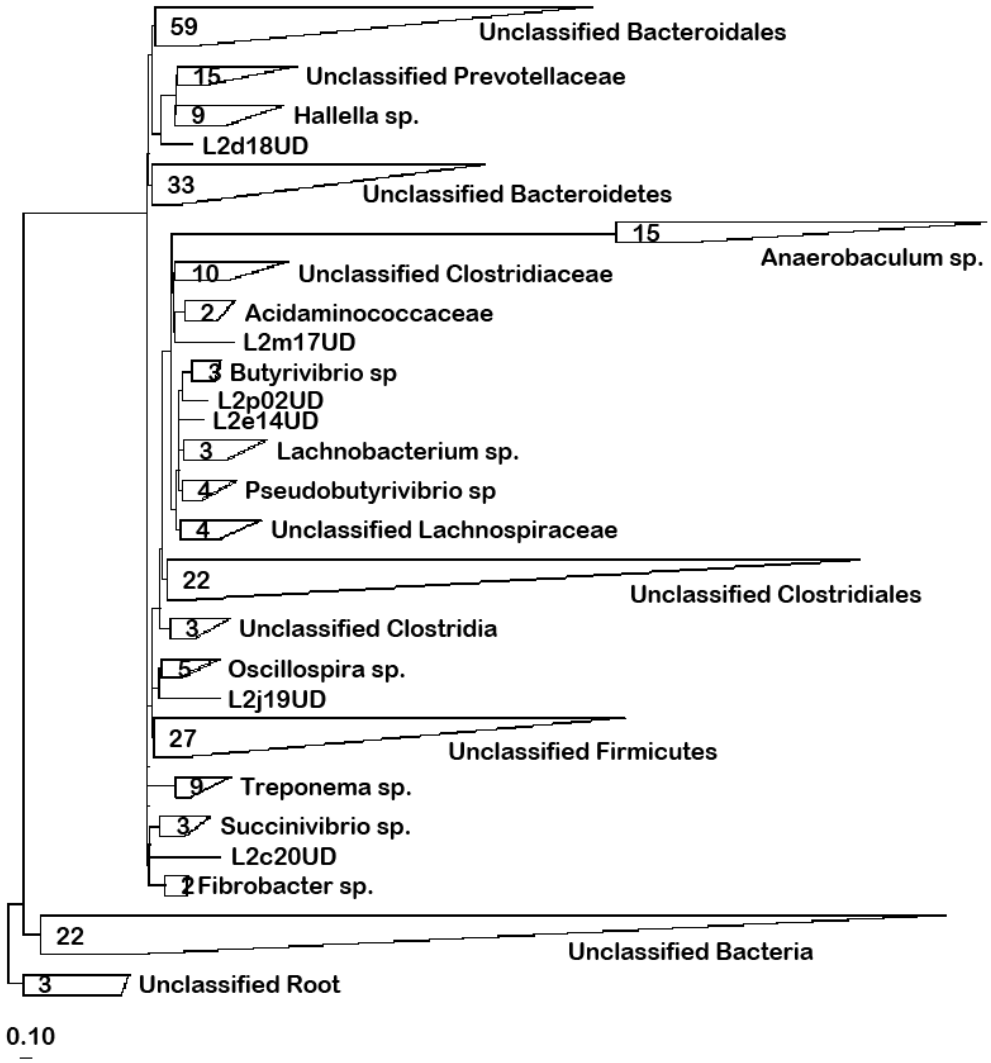

59
Unclassified Bacteroidales
15
Unclassified Prevotellaceae
9
Hallella sp.
L2d18UD
33
Unclassified Bacteroidetes
15
Anaerobaculum sp.
10
Unclassified Clostridiaceae
2
Acidaminococcaceae
L2m17UD
3
Butyrivibrio sp
L2p02UD
L2e14UD
3
Lachnobacterium sp.
4
Pseudobutyrivibrio sp
4
Unclassified Lachnospiraceae
22
Unclassified Clostridiales
3
Unclassified Clostridia
5
Oscillospira sp.
L2j19UD
27
Unclassified Firmicutes
9
Treponema sp.
3
Succinivibrio sp.
L2c20UD
2
Fibrobacter sp.
22
Unclassified Bacteria
3
Unclassified Root
0.10

Figure 7

Phylogenetic analysis of library 3 constructed from steer 65 induced with sub acute ruminal acidosis. A consensus phylogenetic tree constructed using the Neighbour joining method is shown. The scale bars indicates the length of 10 substitutions per 100 residues. The numbers displayed represents the redundancy of each clone.

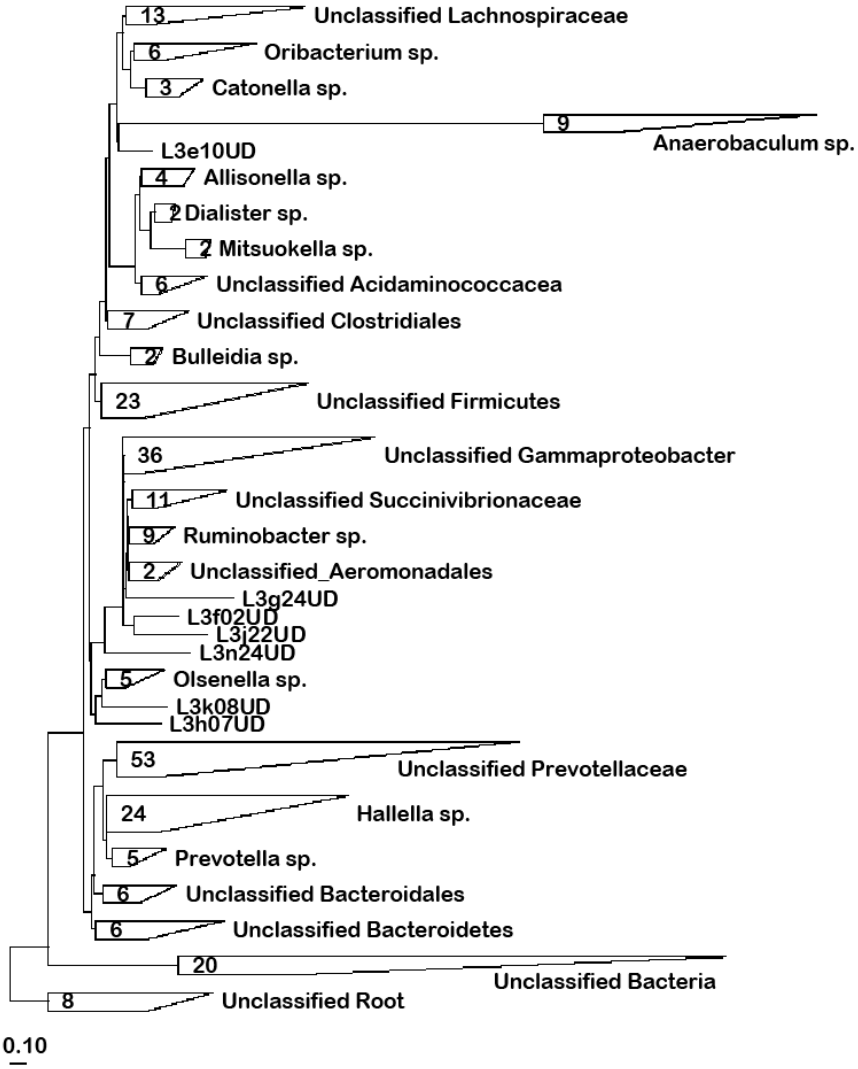

13
Unclassified Lachnospiraceae
6
Oribacterium sp.
3
Catonella sp.
9
Anaerobaculum sp.
L3e10UD
4
Allisonella sp.
2
Dialister sp.
2
Mitsuokella sp.
6
Unclassified Acidaminococcacea
7
Unclassified Clostridiales
2
Bulleidia sp.
23
Unclassified Firmicutes
36
Unclassified Gammaproteobacter
11
Unclassified Succinivibrionaceae
9
Ruminobacter sp.
2
Unclassified_Aeromonadales
L3g24UD
L3f02UD
L3j22UD
L3n24UD
5
Olsenella sp.
L3k08UD
L3h07UD
53
Unclassified Prevotellaceae
24
Hallella sp.
5
Prevotella sp.
6
Unclassified Bacteroidales
6
Unclassified Bacteroidetes
20
Unclassified Bacteria
8
Unclassified Root
0.10

Figure 8

Phylogenetic analysis of library 4 constructed from steer 65 induced with sub acute ruminal acidosis. A consensus phylogenetic tree constructed using the Neighbour joining method is shown. The scale bars indicates the length of 10 substitutions per 100 residues. The numbers displayed represents the redundancy of each clone.

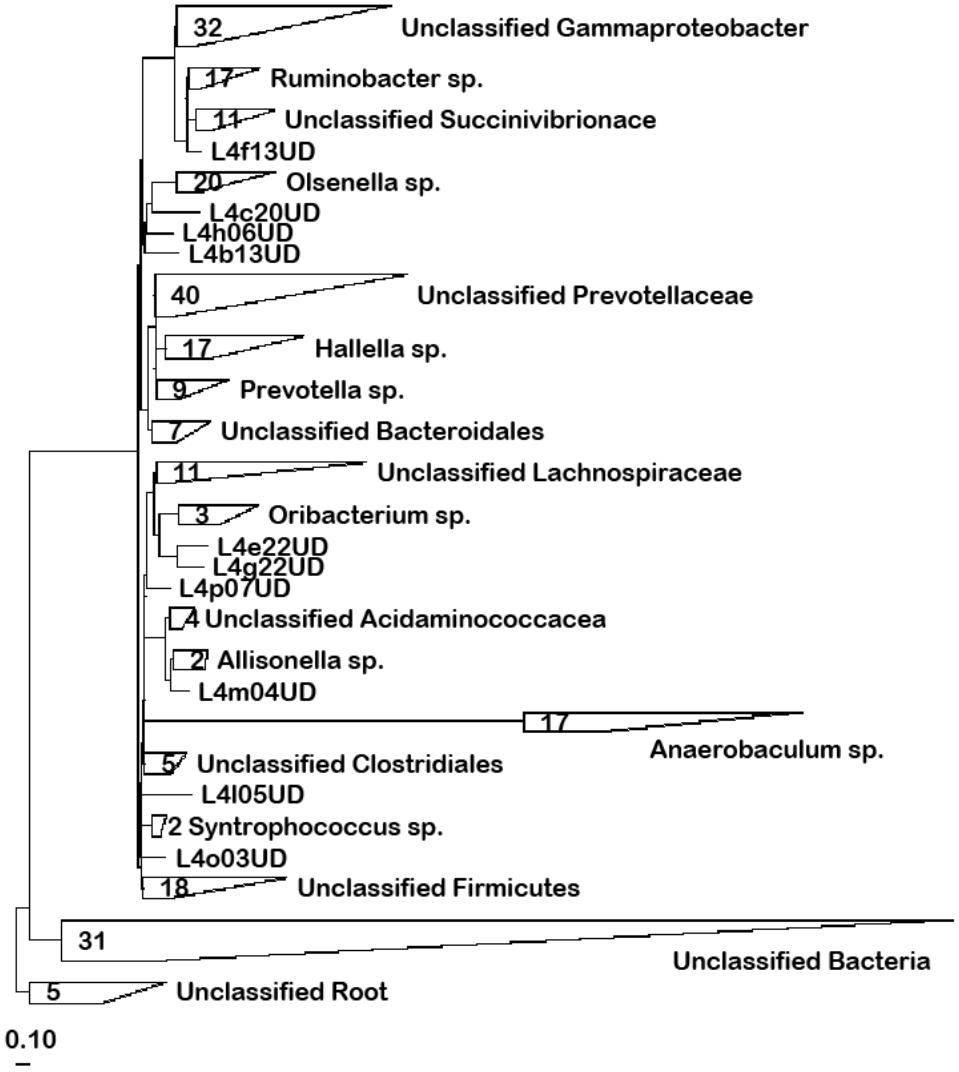
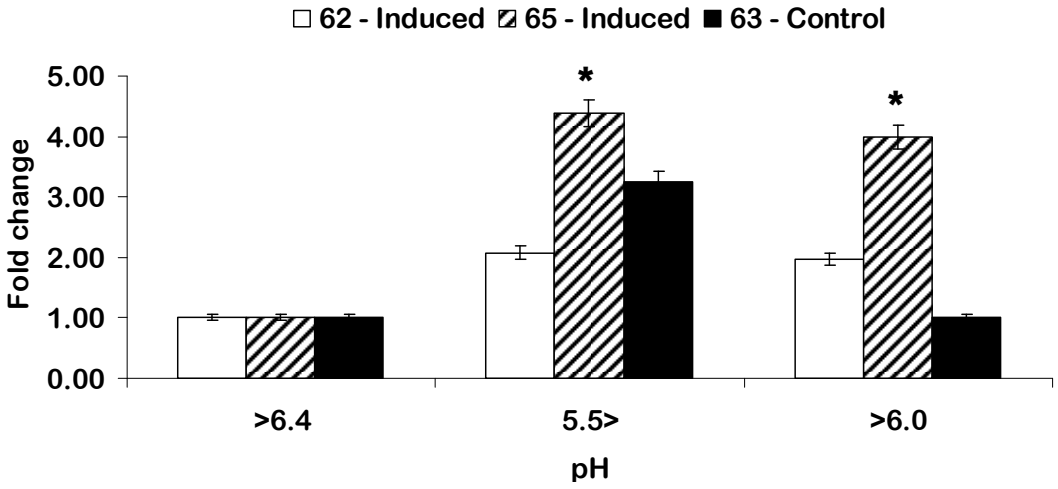

32
Unclassified Gammaproteobacter
17
Ruminobacter sp.
11
Unclassified Succinivibrionace
L4f13UD
20
Olsenella sp.
L4c20UD
L4h06UD
L4b13UD
40
Unclassified Prevotellaceae
17
Hallella sp.
9
Prevotella sp.
7
Unclassified Bacteroidales
11
Unclassified Lachnospiraceae
3
Oribacterium sp.
L4e22UD
L4g22UD
L4p07UD
4
Unclassified Acidaminococcacea
2
Allisonella sp.
L4m04UD
17
Anaerobaculum sp.
5
Unclassified Clostridiales
L4l05UD
2
Syntrophococcus sp.
L4o03UD
18
Unclassified Firmicutes
31
Unclassified Bacteria
5
Unclassified Root
0.10

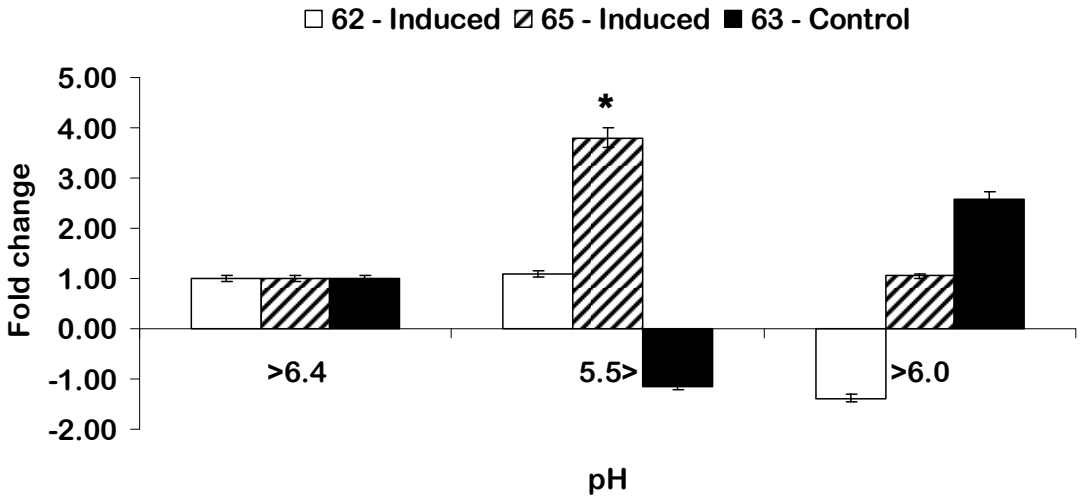
Figure 9

Population changes of selected rumen bacterial species using qRT-PCR in normal and low pH conditions. Microbial population changes were calculated as described in materials and methods. Steer 62 (White) and Steer 65 (Hatched) were induced with acidosis. Steer 63 (Black) was the control animal on high concentrate diet. Values significantly different at $P < 0.05$ is shown with an asterix "*".

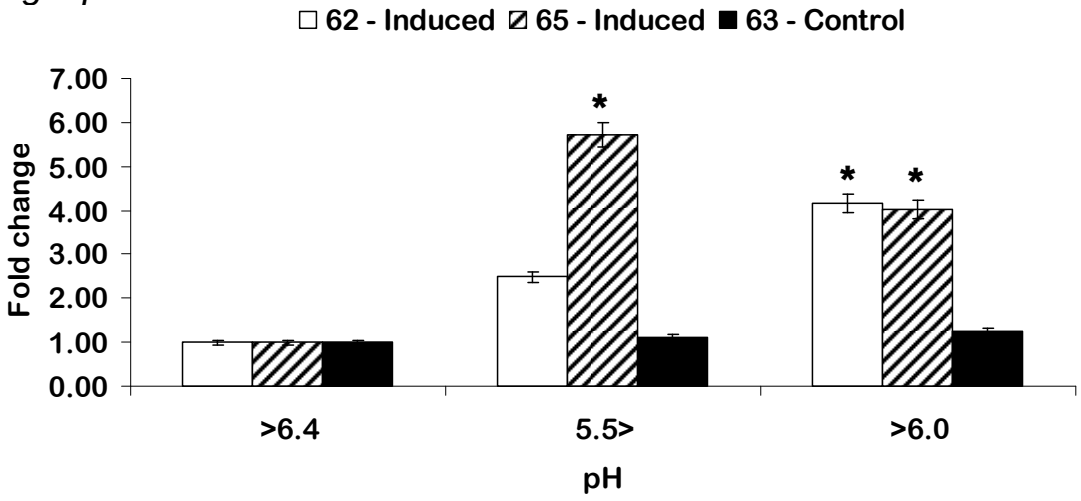
Bifidobacterium ruminantium



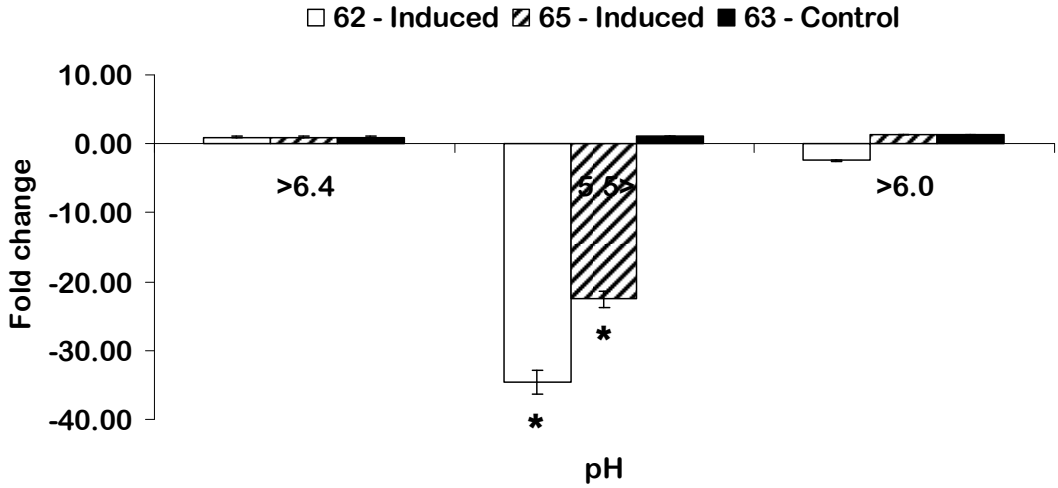
Butyrivibrio fibrosolvens



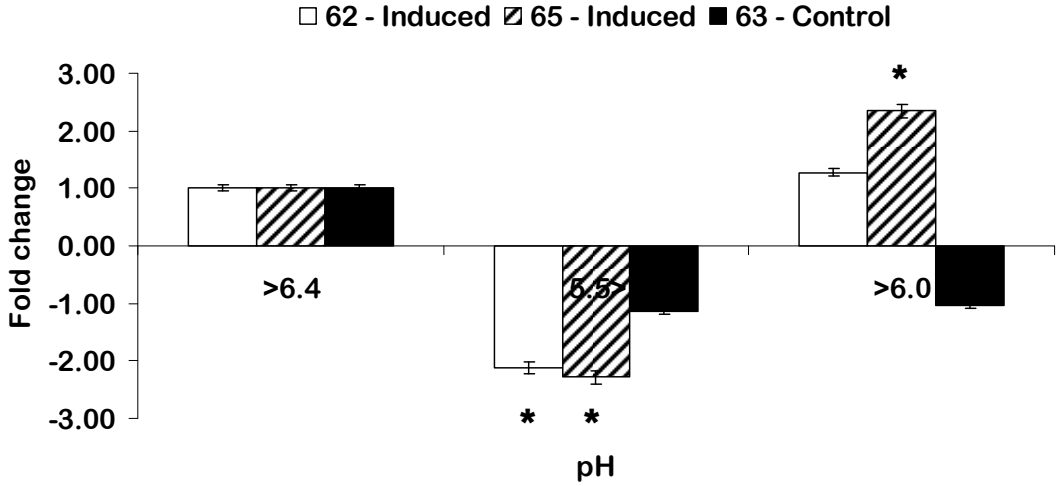
Megasphaeara eslandii



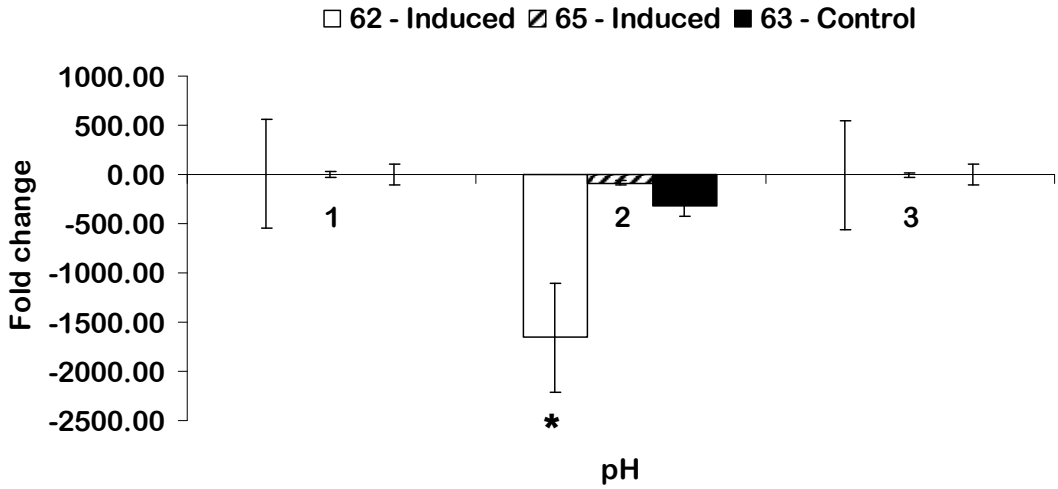
Mitsuokella jalaludinii



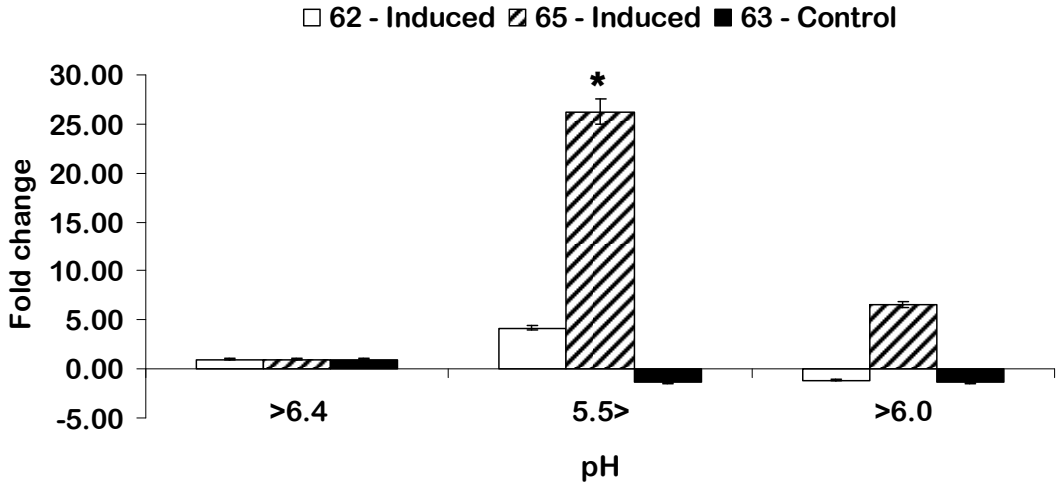
Lactobacillus acidophilus



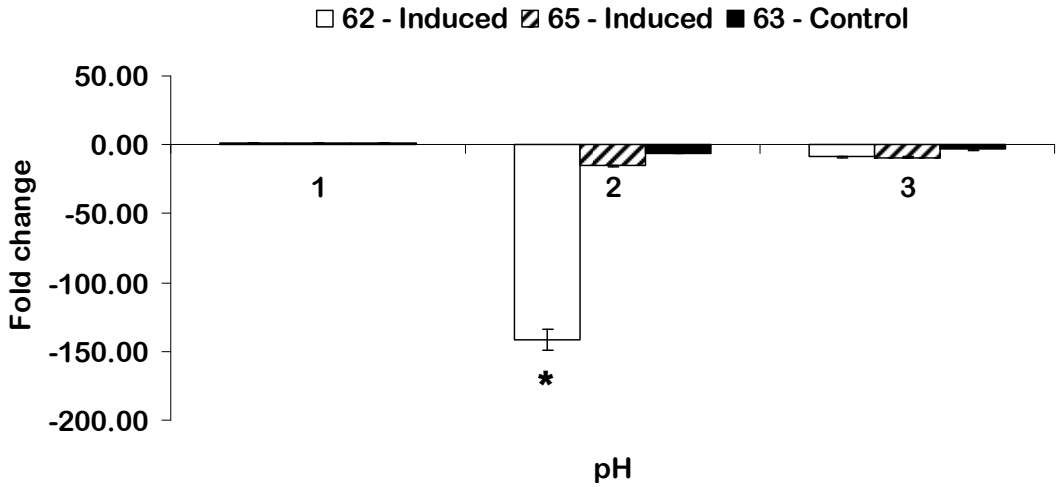
Prevotella bryantii



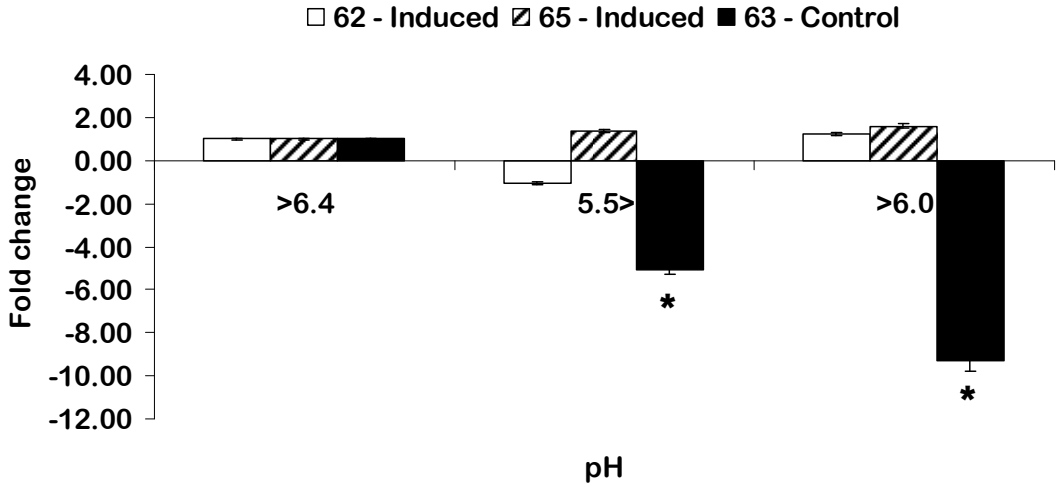
Streptococcus bovis



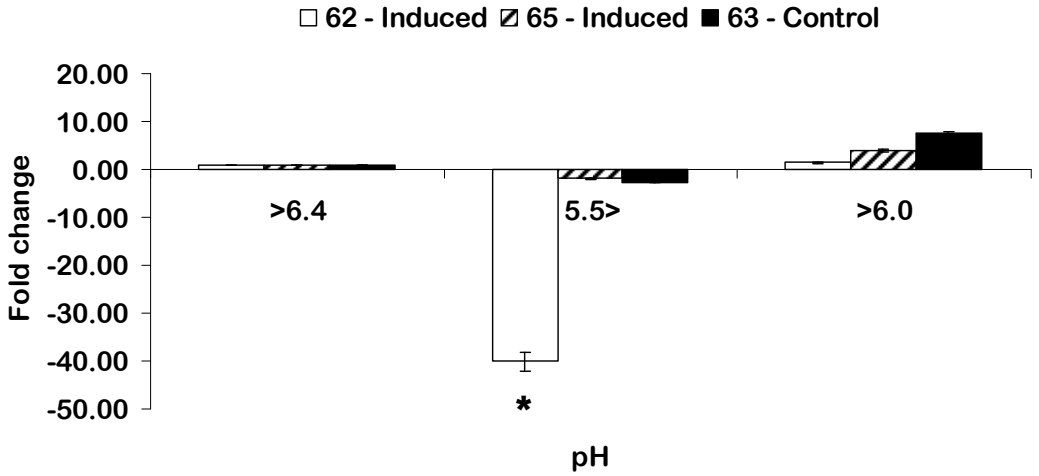
Prevotella ruminicola



Propionibacterium acnes



Prevotella albinensis



Selenomonas ruminantium

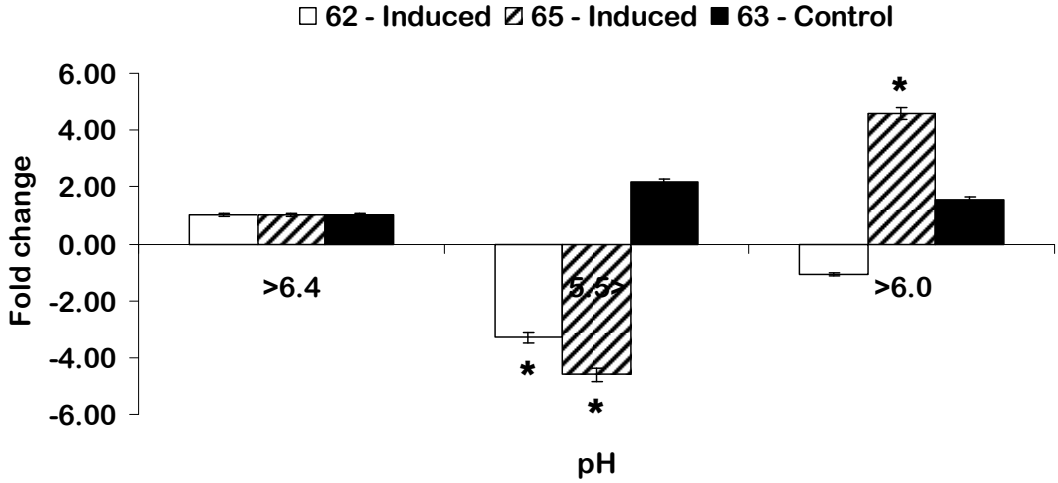


Table 1. Primer sequences used for Real Time PCR analysis.

Primer	Sequence 5'-3'	Product Size (bp)	Tm Used (°C)	Reference
Prevotella ruminicola –For	GGTTATCTTGAGTGAGTT	485	58	Tajima *et al*., 2001
Prevotella ruminicola –Rev	CTGATGGCAACTAAAGAA			
Prevotella bryantii –For	ACTGCAGCGCGAACTGTCAGA	421	58	Tajima *et al*., 2001
Prevotella bryantii –Rev	ACCTTACGGTGGCAGTGTCTC			
Prevotella albensis –For	CAGACGGCATCAGACGAGGAG	400	55	Ozutsumi *et al*., 2006
Prevotella albensis -Rev	ATGCAGCACCTTCACAGGAGC			
Selenomonas ruminantium -For	TGCTAATACCGAATGTTG	513	53	Tajima *et al*., 2001
Selenomonas ruminantium -Rev	TCCTGCACTCAAGAAAGA			
Megasphaeara eslandii -For	GACCGAAACTGCGATGCTAGA	128	60	Ouwerkerk *et al*., 2002
Megasphaeara eslandii -Rev	TCCAGAAAGCCGCTTTCGCCACT			
Streptococcus bovis -For	ATTCTTAGAGATAGGGTTTCTCTT	134	60	This Study
Streptococcus bovis -Rev	ACCTTATGATGGCAACTAACAATA			
Lactobacillus acidophilus -For	GTTCCTTCGGGGACACTAAGACAG	450	58	This Study
Lactobacillus acidophilus -Rev	TCCCGAGTTAGGCCACCGGCTTTG			
Bifidobacterium ruminantium -For	TCCATCGCTTAACGGTGGATCAGC	662	62	This Study
Bifidobacterium ruminantium -Rev	CTCACGGGGTCGCATCCCGTTGTA			
Butyrivibrio fibrisolvens -For	CGCATGATGCAGTGTGAAAAGCTC	625	56	This Study
Butyrivibrio fibrisolvens -Rev	CCTCCCGACACCTATTATTCATCG			
Mitsuokella jalaludinii -For	CTAATACCGAATGTTGTAGAGTTT	462	50	This Study
Mitsuokella jalaludinii -Rev	ACTTTTAAGATAGACTTACCTTCC			

Primer	Sequence 5'-3'	Product Size (bp)	Tm Used (°C)	Reference
Propionibacterium acnes -For	**TCAAGTCATGAAAGTTGGTAACAC**	90	53.5	This Study
Propionibacterium acnes -Rev	**ACTTAGTCCTAATCACCAGTCCCA**			
rpoB –For	**AACATCGGTTTGATCAAC**	371-390	53.5	Dahllof *et al*., 2000
rpoB –Rev	**CGTTGCATGTTGGTACCCAT**			

Table 2. Comparison of Libraries at the phylum level using "Library Compare" software available at the ribosomal database project. Populations that are significantly different at p> 0.01 are shown with an asterix "*". Values are shown as percentage from total population

(D) Library 1 Vs Library 3
(E) Library 2 Vs Library 4
(F) Library 3 Vs Library 4

A

Phylum	Library 1 (Control)	Library 3 (Induced)
Actinobacteria	0.3	2.0
Genera intricate sedis TM7	0.3	0.0
*Proteobacteria	0.6	23.3
*Firmicutes	40.9	24.6
*Spirochaetes	4.4	0.0
*Bacteroidetes	46.7	33.8
Unclassified Bacteria	6.9	16.0

*Significantly different at 0.01

B

Phylum	Library 2 (Control)	Library 4 (Induced)
Spirochaetes	1.5	0.0
Cyanobacteria	0.3	0.0
Fibrobacteres	0.6	0.6
*Proteobacteria	0.9	22.5
*Firmicutes	30.1	18.5
*Actinobacteria	0.0	7.3
*Bacteroidetes	41.7	28.3
Unclassified Bacteria	28.4	22.8

*Significantly different at 0.01

C

Phylum	Library 3 (Early)	Library 3 (Late)
*Actinobacteria	0.2	7.3
Fibrobacteres	0.0	0.6
Proteobacteria	23.3	22.8
Firmicutes	24.8	18.8
Bacteroidetes	33.8	28.3
Unclassified Bacteria	16.0	22.2

*Significantly different at 0.01

Table 3. Classification and comparison of the 16S libraries. The 16S libraries constructed were analyzed using the "Classifier" software tool available at the ribosomal database project.

Rank	Library 1 Early Control	Library 2 Late Control	Library 3 Early Induced	Library 4 Late Induced
Phylum Genera_incertae_sedis_TM7	1	0	0	0
Genus TM7	1	0	0	0
Phylum Actinobacteria	1	0	7	24
Class Actinobacteria	1	0	4	24
Subclass Actinobacteridae	0	0	1	2
Order Bifidobacteriales	0	0	1	2
Family Bifidobacteriaceae	0	0	1	2
Subclass Coriobacteridae	1	0	6	22
Order Coriobacteriales	1	0	6	22
Suborder Coriobacterineae	1	0	6	22
Phylum Lentisphaerae	1	0	0	0
Class Lentisphaerae	1	0	0	0
Order Victivallales	1	0	0	0
Family Victivallaceae	1	0	0	0
Genus Victivallis	1	0	0	0
Phylum Proteobacteria	2	4	82	76
Class Betaproteobacteria	0	0	1	0
Order Neisseriales	0	0	1	0
Family Neisseriaceae	0	0	1	0
Unclassified Neisseriaceae	0	0	1	0
Class Deltaproteobacteria	0	1	1	0
Unclassified Deltaproteobacteria	0	1	1	0
Class Gammaproteobacteria	2	3	80	75
Order Aeromonadales	2	3	49	47
Family Succinivibrionaceae	2	3	43	43
Genus Ruminobacter	0	0	25	25
Genus Succinivibrio	1	3	0	0
Unclassified Succinivibrionaceae	1	0	18	18
Unclassified Aeromonadales	0	0	6	4
Unclassified Gammaproteobacteria	0	0	31	28
Phylum Firmicutes	150	122	95	85
Class Mollicutes	0	0	2	1
Order Incertae sedis 8	0	0	2	1
Family Erysipelotrichaceae	0	01	2	1
Unclassified Erysipelotrichaceae	0	0	2	1
Class Bacilli	0	2	1	2
Order Lactobacillales	0	2	1	2
Family Lactobacillaceae	0	1	1	2
Unclassified Lactobacillaceae	0	0	1	0
Class Clostridia	141	107	88	81
Order Clostridiales	132	103	88	81

Family Eubacteriaceae	0	1	0	0
Genus Anaerovorax	0	1	0	1
Family Syntrophomonadaceae	0	18	7	20
Genus Anaerobaculum	0	18	7	20
Family Clostridiaceae	20	19	3	0
Genus Sporobacter	2	3	1	0
Genus Acetivibrio	1	0	0	0
Unclassified Clostridiaceae	17	16	2	0
Family Acidaminococcaceae	14	4	18	10
Genus Dialister	0	0	1	3
Genus Allisonella	0	0	4	2
Genus Mitsuokella	4	2	2	1
Genus Anaerovibrio	7	1	0	0
Unclassified Acidaminococcaceae	3	0	11	4
Family Eubacteriaceae	4	0	1	0
Genus Anaerovorax	3	0	1	0
Unclassified Eubacteriaceae	1	0	0	0
Family Lachnospiraceae	50	24	48	37
Genus Shuttleworthia	0	0	1	1
Genus Oribacterium	0	1	9	3
Genus Butyrivibrio	5	5	0	1
Genus Catonella	0	0	2	1
Genus Lachnobacterium	13	4	0	0
Genus Roseburia	1	1	0	0
Genus Pseudobutyrivibrio	12	4	0	0
Unclassified Lachnospiraceae	19	9	36	31
Unclassified Clostridiales	44	37	11	13
Unclassified Clostridia	9	4	0	0
Unclassified Firmicutes	9	13	4	1
Phylum Spirochaetes	17	0	0	0
Class Spirochaetes	17	7	0	0
Order Spirochaetales	17	7	0	0
Family Spirochaetaceae	17	7	0	0
Genus Treponema	17	7	0	0
Phylum Bacteroidetes	171	134	115	93
Class Bacteroidetes	96	84	91	81
Order Bacteroidales	96	84	91	81
Family Porphyromonadaceae	0	1	1	1
Genus Tannerella	0	1	1	1
Family Bacteroidaceae	14	6	0	0
Genus Anaerophaga	14	6	0	0
Family Prevotellaceae	21	11	48	47
Genus Prevotella	21	11	48	47
Unclassified Bacteroidales	61	66	42	33
Unclassified Bacteroidetes	75	50	24	12
Unclassified Bacteria	16	46	34	43

Discussion

Ruminal acidosis is one of the most important nutritional disorders that affect the feedlot and dairy cattle industries. Acidosis is caused when too much starch or fermentable sugars are consumed by animals within a short period of time, which leads to a rapid production of organic acids and a decrease in ruminal pH (Nocek, 1997; Osborne et al., 2004; Kleen et al., 2003). This change in pH disrupts the normal microbial environment within the rumen resulting in a reduction in the animal's performance (Nocek, 1997). The pH measurements (Figure 1) displayed a significant drop in ruminal pH in animals induced with acidosis. The drop in pH occurs rapidly within a period of 1.5 hrs to 2.0 hrs post induction, suggesting that the substrate metabolism and utilization within the rumen is rapid and confirms that the animals induced with acidosis were under the influence of sub-acute ruminal acidosis (Garrett et al., 1999; Nocek, 1997; Owens et al., 1998). This decrease in ruminal pH is reported to be due to the increase in lactic acid and other volatile fatty acids within the rumen (Horn et al., 1979; Goad et al., 1998), and has been known to have significant effects on the rumen microbial population (Hungate et al., 1952). The change in rumen microbial populations has been reported to shift the microbial population towards lactic acid utilizes and producers (Owens et al., 1998). Several studies have evaluated the changes in microbial populations in relation to acidosis (Dunlop and Hammond, 1965; Slyter, 1976) but, not much is known about the microbial changes associated with sub-acute ruminal acidosis. Therefore, in this study we present rumen microbial population dynamics using several molecular

techniques and present a broader picture of the rumen microbial changes during sub-acute acidosis and compare these results with animals on high concentrate diet. To survey the changes in microbial population structure and diversity in animals adapted to high concentrate diet and in animals with sub-acute ruminal acidosis, we performed Terminal Restriction Fragment Length polymorphism (T-RFLP) based analysis. T-RFLP analysis demonstrated a great diversity within both treatment groups. But, displayed a greater bacterial diversity during acidosis after 2 hrs of induction when compared to animals on high concentrate diet, and may be due to the increasing fermentable substrates available for the rumen bacteria during initial dosing, leading to increased growth rates in bacterial populations. A similar observation was reported by Nagaraja et. al (1978) in in-vitro experiments where an increase in total number of bacteria was observed on incubation of rumen fluid with corn. But, the consistent increase in bacterial population diversity suggests otherwise, and the increase in bacterial diversity may be due to a change in the bacterial population to more pH tolerant bacterial population. T-RFLP analysis also detected slight fluctuations in microbial populations in the control animals by 3hrs. This may be because the control animals started to feed on the concentrate diet leading to increased population due to feeding. As shown in figure 2, a significant shift in the microbial population was detected during acidosis where several previously unreported bacterial species were detected. Among these bacterial populations, *Tissierella sp., Thermobacillus sp., Thermobiospora sp., Telluria sp., Stenotrophomonas sp., spirilum sp., Rubrivivax sp., Rikenellasp., Rhodococcus sp., Moorella sp., Micrococcus sp., Methylobacillus, Halobacteriodes sp., Herbaspirilum*

sp.,Glaciecola sp., Francisella sp., Frankia sp., Fibrobacter sp., Clavibacter sp., Acidosphaera sp., Actinobacillus sp., Amycolatopsis sp., Anaerofilum sp., Anaeroplasma sp., Aneurinibacillus sp., Prevotella sp., and Actinobacillus sp.. were among a few that increased in population during acidosis. T-RFLP analysis also revealed a decrease in several bacterial populations compared to animals on high concentrate, which included *Buchnera sp., Capnocytophaga sp., Chrysiogenes sp., Clostridium sp., Colwellia sp., Flectobacillus sp., Fusibacter sp., Fusobacterium sp., Kingella sp., Leptospira sp., Leuconostoc sp., Melittangium sp., Methylosinus sp., Microscilla sp., Myxococcus sp., Nitrosomonas sp., Paracoccus sp., Pedobacter sp., Persicobacter sp., Psychroserpens sp., Rhizobium sp., Rhodospirillum sp., Ruminococcus sp., Sphingobacterium sp., Spirochaeta sp., Streptobacillus sp., Thauera sp., and Treponema sp.* We also detected several previously reported bacterial species such as, *Bifidobacterium sp., Butyrivibrio sp., Eubacterium sp. Lactobacillus sp., Prevotella sp., Ruminococcus sp., Selenomonas sp., Streptococcus sp., Fusobacterium sp., and Peptostreptococcus sp.* (Kotarski et al., 1992; Stewart et al., 1997; Tajima et al., 2000; Mackie and Gilchrist, 1979; Russell and Hino, 1985; Mackie and Heath, 1979; Huber, 1976; Huber et al., 1976) during T-RFLP analysis. Using in-vitro and in-vivo experiments Nagaraja et. al (1978) reported an increase in total rumen bacteria during the first 9 hours of lactic acidosis, where they observed a shift in the rumen microbial population structure from gram-negative bacteria to gram-positive bacteria. We observe a similar event occurring during acidosis where the gram-positive bacteria predominated. During our T-RFLP analysis we used three different restriction digestion profiles for identification of

microbial species within the rumen. As Phylogenetic Assignment Tool (PAT) (Kent et al., 2003) requires that all three digests correspond to the predicted restriction fragments of a bacterial species present in the database for accurate assignment, our microbial species identification is very accurate. Although T-RFLP analysis assigned bacterial species, we presented our bacterial assignment in terms of genera identified for two reasons; a) there may be several bacterial species within a genus having the same restriction digestion pattern for all 3 enzymes we used and b) for simplification of the data presentation. T-RFLP analysis enabled us to identify over 300 bacterial species belonging to 116 different bacterial genera. The hierarchical classification of T-RFLP data displayed an increase in *Proteobacteria* and *Actinobacteria* populations during acidosis (Fig. 3), where the *Proteobacteria* were dominated by *Desulfobacterium sp., Methylobacillus sp., Herbaspirilum sp., Pasturella sp., Taylorell sp., Telluria sp., Glaciecola sp.,* and *Haemophilus sp*. While the *Actinobacteria* were dominated by *Brevibacterium sp., Micrococcus sp., Nocardia sp., Rhodococcus sp., Clevibacter sp., Dermatophilus sp., Frankia Sp.,* and *Lentzea sp.*. Hierarchical classification also detected a decrease in *Bacteroidetes* populations of *Microscilla sp., Persicobacter sp., Rikenella sp., Sphingobacterium sp., bacteroides sp., Pedobacter sp.,* and *Flectobacillus sp.*, T-RFLP analysis only allows identification of previously characterized bacterial species in which the 16S rRNA gene has been sequenced. Therefore, to further evaluate the microbial fluctuations during acidosis and to identify new emerging species during acidosis, we constructed four 16S rRNA libraries from control and acidotic animals at critical stages of acidosis and sequenced 384 clones from each

library (4X384). Several previous studies have constructed 16S rRNA libraries from rumen bacteria and have evaluated the microbial community diversity in different diet regimes (Tajima et al., 1999; Tajima et al., 2000; Edwards et al., 2004). But, none have evaluated the microbial changes during sub-acute ruminal acidosis using 16S rDNA libraries, therefore this study provides valuable information towards understanding the rumen microbial fluctuations during acidosis. Initial analysis of the 16S rRNA libraries identified significant changes in microbial population structure and diversity in animals with acidosis when compared to animals on high concentrate diet (Table 2 and Figure 4). The libraries constructed from animals on high concentrate diet displayed significantly greater numbers in bacteria belonging to Phylum, Bacteriodes, Spirochaetes and Firmicutes. Where the predominant bacterial species included, *Anaerophaga sp.,* and *Prevotella sp., Treponema sp., Anaerobaculum sp., Sporobacter sp., Acetivibrio sp., Mitsuokella sp., Anaerovibrio sp., Butyrivibrio sp., Lachnobacterium sp., Pseudobutyrivibrio sp.,* and several unclassified Bacteriodes, Spirochaetes and Firmicutes. Several of the rumen bacterial species we identified have been characterized and are known to be predominant in grain adapted sheep and cattle Mackie and Gilchrist, 1979; Tajima et al., 2000). Tajima *et al*. (2000) reported that during transition to high concentrate diet and when animals are on high concentrate diet, the low G+C gram positive bacteria predominate, which included *Acidomonococcus sp., Clostridium sp., Lactobacillus sp., Megaspera sp., Selenomonas sp., Streptococcus sp.,* and *Ruminococcus sp.* (Krause and Russell, 1996). T-RFLP analysis and sequencing data confirms this observation as we detected increased number of *Clostridium sp., Flectobacillus sp.,*

Fusibacter sp., Fusobacterium sp., Pedobacter sp., Persicobacter sp., Ruminococcus sp., Streptobacillus sp., Treponema sp., Bifidobacterium sp., Butyrivibrio sp., Eubacterium sp. Lactobacillus sp., Prevotella sp., Ruminococcus sp., Selenomonas sp., Streptococcus sp., and Peptostreptococcus sp.. This increasing number of amylolytic and lactic acid utilizing bacteria within the rumen will help maintain ruminal pH. The 16S rRNA libraries constructed from animals induced with sub acute ruminal acidosis displayed significantly different bacterial population structure and diversity compared to animals on high concentrate diet (Table 2 and Figure 4). Animals induced with acidosis displayed large increases in bacteria belonging to phylum *Proteobacteria* and *Actinobacteria*, where *Ruminobacter sp.* belonging to Class; *Gamaproteobacteria* and *Olsenella sp.* belonging to phylum *Actinobacteria* were predominant (Table 3). Interestingly, although *Bacteriodetes* populations decreased during acidosis, *Prevotella sp.* increased significantly suggesting a shift in bacterial population structure. Therefore, the microbial changes during adaptation to high concentrate diet or in different rumen environments may be due minute changes in microbial strains or microbial species in the same genus rather than drastic changes in phylogenetically diverse microbial populations. Hierarchical classification of the 16S rRNA libraries reported the presence of a total of 139 unclassified bacteria in the four libraries sequenced. This suggests that the microbial population within the rumen is far from being known and needs to be investigated. The rumen microbial population may be a great source to identify new microbes of economic importance.

Phylogenetic analysis of the sequenced libraries identified large diversity among the bacterial populations on each library, but a similar organization was observed in Lib 1 & 2 constructed from animals on high concentrate diet and Lib 3 & 4 constructed from animals in acidosis, suggesting that the change in the microbial population during acidosis occurs rapidly and is stable. The number of OTUs detected in the four libraries show greater number of OTUs in animals on high concentrate (133 & 100) compared to animals on acidosis (113 & 70). This observation suggests a decrease in OTUs during acidosis which may be due to the decreasing ruminal pH. Significant change in the *Proteobacteria* was detected during acidosis (Figures 7&8). This increasing in Proteobacterial population was phylogenetically distant to all bacterial species identified, suggesting that this increasing Proteobacterial population may play a significant role during sub acute ruminal acidosis. Sequences representing family *Succinivibrionaceae* displayed higher redundancy in the libraries constructed during acidosis and could serve as an potential species for early detection of acidosis. Sequence analysis of the 16S rRNA libraries constructed, revealed the presence of numerous uncultured rumen bacterial species. As these bacterial species have not been characterized, it is difficult to identify the role of the bacterial species within the rumen. Therefore, we analyzed the 16S rDNA sequences using the “Library Classifier” (Wang et al., 2007) software to predict the hierarchical classification based on the conserved regions of the 16S subunit. Table 3 and the phylogenetic trees show the classification and distribution of the bacterial species sequenced and will enable prediction of the functional role of these bacteria within the rumen. This investigation of rumen microbial population structure and

diversity using 16S rRNA libraries is among the largest sequenced so far and will provide valuable information towards identifying new rumen bacterial species. Previous studies using culture dependent techniques, have reported the presence of several starch digesting organisms within the bovine rumen (Goad et al., 1998), where cellulolytic and gram positive bacteria were predominant during conditions of excess grain and glucose, and *Streptococcus bovis* was the major cause of lactic acidosis (Hungate et al., 1952). To identify the role of previously reported starch digesters, lactate utilizes and lactate producers during sub acute ruminal acidosis, we evaluated the changes in microbial population structure using quantitative real-time PCR (qRT-PCR). One of the major challenges in using qRT-PCR for estimation of microbial population fluctuation was identifying a single copy universally conserved gene that could be reliably used for normalization of bacterial populations. Previous studies have evaluated microbial population changes using absolute quantification (Klieve et al., 2003; Tajima et al., 2001). However, absolute quantification dose not take into account changes in total rumen population size. Estimations of population change using absolute quantification assumes that the total ruminal population is constant throughout the study. In this study we use *rpoB* gene which codes for RNA polymerase beta subunit which is a single copy conserved gene in all eubacterial species (Santos and Ochman, 2004; Dahllof et al., 2000) for normalization. This new approach of normalization using *rpoB* gene may help more accurate evaluation of microbial population changes in different environments as the *rpoB* gene would normalize for any changes in the total bacterial population size.

Realtime PCR analysis detected a 2-5 fold increase in *Bifidobacterium ruminantium* in animals with acidosis when compared to animals on high concentrate diet. *Bifidobacterium sp.* has been reported in animals fed with high starch diets (Biavati and Mattarelli, 1991; Scardovi et al., 1969) and is known to metabolize maltose and glucose which are intermediates or end products of starch digestion (Biavati and Mattarelli, 1991). Furthermore, *Bifidobacterium sp.* has been reported to metabolize glucose using the fructose-6-phosphate shunt to produce acetic acid and lactic acid (Scardovi et al., 1969). Therefore, the increase of *Bifidobacterium sp.* during acidosis may be due to the increase in maltose and glucose which are intermediates and end products of starch digestion. The fermentation of glucose by *Bifidobacterium ruminantium* to lactate using the abundant glucose present within the rumen may have contributed towards the decrease in ruminal pH. Populations of *Butyrivibrio fibrisolvens* increased during acidosis and demonstrated a similar trend to *Bifidobacterium ruminantium. Butyrivibrio sp.* are known to be fibrolytic organisms, but are also known to have high preference towards maltose and sucrose utilization (Russell and Baldwin, 1978). Thus, *Butyrivibrio fibrisolvens* is an organism capable of utilizing both, cellulose and starch. The increase of *Butyrivibrio fibrisolvens* during induction of acidosis suggests that this organism may be involved in the breakdown of starch and the degradation of the grain endosperm. *Prevotella sp. (P. bryanty, P. ruminicola, and P. albenensis*) displayed similar changes in microbial populations. But, the change in populations of *Prevotella sp.* was somewhat inconsistent in the two animals induced with acidosis. Steer 62 showed significant decrease in all 3 *Prevotella* populations, but steer 65 displayed little or no change in the *Prevotella*

populations during acidosis and was similar to the fluctuations in the control animal. This may be due to two reasons; a) Steer 65 may have been better adapted through the step-up diet than steer 62, therefore it was able to tolerate the low ruminal pH or b) the samples used for qRT-PCR had pH values 4.9 and 5.2 respectively and the low pH in steer 62 which was less than pH 5 may have caused a drastic decrease in the Prevotella populations. It is more likely that the significant decrease in Prevotella populations in steer 62 may be due to the pH being very low (pH <5), and that the Prevotella populations were unable to tolerate this low pH. *Mitsuokella amlyophylis* and *Selenomonas ruminantium sub sp. ruminantium* displayed similar trend in microbial population changes where, the microbial populations decreased 25-35 and 3-4 fold respectively during acidosis when compared to the control animal on high concentrate diet. These two amylolytic organisms are known to have similar characteristics and display a similar pattern of substrate utilization (Stewart et al., 1997). *Selenomonas ruminantium* is a propionate producing species and is known to produce propionate through succinate-decarboxylation (Stewart et al., 1997). *Selenomonas ruminantium* has been shown to prefer glucose, maltose, sucrose and xylose as substrates (Russell and Baldwin, 1978) for fermentation and therefore, can utilize a great variety of substrates for energy metabolism. Hence, *Selenomonas ruminantium* may help utilize the increasing fermentable substrates within the rumen. However, *Selenomonas ruminantium sub sp. ruminantium* cannot tolerate the decreasing ruminal pH and has been shown to decrease its' population by 50% at a pH of 5 (Russell and Dombrowski, 1980). Therefore, the decrease in *Selenomonas ruminantium sub sp. ruminantium* may be due to the decreasing pH.

Mitsuokella amlyophylis is a bacterium that can synthesize phytase to help digest phytate to liberate phosphorous (Lan et al., 2002) and utilizes similar substrates to *Selenomonas ruminantium*. The low ruminal pH during sub acute acidosis may also have a similar detrimental effect on the *Mitsuokella* populations as in *Selenomonas ruminantium sub sp. ruminantium* populations causing a decrease in population numbers.

Realtime analysis displayed decrease in *Lactobacillus sp.*. It is reported that *Lactobacillus sp.* are acid tolerant and would increase in acidosis or sub acute acidosis conditions (Goad et al., 1998). However, we see a decrease in Lactobacillus populations during the first 16 hrs of induction. This may be due to the slow growth rate of acid tolerant lactic acid bacteria, as populations of lactic acid bacteria decreased 2-3 fold during early acidosis but increased 3-5 fold by 48 hrs of induction in animals induced with acidosis. Whereas, the populations in the control animal remained low. This suggests a slow but significant increase in lactic acid bacteria during later stages of acidosis. *Megasphaeara elsdenii, Propionibacterium acnes* and *Streptococcus bovis* population increased 3-5 fold, 4-8 fold and 25-fold respectively during acidosis and are known to be lactic acid utilizes and producers. *Megaspaera elsdenii* which is one of the most widely studied rumen organism is somewhat acid tolerant (Russell and Dombrowski, 1980) and known to utilize the lactic acid produced within the rumen to help prevent lactic acid accumulation and acidosis (Counotte et al., 1981). Therefore, an increase in *Megaspaera elsdenii* during acidosis is a mechanism of maintaining ruminal pH by utilizing the increasing lactic acid within the rumen. *Propionibacterium acnes* is also known to be a lactate

utilizer (Huber et al., 1976) like *Megaspaera,* and is predominant in sheep adapted to high concentrate diet (Mackie and Gilchrist, 1979). Therefore the increase of *Propionibacterium acnes* during acidosis suggests that this organism plays a similar role to *Megaspaera elsdenii* by decreasing the available ruminal lactic acid and maintaining ruminal pH. *Streptococcus bovis* is undoubtedly the most heavily studied organism in the rumen. The role of *S. bovis* during acute acidosis is well documented (Dunlop and Hammond, 1965; Slyter, 1976; Owens et al., 1998), but the role of *Streptococcus bovis* during sub acute acidosis has not been evaluated. *S. bovis* is reported to have a very rapid growth rate and can rapidly metabolize cereal grain starch (Mcallister et al., 1990). Therefore, an increase in *S. bovis* population is expected during acidosis. But the increase in population we detected is not as significant as detected in acute acidosis. This may be due to the fact that the animals were adapted to acidosis. Furthermore, although the *S. bovis* populations increased in animals with acidosis compared to the control animal, the fluctuations within the two animals induced with acidosis was also significant and may be due to animal to animal variation.

There is significant animal to animal variation, although steer 62 and 65 were both induced with acidosis steer 65 shows more significant changes in microbial population structure than steer 62. Overall, steer 62 shows decreased bacterial populations than steer 65. As mentioned earlier this may be due to the low ruminal pH in steer 62 (pH<5). This preliminary study of microbial changes during acidosis using T-RFLP analysis, 16S rRNA sequencing and quantitative real-time PCR shows significant changes in rumen microbial population structure during acidosis,

and reports the presence large number of previously unreported organisms within the rumen. As such this study provides new insight into the role and fluctuations of rumen bacteria during sub acute ruminal acidosis.

Reference List

Ashelford,K.E., Weightman,A.J., and Fry,J.C. (2002) PRIMROSE: a computer program for generating and estimating the phylogenetic range of 16S rRNA oligonucleotide probes and primers in conjunction with the RDP-II database. *Nucleic Acids Research* **30:** 3481-3489.

Biavati,B. and Mattarelli,P. (1991) Bifidobacterium-Ruminantium Sp-Nov and Bifidobacterium-Merycicum Sp-Nov from the Rumens of Cattle. *International Journal of Systematic Bacteriology* **41:** 163-168.

Bodenteich,A., Chissoe,S., Wang,Y.F., and Roe,B.A. (1993) Shotgun Cloning as the Strategy of Choice to Generate Templates for High-throughput Dideoxynucleotide Sequencing. In Automated *DNA Sequencing and Analysis Techniques*. Venter,J.C. (ed). Academic Press, London.

Brown,M.S., Krehbiel,C.R., Galyean,M.L., Remmenga,M.D., Peters,J.P., Hibbard,B. et al. (2000) Evaluation of models of acute and subacute acidosis on dry matter intake, ruminal fermentation, blood chemistry, and endocrine profiles of beef steers. *Journal of Animal Science* **78:** 3155-3168.

Chissoe,S.L., Bodenteich,A., Wang,Y.F., Wang,Y.P., Burian,D., Clifton,S.W. et al. (1995) Sequence and Analysis of the Human Abl Gene, the Bcr Gene, and Regions Involved in the Philadelphia Chromosomal Translocation. *Genomics* **27:** 67-82.

Cole,J.R., Chai,B., Farris,R.J., Wang,Q., Kulam,S.A., McGarrell,D.M. et al. (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Research* **33:** D294-D296.

Cole,J.R., Chai,B., Farris,R.J., Wang,Q., Kulam-Syed-Mohideen,A.S., McGarrell,D.M. et al. (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Research* **35:** D169-D172.

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

Counotte,G.H.M., Prins,R.A., Janssen,R.H.A.M., and Debie,M.J.A. (1981) Role of Megasphaera-Elsdenii in the Fermentation of Lactate-2-C-13 in the Rumen of Dairy-Cattle. *Applied and Environmental Microbiology* **42:** 649-655.

Dahllof,I., Baillie,H., and Kjelleberg,S. (2000) rpoB-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. *Applied and Environmental Microbiology* **66:** 3376-3380.

Dunlop,R.H. and Hammond,P.B. (1965) D-Lactic Acidosis of Ruminants. *Annals of the New York Academy of Sciences* **119:** 1109-&.

Edwards,J.E., Mcewan,N.R., Travis,A.J., and Wallace,R.J. (2004) 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **86:** 263-281.

Ewing,B. and Green,P. (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Research* **8:** 186-194.

Ewing,B., Hillier,L., Wendl,M.C., and Green,P. (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Research* **8:** 175-185.

Garrett,E.F., Pereira,M.N., Nordlund,K.V., Armentano,L.E., Goodger,W.J., and Oetzel,G.R. (1999) Diagnostic methods for the detection of subacute ruminal acidosis in dairy cows. *Journal of Dairy Science* **82:** 1170-1178.

Goad,D.W., Goad,C.L., and Nagaraja,T.G. (1998) Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. *Journal of Animal Science* **76:** 234-241.

Gordon,D., Abajian,C., and Green,P. (1998) Consed: A graphical tool for sequence finishing. *Genome Research* **8:** 195-202.

Hettinger,A.M., Allen,M.R., Zhang,B.R., Goad,D.W., Malayer,J.R., and Geisert,R.D. (2001) Presence of the acute phase protein, bikunin, in the endometrium of gilts during estrous cycle and early pregnancy. *Biology of Reproduction* **65:** 507-513.

Horn,G.W., Gordon,J.L., Prigge,E.C., and Owens,F.N. (1979) Dietary Buffers and Ruminal and Blood Parameters of Sub-Clinical Lactic-Acidosis in Steers. *Journal of Animal Science* **48:** 683-691.

Huber,T.L. (1976) Physiological-Effects of Acidosis on Feedlot Cattle. *Journal of Animal Science* **43:** 902-909.

Huber,T.L., Cooley,J.H., Goetsch,D.D., and Das,N.K. (1976) Lactic Acid Utilizing Bacteria in Ruminal Fluid of A Steer Adapted from Hay Feeding to A High-Grain Ration. *American Journal of Veterinary Research* **37:** 611-613.

Hungate,R.E., Dougherty,R.W., Bryant,M.P., and Cello,R.M. (1952) Microbiological and Physiological Changes Associated with Acute Indigestion in Sheep. *Cornell Veterinarian* **42:** 423-449.

Kent,A.D., Smith,D.J., Benson,B.J., and Triplett,E.W. (2003) Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. *Applied and Environmental Microbiology* **69:** 6768-6776.

Kleen,J.L., Hooijer,G.A., Rehage,J., and Noordhuizen,J.P.T.M. (2003) Subacute ruminal acidosis (SARA): a review. *Journal of Veterinary Medicine Series A-Physiology Pathology Clinical Medicine* **50:** 406-414.

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

Kotarski,S.F., Waniska,R.D., and Thurn,K.K. (1992) Starch Hydrolysis by the Ruminal Microflora. *Journal of Nutrition* **122:** 178-190.

Krause,D.O. and Russell,J.B. (1996) How many ruminal bacteria are there? *Journal of Dairy Science* **79:** 1467-1475.

Lan,G.Q., Abdullah,N., Jalaludin,S., and Ho,Y.W. (2002) Culture conditions influencing phytase production of Mitsuokella jalaludinii, a new bacterial species from the rumen of cattle. *Journal of Applied Microbiology* **93:** 668-674.

Ludwig,W., Strunk,O., Westram,R., Richter,L., Meier,H., Yadhukumar et al. (2004) ARB: a software environment for sequence data. *Nucleic Acids Research* **32:** 1363-1371.

Mackie,R.I. and Gilchrist,F.M.C. (1979) Changes in Lactate-Producing and Lactate-Utilizing Bacteria in Relation to Ph in the Rumen of Sheep During Stepwise Adaptation to A High-Concentrate Diet. *Applied and Environmental Microbiology* **38:** 422-430.

Mackie,R.I. and Heath,S. (1979) Enumeration and Isolation of Lactate-Utilizing Bacteria from the Rumen of Sheep. *Applied and Environmental Microbiology* **38:** 416-421.

Marsh,T.L., Saxman,P., Cole,J., and Tiedje,J. (2000) Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. *Applied Environmental Microbiology* **66:** 3616-3620.

Mcallister,T.A., Cheng,K.J., Rode,L.M., and Forsberg,C.W. (1990) Digestion of Barley, Maize, and Wheat by Selected Species of Ruminal Bacteria*. Applied and Environmental Microbiology* **56:** 3146-3153.

Nagaraja,T.G., Bartley,E.E., Fina,L.R., and Anthony,H.D. (1978) Relationship of Rumen Gram-Negative Bacteria and Free Endotoxin to Lactic-Acidosis in Cattle. *Journal of Animal Science* **47:** 1329-1337.

Nagel,S.A. and Broderick,G.A. (1992) Effect of Formic-Acid Or Formaldehyde Treatment of Alfalfa Silage on Nutrient Utilization by Dairy-Cows. *Journal of Dairy Science* **75:** 140-154.

Nocek,J.E. (1997) Bovine acidosis: Implications on laminitis. *Journal of Dairy Science* **80:** 1005-1028.

Olumeyan,D.B., Nagaraja,T.G., Miller,G.W., Frey,R.A., and Boyer,J.E. (1986) Rumen Microbial Changes in Cattle Fed Diets with Or Without Salinomycin. *Applied and Environmental Microbiology* **51:** 340-345.

Osborn,A.M., Moore,E.R.B., and Timmis,K.N. (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environmental Microbiology* **2:** 39-50.

Osborne,J.K., Mutsvangwa,T., Alzahal,O., Duffield,T.F., Bagg,R., Dick,P. et al. (2004) Effects of monensin on ruminal forage degradability and total tract diet digestibility in lactating dairy cows during grain-induced subacute ruminal acidosis. *Journal of Dairy Science* **87:** 1840-1847.

Ouwerkerk,D., Klieve,A.V., and Forster,R.J. (2002) Enumeration of Megasphaera elsdenii in rumen contents by real-time Taq nuclease assay. *Journal of Applied Microbiology* **92:** 753-758.

Owens,F.N., Secrist,D.S., Hill,W.J., and Gill,D.R. (1998) Acidosis in cattle: A review. *Journal of Animal Science* **76:** 275-286.

Ozutsumi,Y., Tajima,K., Takenaka,A., and Itabashi,H. (2006) Real-time PCR detection of the effects of protozoa on rumen bacteria in cattle. *Current Microbiology* **52:** 158-162.

Roe,B.A. (2004) Shotgun Library Construction for DNA Sequencing. In *Methods in Molecular Biology*. Zhao,S. and Stodolsky,M. (eds). Human Press Inc, Totowa, New Jersey, pp. 171-187.

Russell,J.B. and Baldwin,R.L. (1978) Substrate Preferences in Rumen Bacteria - Evidence of Catabolite Regulatory Mechanisms. *Applied and Environmental Microbiology* **36:** 319-329.

Russell,J.B. and Dombrowski,D.B. (1980) Effect of Ph on the Efficiency of Growth by Pure Cultures of Rumen Bacteria in Continuous Culture. *Applied and Environmental Microbiology* **39:** 604-610.

Russell,J.B. and Hino,T. (1985) Regulation of Lactate Production in Streptococcus-Bovis - A Spiraling Effect That Contributes to Rumen Acidosis. *Journal of Dairy Science* **68:** 1712-1721.

Sambrook,J. and Russel,D.W. (2001) *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Santos,S.R. and Ochman,H. (2004) Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. *Environmental Microbiology* **6:** 754-759.

Scardovi,V., Trovatte,L.D., Crociani,F., and Sgorbati,B. (1969) Bifid Bacteria in Bovine Rumen - New Species of Genus Bifidobacterium - B Globosum N.Sp. Andb Ruminale N.Sp. *Archiv fur Mikrobiologie* **68:** 278-&.

Slyter,L.L. (1976) Influence of Acidosis on Rumen Function. *Journal of Animal Science* **43:** 910-929.

Stewart,C.S., Flynt,H.J., and Bryant,M.P. (1997) The Rumen Bacteria. In *The Rumen Microbial Ecosystem*. Hobson,P.N. and Stewart,C.S. (eds). Blackie Academic and Professional, NY, pp. 10-72.

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

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

Tajima,K., Arai,S., Ogata,K., Nagamine,T., Matsui,H., Nakamura,M. et al. (2000) Rumen bacterial community transition during adaptation to high-grain diet. *Anaerobe* **6:** 273-284.

Wang,Q., Garrity,G.M., Tiedje,J.M., and Cole,J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73:** 5261-5267.

CHAPTER III

RUMEN MICROBIAL POPULATION DYNAMICS DURING

ADAPTATION TO HIGH CONCENTRATE DIET

Introduction

Ruminal environment is a complex microbial ecosystem that is composed of a great diversity of microorganisms that include an immense variety of bacteria, protozoa, and fungi (Ozutsumi et al., 2006). Among these microbial populations, the bacterial population predominates and has a significant effect on the animal's performance. Therefore, ruminant nutritionist and microbiologists manipulate the ruminal microbial ecosystem to increase the animal's performance by increasing the efficiency of feed conversion in the animal (Martin, 1998). But, when animals on a forage diet are directly put on a high grain diet a decrease in substrate conversion to volatile fatty acids and microbial protein has been observed (Klieve et al., 2003; Strobel and Russell, 1986) leading to decreased performance of the animal. During this change in diet from a forage diet to a concentrate diet, the ruminal pH decreases (Klieve et al., 2003; Nocek, 1997; Owens et al., 1998) and is accompanied with accumulation of lactic acid leading to sub acute or acute acidosis (Klieve et al., 2003). Therefore, several feeding programs have been implemented to adapt feedlot cattle from a high forage diet to high concentrate diet by gradually increasing the concentration of grain in the diet and decreasing the fiber content (Bevans et al., 2005; Rotger et al., 2006). These attempts have helped improve ruminal fermentation through synchronization of the fermentable energy sources with the available protein supplements present within the rumen (Rotger et al., 2006). During this adaptation to high grain diets, significant changes in the ruminal environment and microbial populations have been reported (Goad et al., 1998; Tajima et al., 2001; Tajima et al., 2000). This adaptation to a high concentrate diet from a high forage diet is

known to help establish a stable microbial population within the rumen and to minimize susceptibility to acidosis (Bevans et al., 2005; Klieve et al., 2003). The shift in rumen microbial population from a forage diet to a high concentrate diet has shown to change the odd-branched chain volatile fatty acids within the rumen and duodenum (Vlaeminck et al., 2006) suggesting that the change in the microbial population helps improve the animal's performance. But, microbial changes during this transition phase is poorly understood as only a few studies have been reported (Coe et al., 1999; Goad et al., 1998; Hungate, 1968; Mackie and Gilchrist, 1979; Tajima et al., 1999; Tajima et al., 2001; Tajima et al., 2000). Many of these studies have utilized culture based techniques to evaluate microbial population changes where only a very few rumen bacterial species have been isolated in pure culture (Tajima et al., 1999; Tajima et al., 2000; Whitford et al., 1998). Due to limitations in culturing rumen bacteria, the use of culture based techniques to evaluate microbial populations substantially underestimates the diversity of rumen microorganisms within the rumen. Therefore, to identify the microbial population changes during adaptation to high concentrate diet, we have utilized culture independent techniques using the 16S rRNA gene. We have evaluated microbial population structure and diversity using Terminal restriction Fragment length Polymorphisms (T-RFLP) and 16S rRNA libraries and have compared microbial population changes in animals on prairie hay and during adaptation to step-up diet. We have also evaluated microbial population changes in previously reported starch digesters, fiber digesters, lactate produces, and lactate utilizes using quantitative real-time PCR (qRT-PCR) to evaluate the role of these organisms during adaptation to high concentrate diet.

Experimental Procedures

Animals and Diets

Eight ruminally cannulated beef steers (837± 60 lbs) were fed prairie hay adlibitum, to have relatively similar background rumen bacterial populations. Following the adaptation phase on prairie hay, four animals were randomly selected and put on a step-up diet containing increasing amounts of metabolic energy with a fiber to concentrate ratio of 80:20, 60:40, 40:60 and 20:80 respectively to adapt the animals to high concentrate diet. The remaining four animals were maintained on prairie hay and were used as control animals. The step up diets were formulated to meet the animals requirements as described in NRC-1996, but was composed with 2.0, 2.4, 2.7 or 3.0 Mcal of ME/kg of dry matter with a fiber to concentrate ratio of 80:20, 60:40, 40:60 and 20:80 respectively. The animals were fed each diet for 7 days and rumen samples were collected and the animals were promoted to the next diet in the step-up diet regime. The control animals were maintained on prairie hay.

Sampling

Samples were collected from both control and animals on high concentrate diet at 7 day intervals to evaluate the microbial population changes during transition from prairie hay to high concentrate diet. The samples collected were snap frozen in liquid nitrogen and were stored at -20°C until DNA extraction.

DNA isolation

Frozen rumen samples were homogenized using a coffee grinder (Walmart, Stillwater, OK, USA), and 0.5 g of the homogenized sample was used to extract total bacterial DNA. Total bacterial DNA was extracted using the QIAamp® DNA mini stool kit (Qiagen, Valencia, CA, USA) according to manufacturers protocol with a few modifications. Briefly, 1.4 ml of buffer ASL was added to 0.5g of rumen sample and was vortexed for 15 sec. Following vortexing the sample was incubated at 95°C for 10 min. The sample was then vortexed again for 15 sec and was centrifuged at 20,000g for 1 min to pellet the cell debris and other suspended particles present in the sample. The supernatant was aspirated to a fresh 2 ml tube and an inhibitorEX tablet was added and vortexed immediately for 1 min, until the tablet was completely dissolved. The sample was then incubated at room temperature for 1 min to bind all inhibitors, and was centrifuged at 20,000g for 3 min to remove the inhibitorEX bound inhibitors. The solution was aspirated and re-centrifuged for an additional 3 min to remove any residual inhibitorEX. To 400 µl of the supernatant, 20 µl of proteinase K and 400 µl of AL buffer was added and incubated at 70°C for 30 min. 400 µl of 100% ethanol was added to the proteinase digested sample. This sample was then added to the QIAamp spin column and centrifuged at 20,000g for 1 min to bind the DNA. The column was washed with 500 µl of buffer AW1 and AW2 respectively. The spin columns were centrifuged at 20,000g to remove residual buffer AW2 and the DNA was eluted using 20 µl of distilled water. The concentration of the extracted DNA was measured using a UV spectrophotometer, and the quality of the DNA was evaluated by agarose gel-electrophoresis using a 1.2% gel.

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis

100 ng of total bacterial genomic DNA isolated from the rumen samples were used for Terminal-Rescticton Fragment Length Polymorphisum (T-RFLP) analysis (Kent et al., 2003a; Marsh et al., 2000b; Osborn et al., 2000b). 100 ng of total bacterial DNA was PCR amplified in a MJ dyad thermocycler (MJ research, Watertown, MA) using FAMBacT0008F 5'-AGAGTTTGATCCTGGCTCAG-3' and BacT0805R 5'-GGACTACCAGGGTATCTAATCCC-3' (Fernando et al., 2008d) to amplify a 809 bp fragment (in *E. coli*) of the 16S rRNA gene in all Eubacteria. The FAMBacT008F primer is labeled at the 5' end with the fluorophore 6-FAM. A 50 µl PCR reaction contained, 100 ng of DNA, 1X PCR buffer (Promega, Madison, WI, USA) 1.5 mM $MgCl_2$, 400 nM of each primer, 200 µM dNTPs, 100 ng/µl BSA and 3 U Taq DNA Polymerase (Promega, Madison, WI, USA). Cycling conditions were: one cycle of 1 min at 95ºC, 30 sec at 52ºC, 1 min at 72ºC followed by 34 additional cycles of 30 sec at 95ºC, 30 sec at 52ºC, 1 min at 72ºC and a final extension of 3 min at 72ºC. The amplified PCR products were ethanol precipitated as follows. The 50 µl PCR product was mixed with 3M sodium acetate to bring the solution to a concentration of 100 nM of sodium acetate. 2.5 volumes of 100% ethanol was added to the solution and was centrifuged at 4000 rpm for 30 min. The supernatant was aspirated and the pellet was washed with 70% ethanol and centrifuged at 4000 rpm for 15 min. The ethanol was removed and the pellet was air dried for 10 min and resuspended in 5 µl of distilled water. The precipitated PCR product was digested separately with *RsaI* (Invitrogen, Carlsbad, CA,USA), *HaeIII (*Invitrogen, Carlsbad, CA,USA) and *MspI (*Promega, Madison, WI, USA) according to the manufacturers protocols. The

restriction enzymes used in T-RFLP analysis were identified using software tools available at the Microbial community analysis web site (MiCA3) (http://mica.ibest.uidaho.edu/default.php). A 10 µl reaction contained 1X buffer, 2.5 U Enzyme and 5 µl of the PCR product. 250 ng/µl of BSA was added to the MSP I digestion. The Digestion reaction was incubated at 37°C for 4 hours, followed by 65°C for 20 min. 2 µl of the digested PCR product was mixed with 0.5 µl of GeneScan ROX 1000 size standard (Applied Biosystems, Foster City, CA) and 3.5 µl of loading dye (Applied Biosystems, Foster City, CA) and was electrophoresed for 8 hours in an ABI 377 sequence analyzer (Applied Biosystems, Foster City, CA). The data collected by the ABI 377 sequence analyzer was analyzed using GeneScan 3.1 analysis software (Applied Biosystems, Foster City, CA). The data set was subsequently normalized by dividing the cumulative peak height of each sample by the sample with the smallest cumulative peak height. The normalized T-RFLP profiles were further analyzed using Phylogenetic Assignment Tool (PAT) (Kent et al., 2003a) to assign species for each restriction fragment generated. The bacterial species assignment was based on the restriction digestion patterns obtained for all 3 enzymes of each sample.

The normalized peak area was used to construct Population fluctuation and microbial diversity plots. The normalized peak areas for the 3 different digests were averaged and were used as an estimate of bacterial population. The representation of each bacterial species within the total bacterial population was calculated as the percentage of peak area of a bacterial species relative to the total peak area of the sample. Therefore, the Population fluctuation and microbial diversity plots show the

bacterial diversity and the relative abundance of each bacterial species identified within the population.

Construction of 16S rRNA libraries and Sequencing

100 ng of total DNA from animals on high concentrate diet and on prairie hay was used for 16S library construction. Total of four 16S rDNA libraries were constructed from animals on prairie hay and high concentrate diet. Out of the four libraries two libraries were constructed by pooling equal amount of DNA from animals on each diet whereas, the other two libraries were constructed using individual animals. DNA from pooled and individual animals on prairie hay and high concentrate were used to PCR amplify an 809 bp fragment (in *E.coli*) using BacT0008F 5'-AGAGTTTGATCCTGGCTCAG-3' and BacT0805R 5'-GGACTACCAGGGTATCTAATCCC-3' primers using a high fidelity taq polymerase. The 50 µl PCR reaction contained, 100 ng of DNA, 1X PCR buffer (Invitrogen, Carlsbad, CA, USA) 1.5 mM $MgCl_2$, 400 nM of each primer, 200 µM dNTPs, and 2 U Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). Cycling conditions were: one cycle of 2 min at 95ºC, 30 sec at 52ºC, 1 min at 68ºC followed by 34 additional cycles of 20 sec at 95ºC, 30 sec at 52ºC, 1 min at 68ºC and a final extension of 3 min at 68ºC. The blunt ended PCR products generated were ligated in to PCR-Blunt® (Invitrogen, Carlsbad, CA) plasmid vector and transformed to DH5α max efficiency competent *E.coli* cells (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol to construct the four different 16S libraries. The transformed cells were grown overnight at 37°C on 22X22 cm Nunc® bioassay plates (Nalge

Nunc International, Rochester, NY, USA) containing 2X YT with 100 µg/ml of kanamycin. Random libraries of approximately 1920 colonies were picked from each library, grown in Terrific Broth (TB) medium (Sambrook and Russel, 2001) supplemented with 100 micrograms of kanamycin for 14 hours at 37°C with shaking at 250 rpm, and the sequencing templates were isolated by a cleared lysate-based protocol (Bodenteich et al., 1993; Roe, 2004).

Sequencing reactions were performed as previously described (Chissoe et al., 1995) using Thermus aquaticus (Taq) DNA polymerase, and either the Amersham ET fluorescent-labeled terminator or the Perkin-Elmer Cetus fluorescent-labeled Big Dye Taq terminator sequencing kits at a 1:16 dilution. The reactions were incubated for 60 cycles in a Perkin-Elmer Cetus DNA Thermocycler 9600 and after removal of unincorporated dye terminators by ethanol precipitation followed by a 70% ethanol wash, the fluorescent-labeled nested fragment sets were resolved by electrophoresis on an ABI 3700 Capillary DNA Sequencers. After base calling with Phred (Ewing et al., 1998), the analyzed data was transferred to a Sun Workstation Cluster, and assembled using Phrap (Ewing et al., 1998; Ewing and Green, 1998) Overlapping sequences (contigs) were analyzed using Consed (Gordon et al., 1998). Each clone was sequenced from both directions to have two fold coverage of each base. The resulting data was cleaned of vector sequences, using the software Crossmatch and was analyzed using analysis tools available at the Ribosomal Database Project (http://rdp.cme.msu.edu). The assembled data was blasted against the 16S RDP database (Cole et al., 2003; Cole et al., 2005) and the NCBI database to identify the bacterial populations present.

Phylogenetic and Comparative Analyses

The data was aligned using the RDP pipline available at *myRDP* space (Cole et al., 2007). The alignment generated was imported to the ARB software package (Ludwig et al., 2004) to generate phylogenetic trees with hierarchical classification of the sequences. Phylogenetic trees were constructed using the neighbor-joining method. Furthermore, "Classifier" (Wang et al., 2007) and "Library Compare" (Cole et al., 2005) software tools available at ribosomal database project (http://rdp.cme.msu.edu) were used to further analyze the sequences and to classify the clones to taxanomic hierarchy. The "Library Compare" software tool was used to compare the libraries with each other and to identify statistically, significant changes in bacterial populations present among the libraries (Cole et al., 2003; Cole et al., 2005).

Quantitative Real-Time PCR analysis

Quantitative analysis of population changes of selected bacterial species were conducted using real-time polymerase chain reaction (qPCR) analysis. Samples collected from both control animals on prarie hay and animals on high concentrate diet were used for realtime PCR analysis. The population changes were evaluated using SYBR green I reporter assay kit available from Roche Diagnosticis (Indianapolis, IN, USA). The primers used for analyzing *Prevotella ruminicola, Prevotella bryantii, Megaspaera elsdenii, Selenomonas ruminantium*, *Streptococcus bovis, Butyrivibrio fibrosolvens, Ruminococcus albus,* and *Fibrobacter succinosolvens* bacterial populations were, primers previously reported by Tajima et

al. (2001), Ouwerkerk et al. (2002), Ozutsumi et al. (2006) and (Fernando et al. (2008).

A 15 µl reaction contained 400 nM forward primer, 400 nM reverse primer, 1X Master mix (Roche Diagnosticis, Indianapolis, IN, USA), and 30 ng of total rumen DNA. Thermal cycling conditions were 95°C for 10 min, followed by 50 additional cycles of 95°C for 30 sec 50-62°C for 30 sec, depending on the annealing temperature of each primer set (see Table 1) and 72°C for 40 sec or 1 min based on the size of the amplicon followed by the melting curve. The single copy universal gene *rpoB* was used for normalization (Dahllof et al., 2000; Fernando et al., 2008d; Santos and Ochman, 2004). The qRT-PCR reactions were performed in an ABI PRISM 7500® sequence detection system (Applied Biosystems, Foster City, CA). Relative quantification of bacterial population changes were performed using the comparative C_T method as previously described (Hettinger et al., 2001). The ΔC_T value was determined by subtracting the 16S C_T value of each sample from the corresponding sample *rpoB* gene C_T value. Calculation of $\Delta\Delta C_T$ was carried out using the zero time point (when all animals were on prairie hay) mean ΔC_T value as an arbitrary constant to subtract from all other ΔC_T mean values. Fold changes in bacterial populations were calculated from the $\Delta\Delta$CT values using the formula $2^{\pm\Delta\Delta Ct}$. The animals on the same diet was then averaged to obtain the average bacterial population on each diet.

Results

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis

To determine the microbial diversity and microbial population dynamics within the rumen in animals fed with prairie hay and to evaluate the changes in microbial populations that occur during adaptation to high concentrate diet, T-RFLP analysis was performed on each rumen sample using 3 different restriction enzymes. Population fluctuation and microbial diversity plots generated based on T-RFLP analysis is shown in Figure 1. Each different bacterial genus identified is represented with a unique color where the width of each colored band represents the abundance of the bacterial genus within the population. The number of genera identified within each sample is shown in parentheses. T-RFLP analysis shows significant changes in microbial population structure and diversity between the control animals on prairie hay and during adaptation to high concentrate diet. This is apparent in the different colored banding pattern and the increasing number of bacterial genera detected during adaptation to high concentrate diet (Figure 1). When the animals on prairie hay was first put on the step up diet which contains 20% Corn and 80% prairie hay, the microbial population diversity decreased, but from the second diet onwards an increase in the microbial population diversity was detected, as more bacterial genera were identified with increasing amounts of metabolic energy in the diet. T-RFLP analysis identified 115 different bacterial genera among the two different diets. The animals on prairie hay displayed increased bacterial numbers in *Xylella sp., Xanthomonas sp., Mycoplasma sp. ,Thauera sp.* and *Bacteroides sp.* Whereas, animals on the step-up diet displayed an increase in *Prevotella sp., Actinobacillus*

sp., Alcanivorax sp., Arthrobacter sp., Bacteroides sp., methylobacillus sp., Megaspaera sp., and Taylorella sp.. As T-RFLP analysis identified a large diversity within the two ruminal environments, we analyzed the microbial population at phylum level by classifying the bacteria identified (Figure 2). This analysis displayed a significant increase in *Proteobacteria* and a significant decrease in *Fermicutes* during stages 3 and 4 of the step up diet.

16S rRNA library and Phylogenetic analysis

T-RFLP analysis only allows the identification of previously reported bacterial species in which the 16S ribosomal subunit has been sequenced. Therefore, to determine the complete microbial population structure in animals on prairie hay and during adaptation to high concentrate diet, we constructed several 16S rDNA libraries. The 16S rDNA libraries from animals on prairie hay and high concentrate diet were constructed by pooling equal amounts of DNA from all animals and using individual animals to compare microbial population structure and diversity within the rumen. 768 clones from each library was sequenced to identify microbial population structure and diversity. The data generated from the sequenced libraries were analyzed using sequence analysis software available at the ribosomal database project. Libraries constructed from animals on prairie hay and high concentrate diet were compared using the “Library compare” software program available at the ribosomal database project. The analysis detected very significant changes in rumen bacterial population diversity and structure among animals on prairie hay and high concentrate diet (Figure 3 and Table 2 & 3). The animals on prairie hay displayed a

significantly high number of *Fibrobacteres*, whereas, the animals on high concentrate displayed large numbers in *Bacteriotedes* (Figure 3a). All the bacteria detected in phylum *Fibrobacteres* belonged to the genus *Fibrobacter*, while most bacterial species in the phylum *Bacteriotedes* belong to the genus *Prevotella*. Although, no significant difference in *Fermicutes* populations were detected between the two libraries, the analysis of *Firmicutes* populations detected the presence of significantly different bacterial species within the two treatments (Figure 3b), where *Clostridiaceae* and *Acidaminococcaceae* populations were significantly different. Hierarchical classification of the 16S rDNA libraries detected the presence of 620 (prairie hay) and 677 (High concentrate) clones belonging to the domain Bacteria. Hierarchical classification also detected 5 clones (prairie hay) belonging to domain Archaea. Among the 620 clones identified on prairie hay, 15 clones belong to *Spirochaetes*, 19 to *Fibrobacteres*, 219 to *Firmicutes*, 15 to *Proteobacteria* and 147 to *Bacteroidetes*, while among the 677 clones identified on high concentrate diet, 24 clones belong to *Spirochaetes*, 2 to *Fibrobacteres*, 271 to *Firmicutes*, 6 to *Proteobacteria* and 303 to *Bacteroidetes*. But most interestingly, we detected 202 and 67 unclassified bacterial species in animals on prairie hay and high concentrate diet respectively.

Phylogenetic analysis of the 16S rRNA libraries sequenced was performed using arb software package (Ludwig et al., 2004). The two libraries constructed from each treatment were pooled to identify a set of non-redundant Operational Taxanomic Units (OTUs) for each treatment. The Non-redundant 16S rRNA sequences identified were used to construct phylogenetic trees for each treatment. Phylogenetic

trees were constructed using the neighbor joining method using the p-distance model with 1000 replications of bootstrapping (Figures 4 & 5). Phylogenetic analysis demonstrated great diversity in both animals on prairie hay and on high concentrate diet. The libraries constructed from prairie hay displayed 261 different OTUs and libraries constructed from high concentrate diet displayed 263 OTUs. A significantly large number of phylogenetically distant *Firmicutes* populations were present in animals on prairie hay. These OTUs displayed very high redundancy accounting to more than 40% of the total sequences. The libraries constructed from animals on high concentrate diet displayed a significantly high number of *Bacteriodetes* sp. that was phylogenetically distinct from other bacteria which accounted for more than 45% of the total sequences. A few Archaea bacterial species were also detected on animals on prairie hay and belong to the genus *Methanobrevibacter sp.*

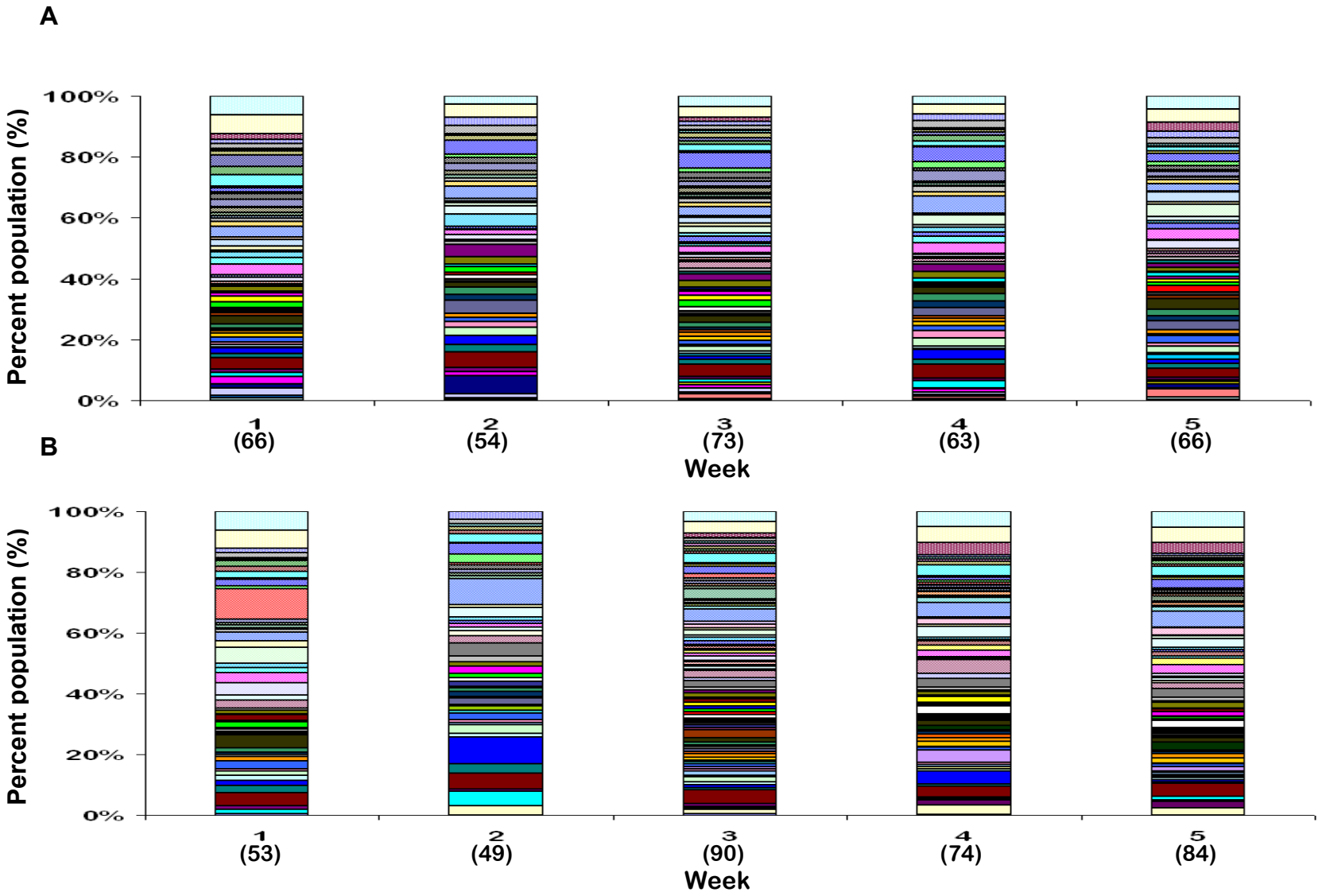
Quantitative Real-Time PCR analysis

Quantitative realtime PCR analysis was performed to evaluate the role of previously reported fiber and starch digesters in animals on prairie hay and high concentrate diet. Primers were developed to amplify specific regions of the 16S ribosomal subunit. Quantitative realtime PCR (qRT-PCR) results are summarized in Figure 6. Quantitative realtime PCR analysis displayed a 11 fold increase in *Megaspaera elsdenii* populations by the 3rd regime of the step up diet but decreased to 6 fold by diet 4. The animals on prairie hay were consistently low and did not change significantly. *Streptococcus bovis* increased 2 fold by the start of the step up diet but decreased by the end of the step up diet regime, and did not show significant

change in population. *Selenomonas ruminantium* increased 30 fold by the second stage of the step up diet and was 30 fold higher there after when compared to the animals on prairie hay. *Prevotella sp.* displayed opposite patterns of population changes, where *Prevotella ruminicola* populations increased 100 fold in both animals on prairie hay and on high concentrate diet on week 2 but decreased by week 3, but *Prevotella bryantii* populations increased gradually and reached 8000 fold by the 3rd regime of the step up diet, and decreased rapidly by diet 4. *Butyrivibrio fibrosolven* and *Fibrobacter succinogenes* populations gradually decreased as animals were adapted to high concentrate diet and were 15 and 40 fold lower respectively. *Ruminococcus flavefaciens* populations also displayed a significant decrease in bacterial populations during adaptation to high concentrate diet when compared to the control animals on prairie hay, where populations decreased 60 fold by diet 4.

Figure 1

Population fluctuation and microbial diversity plots of animals on high concentrate diet and control animals on prairie hay. The Population fluctuation and microbial diversity plots were constructed based on T-RFLP analysis. The different color banding pattern shows significant changes in microbial population diversity. The numbers within parentheses represent the number of different genera identified in each sample. (A) animals on prairie hay, (B) animals on high concentrate diet.


A
Percent population (%)
100%
80%
60%
40%
20%
0%
1
(66)
2
(54)
3
(73)
4
(63)
5
(66)
Week
B
Percent population (%)
100%
80%
60%
40%
20%
0%
1
(53)
2
(49)
3
(90)
4
(74)
5
(84)
Week


Acholeplasma sp.
Alcanivorax sp.
Aranicola sp.
Bacillus sp.
Brevibacillus sp.
Caryophanon sp.
Cytophaga sp.
Desulfotomaculum sp.
Eubacterium sp.
Flexibacter sp.
Gelidibacter sp.
Halobacteroides sp.
Herbaspirillum sp.
Lactobacillus sp.
Melittangium sp.
Microbacterium sp.
Moorella sp.
Nocardia sp.
Paenibacillus sp.
Persicobacter sp.
Prevotella sp.
Rhodococcus sp.
Saccharococcus sp.
Spirillum sp.
Sporosarcina sp.
Streptomyces sp.
Thauera sp.
Thermodesulfovibrio sp.
Vibrio sp.
Acidosphaera sp.
Amphibacillus sp.
Archangium sp.
Bacteroides sp.
Burkholderia sp.
Clostridium sp.
Dermatophilus congolensis
Desulfovibrio sp.
Exiguobacterium sp.
Frankia sp.
Haemophilus sp.
Halomonas sp.
Kineosporia sp.
Leptospira sp.
Methylobacillus sp.
Micrococcus sp.
Mycoplasma sp.
Oceanospirillum sp.
Pasteurella sp.
Photobacterium sp.
Pseudomonas sp.
Rhodospirillum sp.
Salinicoccus sp.
Spirochaeta sp.
Staphylococcus sp.
Sulfobacillus sp.
Thermoactinomyces sp.
Thiobacillus sp.
Xanthomonas sp.
Actinobacillus sp.
Amycolatopsis sp.
Arthrobacter sp.
Bifidobacterium sp.
Butyrivibrio sp.
Colwellia sp.
Desulfitobacterium sp.
Entomoplasma sp.
Flavobacterium sp.
Fusibacter sp.
Haloanaerobacter sp.
Heliobacterium sp.
Kitasatospora sp.
Leptothrix sp.
Methylomicrobium sp.
Microcystis sp.
Myxococcus sp.
Oerskovia sp.
Pedobacter sp.
Planococcus sp.
Pseudoramibacter sp.
Ruminobacter sp.
Selenomonas sp.
Spiroplasma sp.
Streptobacillus sp.
Taylorella sp.
Thermobacillus sp.
Tissierella sp.
Xylella sp.
Agromyces sp.
Angiococcus sp.
Azoarcus sp.
Bordetella sp.
Caldicellulosiruptor sp.
Corynebacterium sp.
Desulfobacterium sp.
Erysipelothrix sp.
Flectobacillus sp.
Fusobacterium sp.
Halobacillus sp.
Heliorestis sp.
Kurthia sp.
Megasphaera sp.
Methylophilus sp.
Microscilla sp.
Nitrosomonas sp.
Oxalophagus sp.
Peptostreptococcus sp.
Porphyromonas sp.
Psychroserpens sp.
Ruminococcus sp.
Sphingobacterium sp.
Sporohalobacter sp.
Streptococcus sp.
Telluria sp.
Thermobispora sp.
Treponema sp.

Figure 2

Summarized, hierarchical classification of Terminal Restriction Fragment length Polymorphism (T-RFLP) data. The data displayed is classified based on phylum. The predominant phyla are depicted. The remaining phyla are pooled and displayed as “others”.

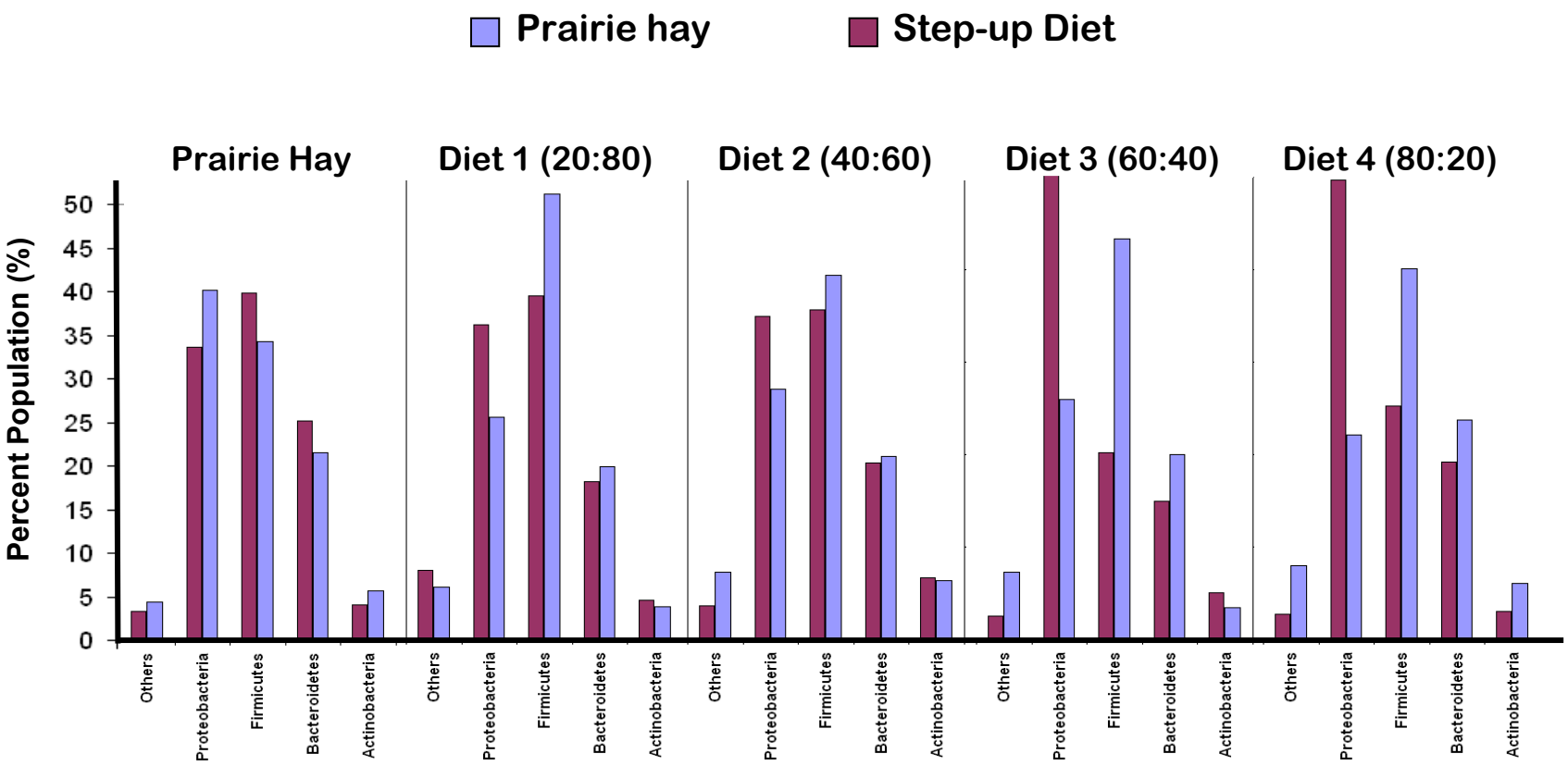

Prairie hay
Step-up Diet
Prairie Hay
Diet 1 (20:80)
Diet 2 (40:60)
Diet 3 (60:40)
Diet 4 (80:20)
Percent Population (%)
0
5
10
15
20
25
30
35
40
45
50
Others
Proteobacteria
Firmicutes
Bacteroidetes
Actinobacteria

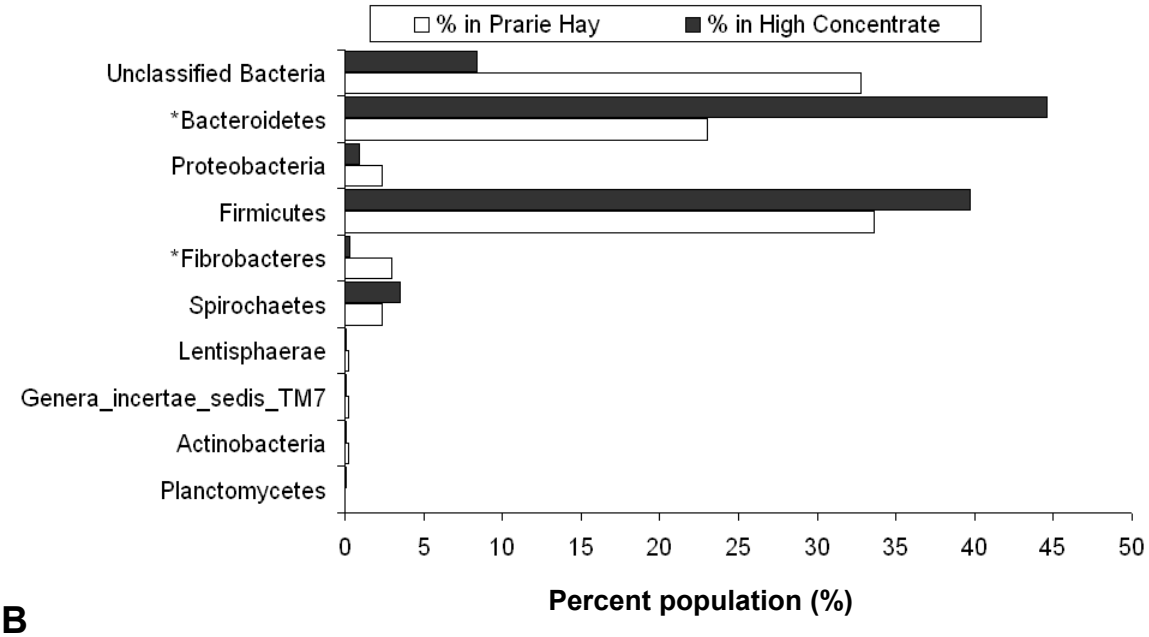
Figure 3

Comparison of Libraries at the phylum level using “Library Compare” software available at the ribosomal database project. Populations that are significantly different at p> 0.01 are shown with an asterix “*”.

(A) Total population (Prairie hay Vs High concentrate)

(B) Distribution of *Firmicutes* among animals on prairie hay Vs high concentrate

A


% in Prarie Hay
% in High Concentrate
Unclassified Bacteria
*Bacteroidetes
Proteobacteria
Firmicutes
*Fibrobacteres
Spirochaetes
Lentisphaerae
Genera_incertae_sedis_TM7
Actinobacteria
Planctomycetes
0
5
10
15
20
25
30
35
40
45
50
Percent population (%)

B

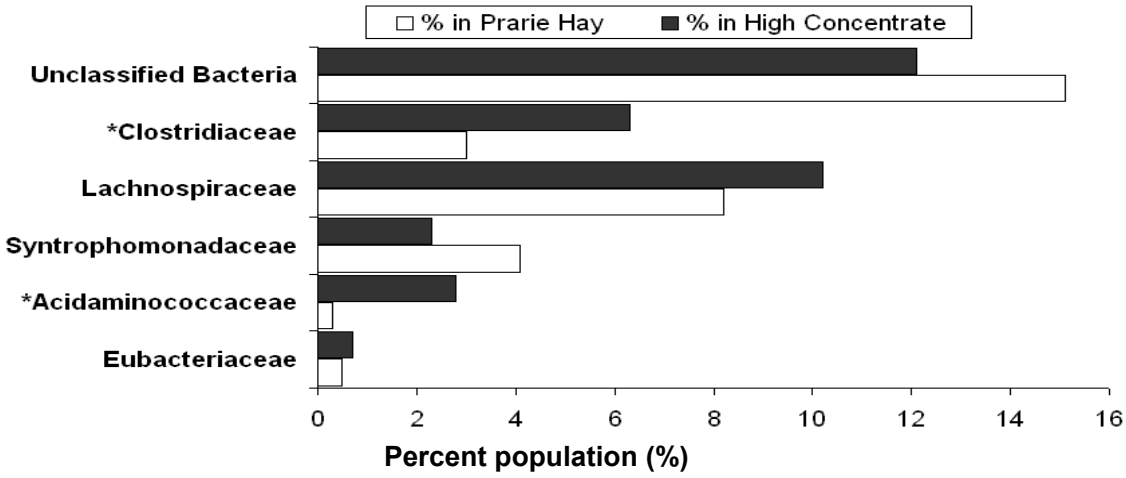

% in Prarie Hay
% in High Concentrate
Unclassified Bacteria
*Clostridiaceae
Lachnospiraceae
Syntrophomonadaceae
*Acidaminococcaceae
Eubacteriaceae
0
2
4
6
8
10
12
14
16
Percent population (%)

Figure 4

Phylogenetic analysis of libraries constructed from prairie hay. A consensus phylogenetic tree constructed using the Neighbour joining method is shown. The scale bars indicates the length of 10 substitutions per 100 residues. The numbers represent the redundancy of each clone.

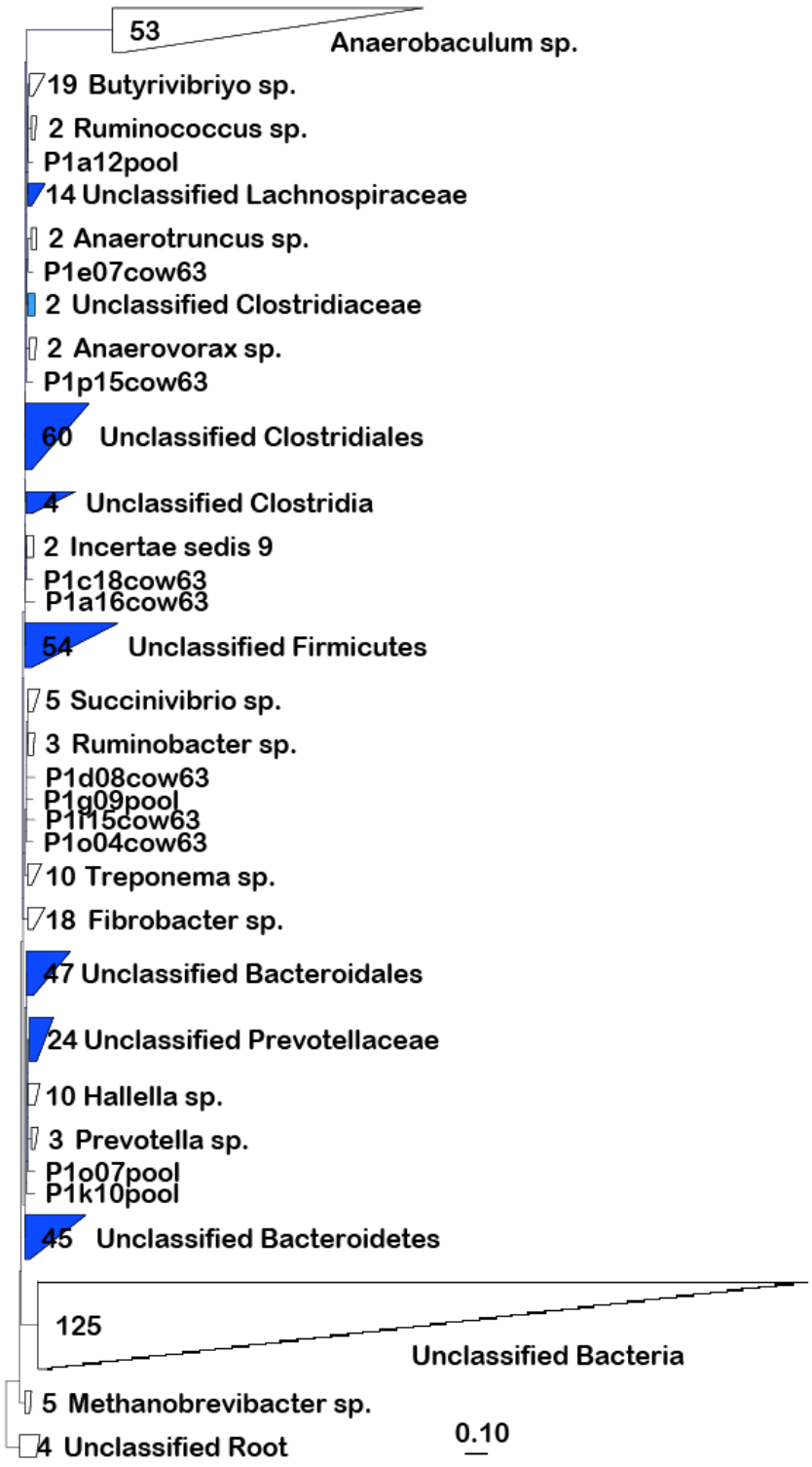

53 Anaerobaculum sp.
19 Butyrivibriyo sp.
2 Ruminococcus sp.
P1a12pool
14 Unclassified Lachnospiraceae
2 Anaerotruncus sp.
P1e07cow63
2 Unclassified Clostridiaceae
2 Anaerovorax sp.
P1p15cow63
60 Unclassified Clostridiales
4 Unclassified Clostridia
2 Incertae sedis 9
P1c18cow63
P1a16cow63
54 Unclassified Firmicutes
5 Succinivibrio sp.
3 Ruminobacter sp.
P1d08cow63
P1g09pool
P1f15cow63
P1o04cow63
10 Treponema sp.
18 Fibrobacter sp.
47 Unclassified Bacteroidales
24 Unclassified Prevotellaceae
10 Hallella sp.
3 Prevotella sp.
P1o07pool
P1k10pool
45 Unclassified Bacteroidetes
125 Unclassified Bacteria
5 Methanobrevibacter sp.
4 Unclassified Root
0.10

Figure 5

Phylogenetic analysis of libraries constructed from animals on high concentrate diet. A consensus phylogenetic tree constructed using the Neighbour joining method is shown. The scale bars indicates the length of 10 substitutions per 100 residues. The numbers represents the redundancy of each clone.

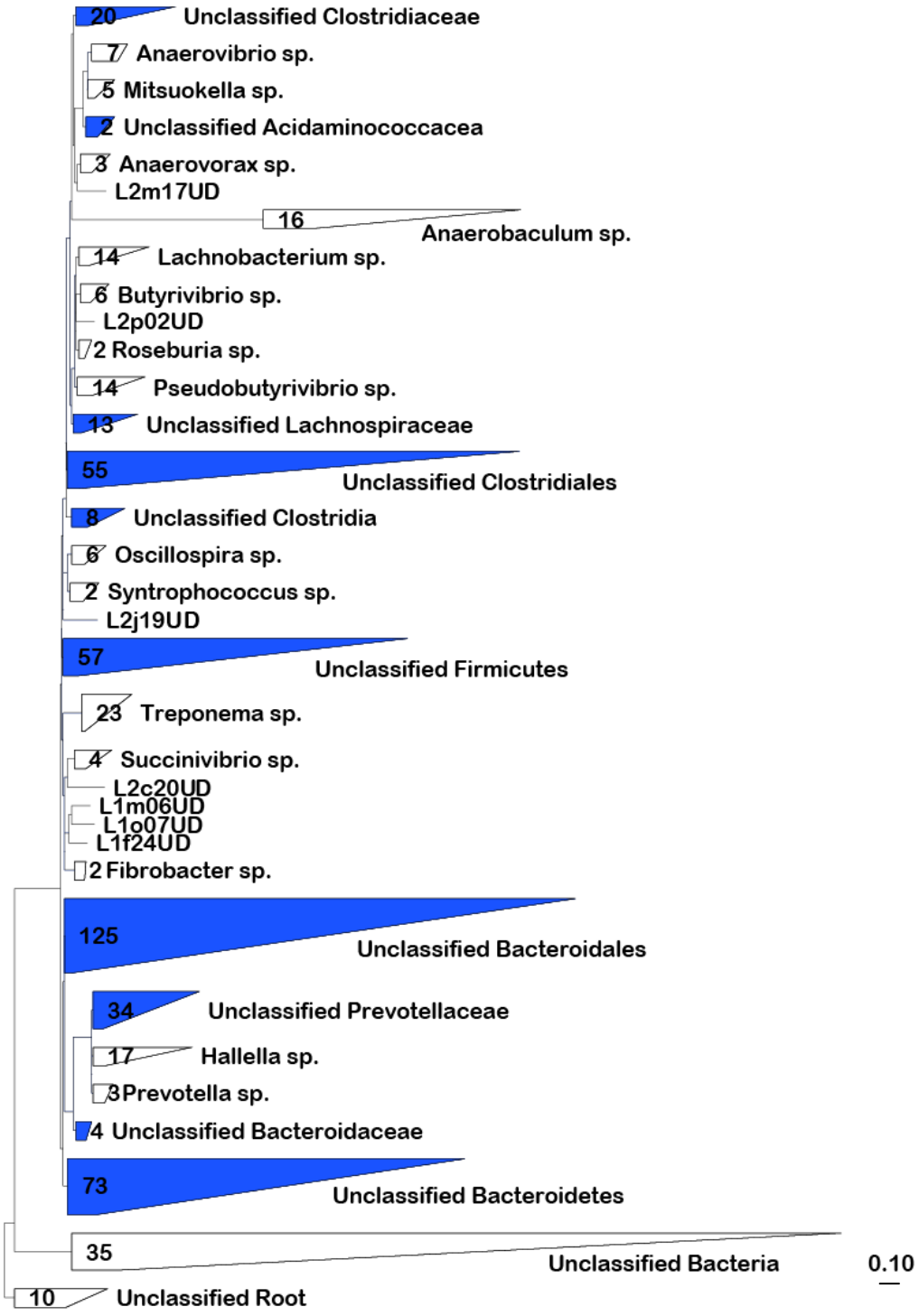
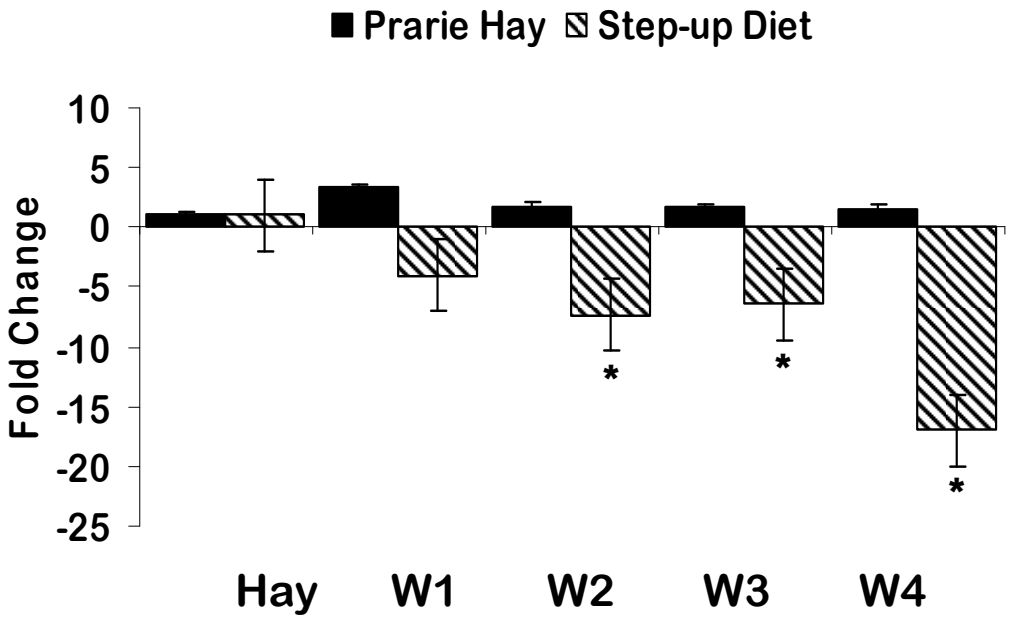

20 Unclassified Clostridiaceae
7 Anaerovibrio sp.
5 Mitsuokella sp.
2 Unclassified Acidaminococcacea
3 Anaerovorax sp.
L2m17UD
16 Anaerobaculum sp.
14 Lachnobacterium sp.
6 Butyrivibrio sp.
L2p02UD
2 Roseburia sp.
14 Pseudobutyrivibrio sp.
13 Unclassified Lachnospiraceae
55 Unclassified Clostridiales
8 Unclassified Clostridia
6 Oscillospira sp.
2 Syntrophococcus sp.
L2j19UD
57 Unclassified Firmicutes
23 Treponema sp.
4 Succinivibrio sp.
L2c20UD
L1m06UD
L1o07UD
L1f24UD
2 Fibrobacter sp.
125 Unclassified Bacteroidales
34 Unclassified Prevotellaceae
17 Hallella sp.
3 Prevotella sp.
4 Unclassified Bacteroidaceae
73 Unclassified Bacteroidetes
35 Unclassified Bacteria
0.10
10 Unclassified Root

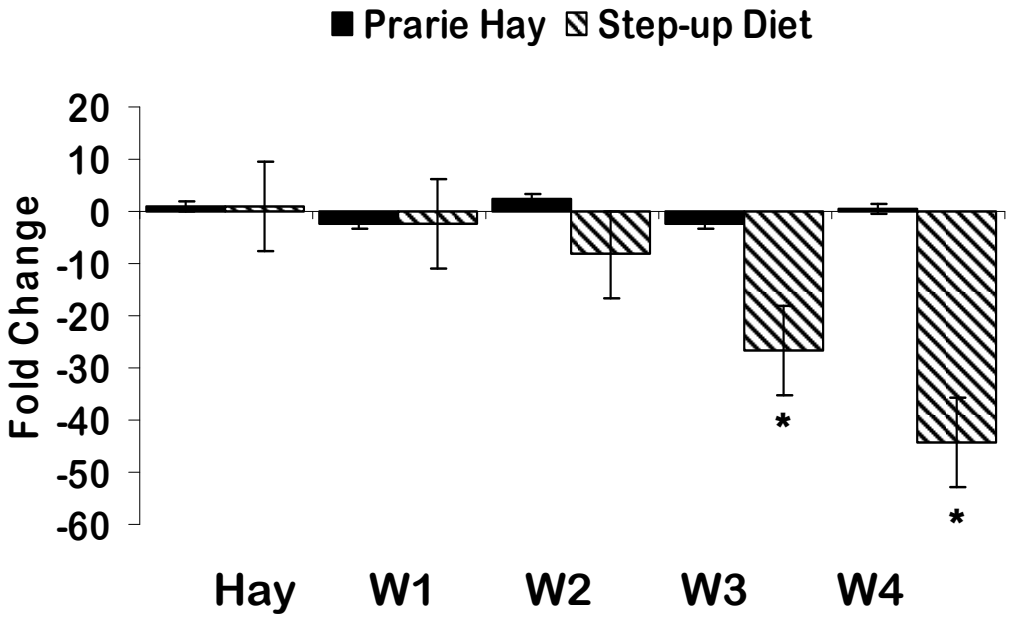
Figure 6

Population changes of selected rumen bacterial species using qRT-PCR during adaptation to high concentrate diet when compared to prairie hay. Microbial population changes were calculated as described in materials and methods. The microbial population changes that were significant at $P<0.05$ are shown with an asterix"*".

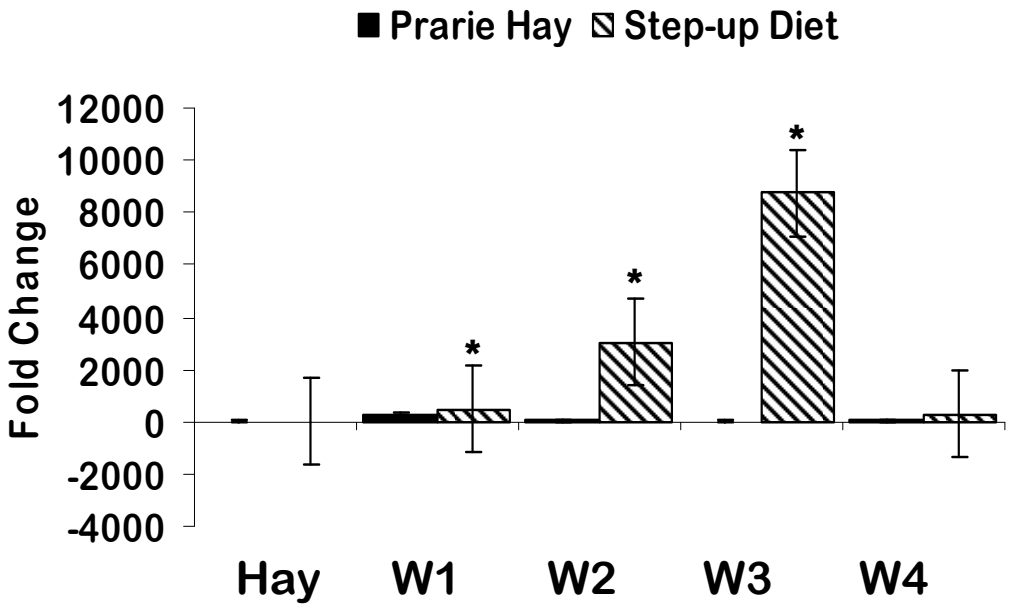
Butyrivibrio fibrosolvans



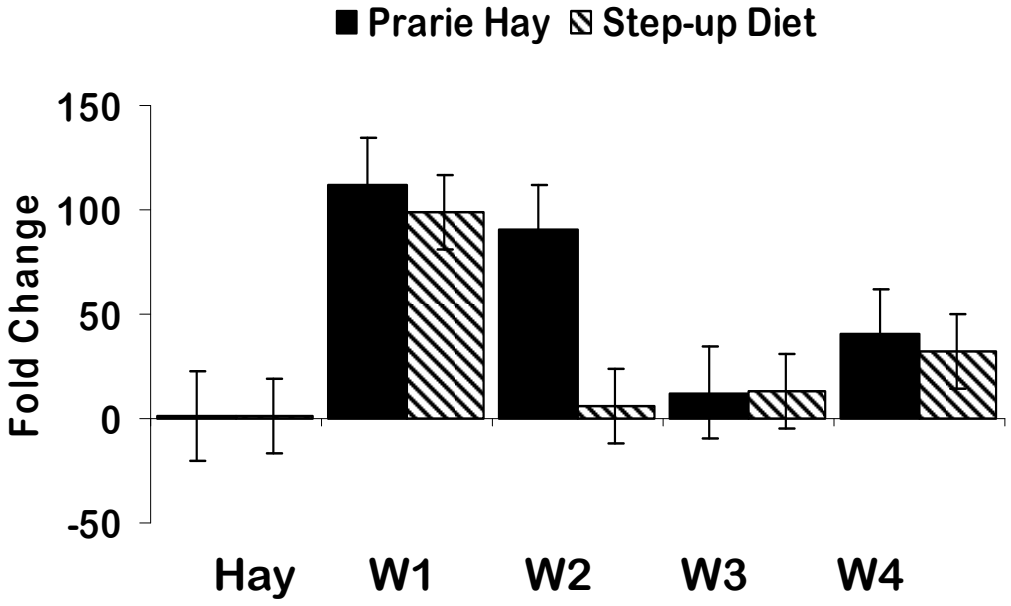
Fibrobacter succinogenes



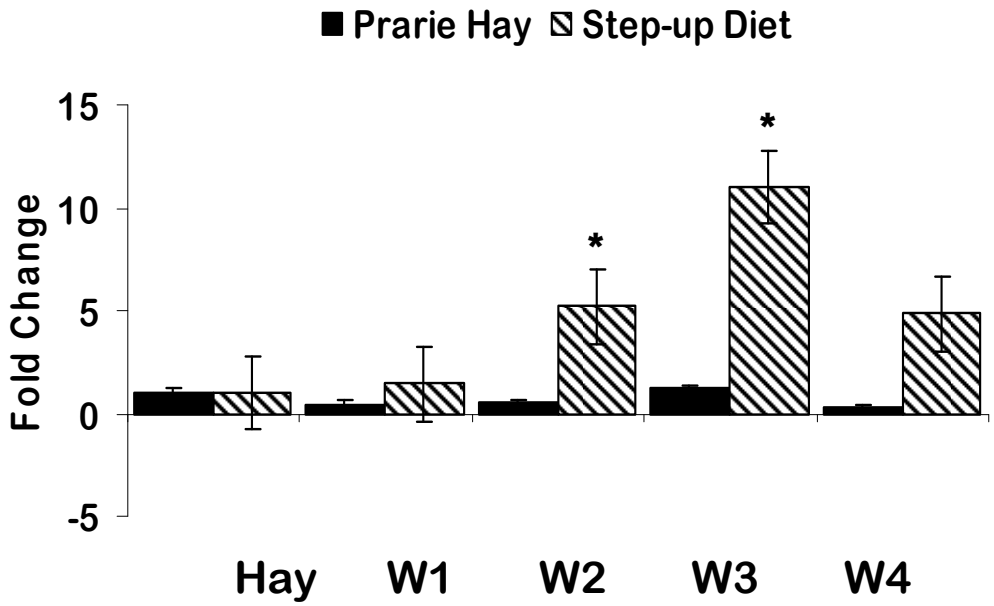
Prevotella bryantii



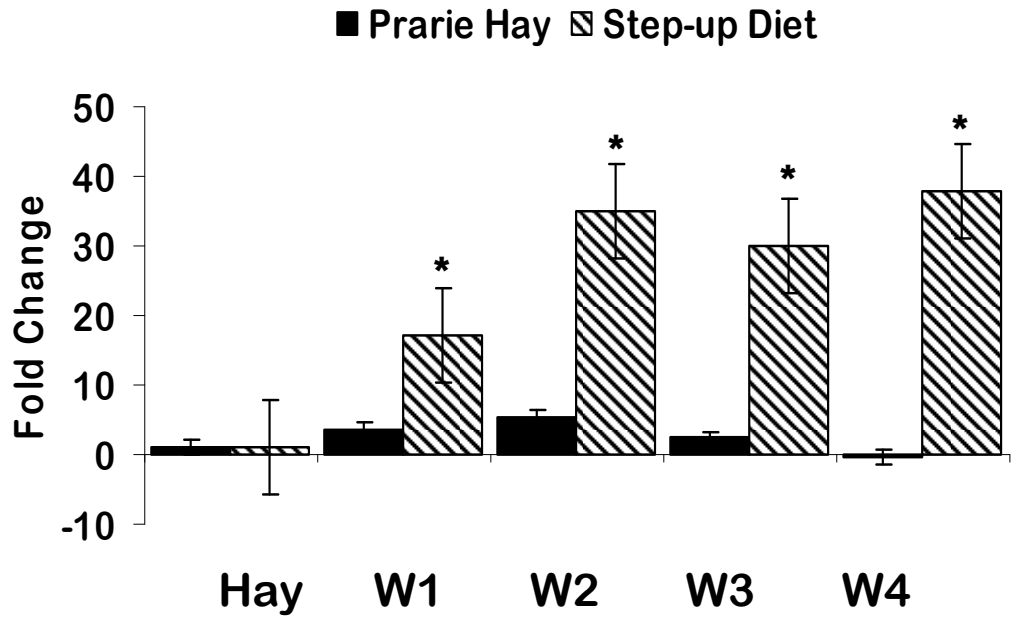
Prevotella ruminicola



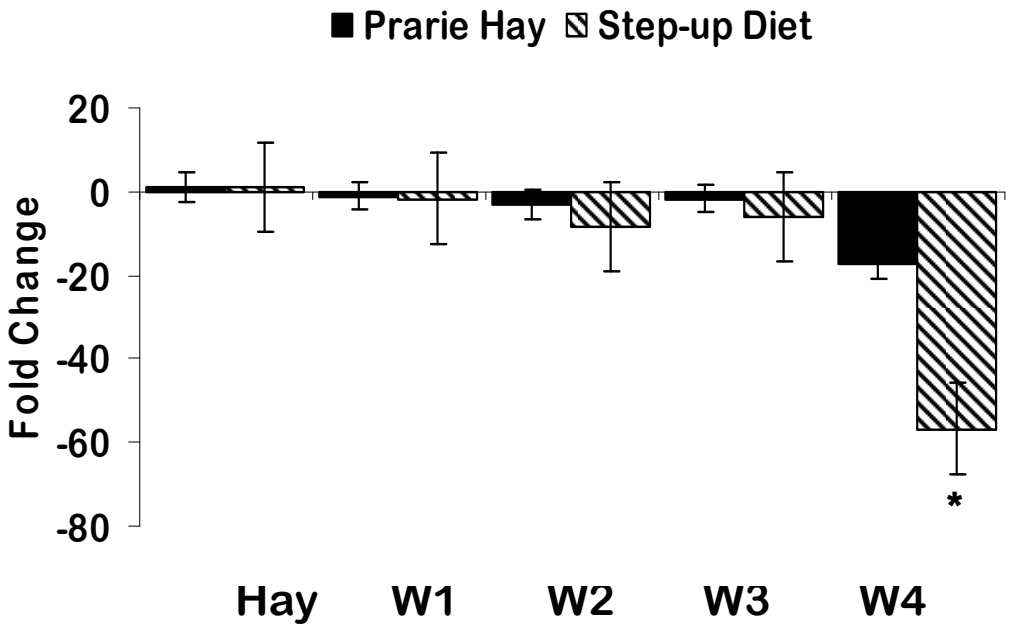
Megasphera elsdenii



Selenomonas ruminantium



Ruminococcus flavefasciens



Streptococcus bovis

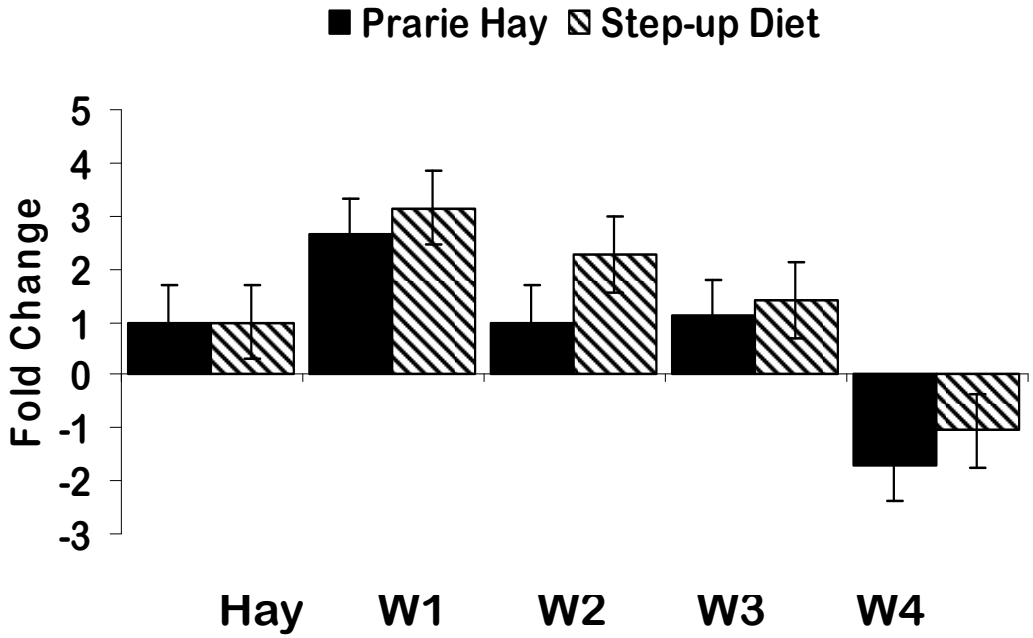


Table 1. Primer sequences used for Real Time PCR analysis.

Primer	Sequence 5'-3'	Product Size (bp)	Tm Used (°C)	Reference
Prevotella ruminicola -For	GGTTATCTTGAGTGAGTT	485	58	(Tajima et al., 2001)
Prevotella ruminicola -Rev	CTGATGGCAACTAAAGAA			
Prevotella bryantii -For	ACTGCAGCGCGAACTGTCAGA	421	58	(Tajima et al., 2001)
Prevotella bryantii -Rev	ACCTTACGGTGGCAGTGTCTC			
Fibrobacter succinogenes -For	GGTATGGGATGAGCTTGC	445	62	(Tajima et al., 2001)
Fibrobacter succinogenes -Rev	GCCTGCCCCTGAACTATC			
Selenomonas ruminantium -For	TGCTAATACCGAATGTTG	513	53	(Tajima et al., 2001)
Selenomonas ruminantium -Rev	TCCTGCACTCAAGAAAGA			
Megasphaeara eslandi i-For	GACCGAAACTGCGATGCTAGA	128	60	(Ouwerkerk et al., 2002)
Megasphaeara eslandii -Rev	TCCAGAAAGCCGCTTTCGCCACT			
Streptococcus bovis -For	ATTCTTAGAGATAGGGTTTCTCTT	134	60	(Fernando et al., 2008d)
Streptococcus bovis -Rev	ACCTTATGATGGCAACTAACAATA			
Ruminococcus flavefaciens -For	TCTGGAAACGGATGGTA	295	55	(Koike and Kobayashi, 2001)
Ruminococcus flavefaciens -Rev	CCTTTAAGACAGGAGTTTACAA			
Butyrivibrio fibrisolvens -For	CGCATGATGCAGTGTGAAAAGCTC	625	56	(Fernando et al., 2008d)
Butyrivibrio fibrisolvens -Rev	CCTCCCGACACCTATTATTCATCG			
rpoB –For	AACATCGGTTTGATCAAC	371-390	53.5	(Dahllof et al., 2000)
rpoB –Rev	CGTTGCATGTTGGTACCCAT			

Table 2. Classification and comparison of the 16S libraries constructed from animals on prairie hay and high concentrate diet. The 16S libraries constructed were analyzed using software tools available at the ribosomal database project using a confidence threshold value of 80%.

Rank	Library 1 Prairie Hay	Library 2 High Concentrate
Domain Archaea	5	0
Phylum Euryarchaeota	5	0
Class Methanobacteria	5	0
Order Methanobacteriales	5	0
Family Methanobacteriaceae	5	0
Genus Methanobrevibacter	5	0
Domain Bacteria	620	677
Phylum Planctomycetes	0	1
Class Planctomycetacia	0	1
Order Planctomycetales	0	1
Family Planctomycetaceae	0	1
Unclassified Planctomycetaceae	0	1
Phylum Actinobacteria	1	1
Class Actinobacteria	1	1
Subclass Coriobacteridae	1	1
Order Coriobacteriales	1	1
Suborder Coriobacterineae	1	1
Family Coriobacteriaceae	1	1
Phylum Genera_incertae_sedis_TM7	1	1
Genus TM7	1	1
Phylum Lentisphaerae	1	1
Class Lentisphaerae	1	1
Unclassified Lentisphaerae	1	1
Phylum Spirochaetes	15	24
Class Spirochaetes	15	24
Order Spirochaetales	15	24
Family Spirochaetaceae	14	24
Genus Treponema	14	24
Unclassified Spirochaetales	1	0
Phylum Fibrobacteres	19	2
Class Fibrobacteres	19	2
Order Fibrobacterales	19	2
Family Fibrobacteraceae	19	2
Genus Fibrobacter	19	2
Phylum Firmicutes	219	271
Class Mollicutes	2	0
Order Anaeroplasmatales	2	0
Family Anaeroplasmataceae	2	0
Genus Anaeroplasma	2	0
Class Bacilli	0	2

Order Lactobacillales	0	2
Family Incertae sedis 9	0	1
Genus Syntrophococcus	0	1
Unclassified Lactobacillales	0	1
Class Clostridia	205	247
Order Clostridiales	199	234
Family Eubacteriaceae	3	5
Genus Anaerovorax	3	4
Unclassified Eubacteriaceae	0	1
Family Acidaminococcaceae	3	20
Genus Schwartzia	0	1
Genus Mitsuokella	0	6
Genus Papillibacter	2	1
Genus Anaerovibrio	1	9
Unclassified Acidaminococcaceae	0	3
Family Syntrophomonadaceae	30	19
Genus Anaerobaculum	30	19
Family Lachnospiraceae	48	72
Genus Pseudobutyrivibrio	1	16
Genus Shuttleworthia	0	1
Genus Lachnobacterium	0	17
Genus Oribacterium	0	1
Genus Roseburia	0	2
Genus Butyrivibrio	23	10
Unclassified Lachnospiraceae	24	25
Family Clostridiaceae	21	38
Genus Anaerotruncus	2	0
Genus Acetivibrio	8	1
Genus Sporobacter	5	5
Unclassified Clostridiaceae	6	32
Unclassified Clostridiales	94	80
Unclassified Clostridia	6	13
Unclassified Firmicutes	12	22
Phylum Proteobacteria	15	6
Class Deltaproteobacteria	2	1
Order Desulfovibrionales	1	0
Unclassified Desulfovibrionales	1	0
Order Myxococcales	1	0
Suborder Cystobacterineae	1	0
Unclassified Cystobacterineae	1	0
Unclassified Deltaproteobacteria	0	1
Class Gammaproteobacteria	9	5
Order Aeromonadales	9	5
Family Succinivibrionaceae	9	5
Genus Succinivibrio	6	4
Genus Ruminobacter	3	0
Unclassified Succinivibrionaceae	0	1

Unclassified Proteobacteria	4	0
Phylum Bacteroidetes	147	303
Class Sphingobacteria	1	0
Order Sphingobacteriales	1	0
Unclassified Sphingobacteriales	1	0
Class Bacteroidetes	82	18
Order Bacteroidales	82	18
Family Bacteroidaceae	1	18
Genus Anaerophaga	1	18
Family Porphyromonadaceae	3	2
Genus Tannerella	3	2
Family Prevotellaceae	22	33
Genus Prevotella	22	33
Unclassified Bacteroidales	56	127
Unclassified Bacteroidetes	64	122
Unclassified Bacteria	202	67

Table 3. Comparison of Libraries constructed using prairie hay and high concentrate diet at the phylum level using "Library Compare" software available at the ribosomal database project. Populations that are significantly different at p> 0.01 are shown with an asterix "*". Values are shown as percentage from total population

(A) Total population (Prairie hay Vs High concentrate)

(B) Distribution of *Firmicutes* among animals on prairie hay Vs high concentrate

Phylum	% in Prarie Hay	% in Concentrate
Planctomycetes	0.0	0.1
Actinobacteria	0.2	0.1
Genera_incertae_sedis_TM7	0.2	0.1
Lentisphaerae	0.2	0.1
Spirochaetes	2.4	3.5
*Fibrobacteres	3.0	0.3
Firmicutes	33.6	39.7
Proteobacteria	2.4	0.9
*Bacteroidetes	23.0	44.6
Unclassified Bacteria	32.8	8.4

*Significantly different at 0.01

Phylum	% in Prarie Hay	% in High Concentrate
Eubacteriaceae	0.5	0.7
*Acidaminococcaceae	0.3	2.8
Syntrophomonadaceae	4.1	2.3
Lachnospiraceae	8.2	10.2
*Clostridiaceae	3.0	6.3
Unclassified Bacteria	15.1	12.1

*Significantly different at 0.01

Discussion

High grain adaptation programs for feedlot cattle from a high forage diet to high concentrate diet are widely used to balance enhanced growth performance against the risk of acidosis (Bevans et al., 2005). During adaptation to high grain diets significant changes in the ruminal environment and microbial populations have been reported (Goad et al., 1998; Tajima et al., 2001; Tajima et al., 2000). This adaptation to a high concentrate diet from a high forage diet is known to help establish a stable microbial population within the rumen and to help minimize susceptibility to acidosis (Bevans et al., 2005; Klieve et al., 2003). The shift in rumen microbial population from a forage diet to a high concentrate diet is of great interest as it helps improves the animal's performance by increasing gain efficiency and average daily gain (ADG) (Bevans et al., 2005). But, microbial changes during this transition phase is poorly understood as only a few studies have been reported (Coe et al., 1999; Goad et al., 1998; Hungate, 1968; Mackie and Gilchrist, 1979; Tajima et al., 1999; Tajima et al., 2001; Tajima et al., 2000) and many of these studies have utilized culture based techniques where only a very few rumen bacterial species have been isolated in pure culture (Tajima et al., 1999; Tajima et al., 2000; Whitford et al., 1998). Therefore, in this study we present rumen microbial population dynamics using several molecular techniques and present a broader picture of the rumen microbial changes during adaptation to high concentrate diet from a forage diet.

The survey of rumen microbial species using T-RFLP analysis demonstrated significant changes in rumen microbial population during adaptation to high

concentrate diet when compared to animals on high concentrate diet. As shown in figure 1, when the animals on prairie hay was first put on the step-up diet the microbial population diversity dropped. This may be because when the animals were first shifted to the step-up diet, the microbial population within the rumen was not adapted to utilize the 20% corn present in the diet as the microbial population was mainly composed of fiber digesters and cellulolytic bacteria. This observation of a drop in fiber digesters when shifted to a grain diet is similar to the observation reported by Tajima et. al. in 2001, where he reported the decrease of *Treponema bryantii, Fibrobacter succinogenes* and *Ruminococcus falvefaciens* when feeding high grain diets. But, during the second diet of the step-up diet the microbial population diversity started to increase in animals on the step-up diet compared to animals on prairie hay and was at the highest diversity. This increase in microbial diversity may be due to the increase in metabolizable substrate within the rumen promoting the growth of amylolytic bacteria and other bacterial species. Goad and coworkers (1998) also detected a similar change in the rumen microbial population numbers where they observed an increase of total viable anaerobic and amylolytic bacteria when compared to animals on prairie hay. On diets 3 and 4 the microbial population decreased slightly, this may be due to the increasing fermentable substrates in the diet causing a decrease in ruminal pH leading to sub acute acidosis. This survey of the rumen microbial changes during adaptation to high concentrate diet using T-RFLP analysis is among the first reported and identifies a large number of bacterial species within the rumen (Figure 1). The presence of many of these bacterial species has not been reported from the bovine rumen. The

phylogenetic analysis of the T-RFLP data suggests an increase in *Proteobacteria* and a decrease in *Firmicutes* during adaptation to high concentrate diet (Figure 2). Among the increasing proteobacterial populations T*aylorella sp., Alcanivorax sp., Colwellia sp., Methylobacillus sp., Actinobacillus sp., Vibrio sp., Xanthomonas sp.,* and *Xylella sp.,* were among the predominant species, while the decreasing Firmicutes population was predominantly composed of *Amphibacillus sp., Eubacterium sp., Ruminococcus sp., Butyrivibrio sp.,* and *Brevibacillus sp..* Previously reported, *Bifidobacterium sp., Butyrivibrio sp., Eubacterium sp. Lactobacillus sp., Prevotella sp., Ruminococcus sp., Selenomonas sp., Streptococcus sp., Fusobacterium sp., and Peptostreptococcus sp.* (Huber et al., 1976; Huber, 1976; Kotarski et al., 1992; Mackie and Gilchrist, 1979; Mackie and Heath, 1979; Russell and Hino, 1985; Stewart et al., 1997; Tajima et al., 2000) was also identified by T-RFLP analysis. Three different restriction digestion profiles were used for identification of microbial species within the rumen in T-RFLP analysis. As Phylogenetic Assignment Tool (PAT) (Kent et al., 2003) requires that all three digests correspond to the predicted restriction fragments of a bacterial species present in the database for accurate assignment, our microbial species identification is very accurate. Although T-RFLP analysis assigned bacterial species based on previously reported organisms, we present our bacterial assignment in terms of genera identified for two reasons; a) there may be several bacterial species within a genus having the same restriction digestion pattern for all 3 enzymes we used and b) for simplification of the data presentation. T-RFLP analysis enabled us to identify over 350 bacterial species belonging to over 115 different bacterial genera, where

we see a shift in the rumen microbial population structure from gram-negative bacteria to more gram-positive bacteria.

T-RFLP analysis provides a snapshot of the rumen bacterial populations that exist within the rumen at a given time and is a reliable and reproducible technique to survey the rumen microbial population. But, T-RFLP analysis only allows the identification of bacterial species that have been identified and characterized thus far. Therefore, to further evaluate the microbial fluctuations during adaptation to high concentrate diet and on prairie hay, we constructed several 16S rDNA libraries from animals on prairie hay and on high concentrate diet and sequenced 768 clones from each library. Previously several 16S rRNA libraries have been constructed from animals on high concentrate diet, but only reported sequence analysis of less than 100 clones (Edwards et al., 2004; Tajima et al., 1999; Tajima et al., 2000), also microbial changes in animals on prairie hay has not been evaluated using 16S rRNA libraries. Therefore this study provides valuable information towards understanding the rumen microbial changes during adaptation high concentrate diet from a forage diet. Initial analysis of the 16S rRNA libraries identified show significant changes in microbial population structure and diversity in animals on prairie hay when compared to animals on high concentrate diet (Table 2 & 3 and Figure 3a). The libraries constructed from animals on high concentrate displayed significantly greater numbers in *Bacteriodes* species which accounted for more than 44% of the clones sequenced. This *Bacteriodes* population was composed of bacterial species belonging to genera *Prevotella, Anaerophaga,* and *Tannerella,* but also contained a large number of unclassified *Bacteriodes sp.*, suggesting that a greater number of

bacterial species are still unknown and need to be characterized from the bovine rumen. The libraries on prairie hay displayed significantly greater numbers of *Fibrobacteres* species which all belong to the genus *Fibrobacter*. *Fibrobacter sp.* have been identified as major cellulolytic bacterial species present within the rumen that help in fiber digestion (Forsberg et al., 1997; Koike and Kobayashi, 2001; Tajima et al., 2001), therefore this observation of *Fibrobacter* species within our library is consistent with previous reports. Although we did not detect significant changes in total rumen *Firmicutes* populations, the hierarchical classification of the microbial populations within the phylum *Firmicutes* revealed the presence of significantly different microbial populations (Table 2 & 3b and Figure 3b). The microbial population in animals on high concentrate diet contained more bacterial species belonging to the genera *Mitsuokella* and *Anaerovibrio*, and a significantly greater number of unclassified *Clostridiaceae* species. *Mitsuokella* is known to be an amylolytic organism and has been reported to increase during increase of fermentable sugars (Stewart et al., 1997), therefore, the increase of *Mitsuokell* during adaptation to high concentrate diet is expected. We have also detected the presence of a few *Archeae* bacteria within our library from animals on prairie hay. The identified *Archeae* bacteria belong to the genus *Methnobrevibacter*. Previous studies using *Archeae* specific primers have demonstrated that *Methnobrevibacter* is a common inhabitant in the bovine rumen and is present in forage diets (Skillman et al., 2006). Hierarchical classification of the 16S rRNA libraries reported the presence of 202 and 67 unclassified bacterial species in animals on prairie hay and high concentrate diet respectively. This suggests that the microbial population within the

rumen is far from being known and needs to be studied extensively. The rumen microbial population has great potential as a source to identify new microbes of great economic importance.

Phylogenetic analysis was performed on the sequenced 16S rRNA libraries to identify the phylogenetic relatedness among the bacterial sequences (Figure 4 & 5). Phylogenetic analysis demonstrated great diversity in both animals on prairie hay and on high concentrate diet suggesting that the microbial population is not restricted to a few species, but a compilation of a bacterial population that has organisms with different capabilities. The libraries constructed from prairie hay displayed 261 different OTUs and libraries constructed from high concentrate diet displayed 263 OTUs. Although the number of OTUs identified within each library was similar, the distribution and relatedness of the 16S sequences were significantly different among the two ruminal environments. This suggests that when animals are shifted from a forage diet to a high concentrate diet the microbial diversity in terms of the number of different species remain somewhat similar but the composition or the species makeup change significantly to adapt to the new ruminal environment. The significantly large group of phylogenetically distant *Firmicutes* population observed on prairie hay corresponded to more than 30% of the total sequences and was mainly composed of *Anaerobaculum, Butyrivibrio, Acetivibrio* and other Clostridia sp. The few *Archaea* identified on prairie hay belonged to the genus *Methanobrevibacter,* but to our surprise they clustered in-between the bacterial genera. At first we were surprised to identify any *Archaea* at all, as the primers we used to amplify the 16S rRNA gene was specific to all Eubacterial species. But as it

amplified *Methanobrevibacter,* this suggests that the region we have amplified from *Methanobrevibacter* has high similarity to the *Eubacterial* sequence. The phylogenetic analysis of the 16S rRNA sequences generated from animals on High concentrate diet displayed the presence of a phylogenetically diverse *Bacteriodetes* population. This *Bacteriodetes* population was twice the size of the population found in animals on prairie hay and was mainly composed of unclassified *Bacteroidetes sp. (*122), but also contained high number of bacteria belonging to the genera *Anaerophaga* and *Prevotella*. Sequence and phylogenetic analysis of the 16S rRNA libraries constructed, reveals the presence of numerous uncultured rumen bacterial species. As these bacterial species have not been characterized, it is difficult to identify the role of these bacterial species within the rumen. Therefore, the hierarchical classification based on the conserved regions of the 16S rRNA sequences will enable prediction of the functional role of these bacteria within the rumen. This investigation of rumen microbial population structure and diversity using 16S rRNA libraries is among the largest sequenced thus far and will provide valuable information towards identifying new rumen bacterial species.

Previous studies have evaluated and quantified rumen microbial population changes during adaptation to a high concentrate diet using culture dependent techniques and molecular techniques such as quantitative realtime PCR and 16S probes (qRT-PCR) (Attwood et al., 1988; Stahl et al., 1988; Tajima et al., 2001; Tajima et al., 2000). But, culture dependent techniques have failed to provide accurate estimates of microbial population changes as these experiments has demonstrated bias towards overrepresentation of easy to culture bacterial species (Tajima et al., 2001).

Therefore currently, molecular techniques are widely used to quantify population changes (Ozutsumi et al., 2006; Tajima et al., 2001). To identify the microbial changes during adaptation to a high concentrate diet we quantified rumen microbial population changes using qRT-PCR. Population of *Fibrobacter succinogenes* gradually decreased as animals were adapted to high concentrate diet and were 40-fold lower compared to animals on prairie hay. *Fibrobacter succinogenes* is reported to be a fibrolytic bacterium that digests fiber (Koike and Kobayashi, 2001; Tajima et al., 2001) and is predominantly present in diets high in fiber and is therefore expected to drop in population size during adaptation to high grain diet. This decrease in population is similar to the observation reported by Tajima et. al. (2001) who reported a 20-fold decrease in population size by day 3, which decreased further by 57-fold by day 28 (Tajima et al., 2001). *Butyrivibrio fibrisolven* population also declined 20-fold during adaptation to high concentrate diet. *Butyrivibrio fibrisolvens* is known to be fibrolytic organisms, but is also known to have high preference towards maltose and sucrose utilization (Russell and Baldwin, 1978) and produce butyrate (Goad et al., 1998). Hence, *Butyrivibrio fibrisolvens* is an organism capable of utilizing both, cellulose and starch. But, *Butyrivibrio fibrisolvens* only decreased slightly during the first 3 stages of the step-up diet (~5-fold) and decreased 20-fold on the 4^{th} diet. This may be due to the increasing pH within the rumen with the increasing amount of fermentable substrates present in the diet. But a recent study also reports that *Butyrivibrio fibrisolvens* population increase in high fiber diets and that high energy diets decrease population numbers (Mrazek et al., 2006). This observation is similar to our observation. *Ruminococcus albus*

population also displayed a significant decrease in bacterial population during adaptation to high concentrate diet when compared to animals on prairie hay, where populations decreased 60 fold by diet 4. *Ruminococcus flavefaciens* is also reported as a cellulolytic bacterium that has fibrolytic activities (Michalet-Doreau et al., 2001), therefore the decrease in the *Ruminococcus flavefaciens* population is expected during adaptation to high concentrate diet.

Quantitative realtime PCR analysis displayed an 11 fold increase in *Megaspaera elsdenii* populations by the 3rd regime of the step up diet but decreased to 6 fold by diet 4. *Megaspaera elsdenii* which is one of the most widely studied rumen organism (Russell and Dombrowski, 1980) and is known to utilize the lactic acid produced within the rumen to help prevent against lactic acid accumulation and acidosis (Counotte et al., 1981). Therefore, an increase in *Megaspaera elsdenii* during acidosis is a mechanism of maintaining ruminal pH by utilizing the increasing lactic acid within the rumen. The 5-fold decrease in *Megaspaera elsdenii* during the 4th stage may be due to the increasing ruminal pH due to the increased amount of fermentable substrates in the diet. *Streptococcus bovis* increased 2 fold by the start of the step up diet but decreased by the end of the step up diet regime, and did not show significant change in population. *Streptococcus bovis* is a facultative anaerobe and is known to predominate during acidosis (Dunlop and Hammond, 1965; Owens et al., 1998; Slyter, 1976). The rapid growth of *Streptococcus bovis* has not been reported in animals adapted to grain, and has been reported to have similar numbers to animals on a forage diet (Nagaraja and Titgemeyer, 2007). Therefore, our observation of no significant change in *S. bovis* population is consistant with

previous reports. *Selenomonas ruminantium* population increased 30 fold by the second stage of the step up diet and was 30 fold higher there after when compared to the animals on prairie hay. *Selenomonas ruminantium* is a propionate producing species and is known to produce propionate through succinate-decarboxylation (Stewart et al., 1997). *Selenomonas ruminantium* also has the capability to utilize a wide range of substrates including lactate (Russell and Baldwin, 1978). Hence, the increasing *Selenomonas ruminantium* population may help utilize the increasing fermentable substrates within the rumen during adaptation to high concentrate and protect against acidosis. The *Prevotella sp.* displayed opposite patterns of population changes, where *Prevotella ruminicola* populations increased 100 fold in both animals on prairie hay and on high concentrate diet on week 2 but decreased by week 3, but *Prevotella bryantii* populations increased gradually and reached 8000 fold by the 3rd regime of the step up diet, and decreased rapidly by diet 4. A similar trend in population changes was also reported by Tajima et. al (2001), where they reported a 7-fold increase of *P. ruminicola* and a 263-fold increase of *P. bryantii* by day 3 of the step-up program. But, reported a 3-fold decrease of *P. ruminicola* and a large decrease in *P. bryantii* (Tajima et al., 2001). This decrease in *P. bryantii* population on diet 4 may be due to the increasing pH due to the excess fermentable substrates in the diet.

This preliminary study of microbial population changes during adaptation to high concentrate diet using T-RFLP analysis, 16S rRNA sequencing and quantitative real-time PCR shows significant changes in rumen microbial population structure and diversity, and reports the presence large number of previously unreported

organisms within the rumen. Therefore this study will provide new insight into the role and changes of rumen bacteria during adaptation to high energy diets from a forage diet.

Reference List

Attwood,G.T., Lockington,R.A., Xue,G.P., and Brooker,J.D. (1988) Use of A Unique Gene Sequence As A Probe to Enumerate A Strain of Bacteroides-Ruminicola Introduced Into the Rumen. *Applied and Environmental Microbiology* **54:** 534-539.

Bevans,D.W., Beauchemin,K.A., Schwartzkopf-Genswein,K.S., McKinnon,J.J., and Mcallister,T.A. (2005) Effect of rapid or gradual grain adaptation on subacute acidosis and feed intake by feedlot cattle. *Journal of Animal Science* **83:** 1116-1132.

Bodenteich,A., Chissoe,S., Wang,Y.F., and Roe,B.A. (1993) Shotgun Cloning as the Strategy of Choice to Generate Templates for High-throughput Dideoxynucleotide Sequencing. In *Automated DNA Sequencing and Analysis Techniques*. Venter,J.C. (ed). Academic Press, London.

Chissoe,S.L., Bodenteich,A., Wang,Y.F., Wang,Y.P., Burian,D., Clifton,S.W. et al. (1995) Sequence and Analysis of the Human Abl Gene, the Bcr Gene, and Regions Involved in the Philadelphia Chromosomal Translocation. *Genomics* **27:** 67-82.

Coe,M.L., Nagaraja,T.G., Sun,Y.D., Wallace,N., Towne,E.G., Kemp,K.E., and Hutcheson,J.P. (1999) Effect of virginiamycin on ruminal fermentation in cattle during adaptation to a high concentrate diet and during an induced acidosis. *Journal of Animal Science* **77:** 2259-2268.

Cole,J.R., Chai,B., Farris,R.J., Wang,Q., Kulam,S.A., McGarrell,D.M. et al. (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Research* **33:** D294-D296.

Cole,J.R., Chai,B., Farris,R.J., Wang,Q., Kulam-Syed-Mohideen,A.S., McGarrell,D.M. et al. (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Research* **35:** D169-D172.

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

Counotte,G.H.M., Prins,R.A., Janssen,R.H.A.M., and Debie,M.J.A. (1981) Role of Megasphaera-Elsdenii in the Fermentation of Lactate-2-C-13 in the Rumen of Dairy-Cattle. *Applied and Environmental Microbiology* **42:** 649-655.

Dahllof,I., Baillie,H., and Kjelleberg,S. (2000) rpoB-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. *Applied and Environmental Microbiology* **66:** 3376-3380.

Dunlop,R.H. and Hammond,P.B. (1965) D-Lactic Acidosis of Ruminants. *Annals of the New York Academy of Sciences* **119:** 1109-&.

Edwards,J.E., Mcewan,N.R., Travis,A.J., and Wallace,R.J. (2004) 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **86:** 263-281.

Ewing,B. and Green,P. (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Research* **8:** 186-194.

Ewing,B., Hillier,L., Wendl,M.C., and Green,P. (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Research* **8:** 175-185.

Fernando,S.C., Purvis II,H.T., Najar,F.Z., Sukharnikov,L., Nagaraja,T.G., Krehbiel,C.R. et al. Rumen microbial population dynamics during sub acute acidosis. 2008.
Ref Type: Unpublished Work

Forsberg,C.W., Cheng,K.J., and White,B.A. (1997) Polysaccharide degradation in the rumen and large intestine. In *Gastrointestinal microbiology*. Mackie,R.I. and White,B.A. (eds). New York: Chapman and Hall, pp. 319-379.

Goad,D.W., Goad,C.L., and Nagaraja,T.G. (1998) Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. *Journal of Animal Science* **76:** 234-241.

Gordon,D., Abajian,C., and Green,P. (1998) Consed: A graphical tool for sequence finishing. *Genome Research* **8:** 195-202.

Hettinger,A.M., Allen,M.R., Zhang,B.R., Goad,D.W., Malayer,J.R., and Geisert,R.D. (2001) Presence of the acute phase protein, bikunin, in the endometrium of gilts during estrous cycle and early pregnancy. *Biology of Reproduction* **65:** 507-513.

Huber,T.L. (1976) Physiological-Effects of Acidosis on Feedlot Cattle. *Journal of Animal Science* **43:** 902-909.

Huber,T.L., Cooley,J.H., Goetsch,D.D., and Das,N.K. (1976) Lactic Acid Utilizing Bacteria in Ruminal Fluid of A Steer Adapted from Hay Feeding to A High-Grain Ration. *American Journal of Veterinary Research* **37:** 611-613.

Hungate,R.E. (1968) Ruminal fermentation. In *Handbook of Physiology*. Code,C.F. (ed). Washington, D.C.: American Physiology Society, pp. 2725-2745.

Kent,A.D., Smith,D.J., Benson,B.J., and Triplett,E.W. (2003) Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. *Applied and Environmental Microbiology* **69:** 6768-6776.

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

Koike,S. and Kobayashi,Y. (2001) Development and use of competitive PCR assays for the rumen cellulolytic bacteria: Fibrobacter succinogenes, Ruminococus albus and Ruminococcus flavefaciens. *Fems Microbiology Letters* **204:** 361-366.

Kotarski,S.F., Waniska,R.D., and Thurn,K.K. (1992) Starch Hydrolysis by the Ruminal Microflora. *Journal of Nutrition* **122:** 178-190.

Ludwig,W., Strunk,O., Westram,R., Richter,L., Meier,H., Yadhukumar et al. (2004) ARB: a software environment for sequence data. *Nucleic Acids Research* **32:** 1363-1371.

Mackie,R.I. and Gilchrist,F.M.C. (1979) Changes in Lactate-Producing and Lactate-Utilizing Bacteria in Relation to Ph in the Rumen of Sheep During Stepwise Adaptation to A High-Concentrate Diet. *Applied and Environmental Microbiology* **38:** 422-430.

Mackie,R.I. and Heath,S. (1979) Enumeration and Isolation of Lactate-Utilizing Bacteria from the Rumen of Sheep. *Applied and Environmental Microbiology* **38:** 416-421.

Marsh,T.L., Saxman,P., Cole,J., and Tiedje,J. (2000) Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. *Applied and Environmental Microbiology* **66:** 3616-3620.

Martin,S.A. (1998) Manipulation of ruminal fermentation with organic acids: A review. *Journal of Animal Science* **76:** 3123-3132.

Michalet-Doreau,B., Fernandez,I., Peyron,C., Millet,L., and Fonty,G. (2001) Fibrolytic activities and cellulolytic bacterial community structure in the solid and liquid phases of rumen contents. *Reproduction Nutrition Development* **41:** 187-194.

Mrazek,J., Tepsic,K., Avgustin,G., and Kopecny,J. (2006) Diet-dependent shifts in ruminal butyrate-producing bacteria. *Folia Microbiologica* **51:** 294-298.

Nagaraja,T.G. and Titgemeyer,E.C. (2007) Ruminal acidosis in beef cattle: The current microbiological and nutritional outlook. *Journal of Dairy Science* **90:** E17-E38.

Nocek,J.E. (1997) Bovine acidosis: Implications on laminitis. *Journal of Dairy Science* **80:** 1005-1028.

Osborn,A.M., Moore,E.R.B., and Timmis,K.N. (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environmental Microbiology* **2:** 39-50.

Ouwerkerk,D., Klieve,A.V., and Forster,R.J. (2002) Enumeration of Megasphaera elsdenii in rumen contents by real-time Taq nuclease assay. *Journal of Applied Microbiology* **92:** 753-758.

Owens,F.N., Secrist,D.S., Hill,W.J., and Gill,D.R. (1998) Acidosis in cattle: A review. *Journal of Animal Science* **76:** 275-286.

Ozutsumi,Y., Tajima,K., Takenaka,A., and Itabashi,H. (2006) Real-time PCR detection of the effects of protozoa on rumen bacteria in cattle. *Current Microbiology* **52:** 158-162.

Roe,B.A. (2004) Shotgun Library Construction for DNA Sequencing. In *Methods in Molecular Biology*. Zhao,S. and Stodolsky,M. (eds). Human Press Inc, Totowa, New Jersey, pp. 171-187.

Rotger,A., Ferret,A., Calsamiglia,S., and Manteca,X. (2006) Effects of nonstructural carbohydrates and protein sources on intake, apparent total tract digestibility, and ruminal metabolism in vivo and in vitro with high-concentrate beef cattle diets. *Journal of Animal Science* **84:** 1188-1196.

Russell,J.B. and Baldwin,R.L. (1978) Substrate Preferences in Rumen Bacteria - Evidence of Catabolite Regulatory Mechanisms. *Applied and Environmental Microbiology* **36:** 319-329.

Russell,J.B. and Dombrowski,D.B. (1980) Effect of Ph on the Efficiency of Growth by Pure Cultures of Rumen Bacteria in Continuous Culture. *Applied and Environmental Microbiology* **39:** 604-610.

Russell,J.B. and Hino,T. (1985) Regulation of Lactate Production in Streptococcus-Bovis - A Spiraling Effect That Contributes to Rumen Acidosis. *Journal of Dairy Science* **68:** 1712-1721.

Sambrook,J. and Russel,D.W. (2001) *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Santos,S.R. and Ochman,H. (2004) Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. *Environmental Microbiology* **6:** 754-759.

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

Slyter,L.L. (1976) Influence of Acidosis on Rumen Function. *Journal of Animal Science* **43:** 910-929.

Stahl,D.A., Flesher,B., Mansfield,H.R., and Montgomery,L. (1988) Use of Phylogenetically Based Hybridization Probes for Studies of Ruminal Microbial Ecology. *Applied and Environmental Microbiology* **54:** 1079-1084.

Stewart,C.S., Flynt,H.J., and Bryant,M.P. (1997) The Rumen Bacteria. In *The Rumen Microbial Ecosystem*. Hobson,P.N. and Stewart,C.S. (eds). Blackie Academic and Professional, NY, pp. 10-72.

Strobel,H.J. and Russell,J.B. (1986) Effect of Ph and Energy Spilling on Bacterial Protein-Synthesis by Carbohydrate-Limited Cultures of Mixed Rumen Bacteria. *Journal of Dairy Science* **69:** 2941-2947.

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

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

Tajima,K., Arai,S., Ogata,K., Nagamine,T., Matsui,H., Nakamura,M. et al. (2000) Rumen bacterial community transition during adaptation to high-grain diet. *Anaerobe* **6:** 273-284.

Vlaeminck,B., Fievez,V., Demeyer,D., and Dewhurst,R.J. (2006) Effect of forage: Concentrate ratio on fatty acid composition of rumen bacteria isolated from ruminal and duodenal digesta. *Journal of Dairy Science* **89:** 2668-2678.

Wang,Q., Garrity,G.M., Tiedje,J.M., and Cole,J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73:** 5261-5267.

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

CHAPTER IV

RUMEN MICROBIAL POPULATION DYNAMICS DURING

IONOPHORE TREATMENT

Introduction

Ionophore treatment has been used as an effective management tool in feedlot and grazing cattle (Russell and Strobel, 1989) to improve the efficiency of ruminal fermentation. This change in ruminal fermentation by Ionophore treatment has shown to decline molar proportion of acetate and butyrate in the rumen (Chalupa, 1980; Richardson et al., 1976; Russell, 1987). The decline in acetate and butyrate in the rumen is associated with a decline in methane production and improved carbon and energy retention (Chalupa, 1980; Richardson et al., 1976). Treatment of monensin also effects nitrogen metabolism in ruminants and has a protein sparing effect (Chalupa, 1980), where dietary protein is available for digestion and uptake in the small intestine. Apart from the improved ruminal fermentation, ionophore treatment has also been shown to increase efficiency of energy and nitrogen metabolism, and reduce feedlot disorders, such as lactic acidosis and bloating (Russell and Strobel, 1989). It is believed that ionophores alter rumen microflora via changes in the microbial membrane's permeability to specific ions, where the ionophore destroys the primary membrane transport system of the cell, and interferes with the cellular uptake (Bergen and Bates, 1984; Ipharraguerre and Clark, 2003; Russell and Strobel, 1989). This change in membrane permeability has been reported to be more detrimental to Gram-positive bacteria which leads to selective elimination of Gram-positive bacteria (Bergen and Bates, 1984; Ipharraguerre and Clark, 2003; Russell, 1987; Russell and Strobel, 1989). It is widely accepted that this change in rumen microflora, where Gram-negative bacteria predominate, is the main reason for the increased fermentation efficiency within the rumen (Bergen and

Bates, 1984; Chow et al., 1994; Ipharraguerre and Clark, 2003; Wallace et al., 1981). But, these changes in Gram-positive and negative bacteria detected in earlier studies have been conducted using pure cultures and in-vitro experiments (Bergen and Bates, 1984; Chen and Wolin, 1979; Chow et al., 1994; Ipharraguerre and Clark, 2003; Wallace et al., 1981), where the interactions among the bacteria within the rumen, the ability of ionophores to concentrate in bacterial membranes, ionophores being absorbed by the animal and the higher concentration of bacteria within the rumen were ignored (Callaway et al., 1997; Chow et al., 1994). Recent studies have also reported that bacteria defer considerably with respect to their susceptibility to ionophore treatment (Callaway et al., 1997; Russell and Strobel, 1989), where certain Gram-negative bacteria such as *Butryrivibrio fibrosolvens* (Bergen and Bates, 1984) and certain strains of *Prevotella* (Callaway and Russell, 2000) are susceptible to ionophore treatment. Furthermore, Russell and Houlihan (2003) reports that the model of Gram-positive bacteria being susceptible and Gram-negative bacteria being resistant to ionophore treatment is not “clear-cut” and that Gram positive bacteria can adapt and become ionophore resistant. These recent reports suggest that the effect of ionophore treatment on ruminal bacteria is more complex than currently believed. Therefore as a first step towards evaluating global microbial population changes during ionophore treatment in wheat pasture cattle, we have evaluated bacterial population changes in cattle dosed with monensin and lasalosid using T-RFLP (Terminal Restriction Fragment Length Polymorphism), 16S rDNA Libraries and quantitative realtime PCR.

Experimental Procedures

Animals and Diets

Twelve ruminally cannulated beef steers (510 $\pm$ 20 Kg) were maintained at the Oklahoma State University wheat pasture research facility. The steers were allowed to have free access to wheat pasture, and adlibitum water and salt during the entire duration of the study. To have similar background rumen bacterial populations all twelve steers were maintained on wheat pasture for a week. Following the adaptation phase on wheat pasture, the steers were randomly assigned to one of three treatment groups. The “Control” group (n=4) were maintained on wheat pasture with no ionophore, while groups “Monensin” (n=4) and “Lasalosid” (n=4) were ruminally dosed with 200mg of Monensin and Lasalosid respectively.

Sampling

Rumen samples were collected before ionophore feeding (BEFORE), following 8 days of ionophore dosing (DURING) and 11 days after removal of the ionophore dosing (AFTER) and was used to evaluate the microbial population changes during ionophore feeding. The samples collected were snap frozen in liquid nitrogen and were stored at -20°C until DNA extraction.

DNA isolation

Frozen rumen samples were ground in liquid nitrogen using a motar and pestal, and 0.5 g of the homogenized sample was used to extract total bacterial DNA. Total

bacterial DNA was extracted using the QIAamp® DNA mini stool kit (Qiagen, Valencia, CA, USA) according to manufacturers protocol with a few modifications as described by Fernando et al. (2008). The concentration of the extracted DNA was measured using a UV spectrophotometer, and the quality of the DNA was evaluated by agarose gel-electrophoresis using a 1.2% gel.

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis

T-RFLP analysis was performed as described by Fernando et al. (2008). Breifly, 100 ng of total bacterial genomic DNA was used for Terminal-Rescticton Fragment Length Polymorphisum (T-RFLP) analysis. Total bacterial DNA was PCR amplified in a MJ dyad thermocycler (MJ research, Watertown, MA) using FAMBacT0008F 5'-AGAGTTTGATCCTGGCTCAG-3' and BacT0805R 5'-GGACTACCAGGGTATCTAATCCC-3' (Fernando et al., 2008) primers which amplify a ~800 bp fragment of the 16S rRNA gene present in all Eubacteria and some Archaea. The FAMBacT008F primer was labeled at the 5' end with the fluorophore 6-FAM to help detection using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA). A 50 µl PCR reaction contained, 100 ng of DNA, 1X PCR buffer (Promega, Madison, WI, USA) 1.5 mM $MgCl_2$, 400 nM of each primer, 200 µM dNTPs, 100 ng/µl BSA and 3 U Taq DNA Polymerase (Promega, Madison, WI, USA). Cycling conditions were: one cycle of 1 min at 95ºC, 30 sec at 52ºC, 1 min at 72ºC followed by 34 additional cycles of 30 sec at 95ºC, 30 sec at 52ºC, 1 min at 72ºC and a final extension of 3 min at 72ºC.

The amplified PCR products were ethanol precipitated as described by Fernando et al. (2008). The precipitated PCR product was digested separately with RSA I *(*Invitrogen, Carlsbad, CA,USA), HAE III *(*Invitrogen, Carlsbad, CA,USA) and MSP I *(*Promega, Madison, WI, USA) according to the manufacturers protocols. A 10 µl digestion reaction contained 1X buffer, 2.5 U Enzyme and 5 µl of the PCR product. 250 ng/µl of BSA was added to the MSP I digestion. The Digestion reaction was incubated at 37°C for 4 hours, followed by 65°C for 20 min. 2 µl of the digested PCR product was mixed with 0.5 µl of GeneScan ROX 1000 size standard (Applied Biosystems, Foster City, CA) and 3.5 µl of loading dye (Applied Biosystems, Foster City, CA) and was electrophoresed for 8 hours in an ABI 377 sequence analyzer (Applied Biosystems, Foster City, CA). The data collected by the ABI 377 sequence analyzer was analyzed using GeneScan 3.1 analysis software (Applied Biosystems, Foster City, CA). The data set was subsequently normalized by dividing the cumulative peak height of each sample by the sample with the smallest cumulative peak height. The normalized T-RFLP profiles were further analyzed using Phylogenetic Assignment Tool (PAT) (Kent et al., 2003) to assign species for each restriction fragment generated. The bacterial species assignment was based on the restriction digestion patterns obtained for all 3 enzymes of each sample.

The normalized peak area was used to calculate population fluctuations and to construct microbial diversity plots. The normalized peak areas for the 3 different digests were averaged and were used as an estimate of bacterial population. The representation of each bacterial species within the total bacterial population was calculated as the percentage of peak area of a bacterial species relative to the total

peak area of the sample. Each bacterial species identified was further classified and was plotted based on phylum.

Construction of 16S rRNA libraries and Sequencing

100 ng of total DNA from each treatment (Monensin, Lasalosid and Control) was used for the construction of three 16S libraries. The three libraries were constructed by pooling equal amount of DNA from all animals within each treatment. The pooled DNA was used to PCR amplify an ~800 bp fragment using BacT0008F 5'-AGAGTTTGATCCTGGCTCAG-3' and BacT0805R 5'-GGACTACCAGGGTATCTAATCCC-3' primers using a high fidelity taq polymerase. The 50 µl PCR reaction contained, 100 ng of DNA, 1X PCR buffer (Invitrogen, Carlsbad, CA, USA) 1.5 mM $MgCl_2$, 400 nM of each primer, 200 µM dNTPs, and 2 U Pfx DNA Polymerase (Invitrogen, Carlsbad, CA, USA). Cycling conditions were: one cycle of 2 min at 95ºC, 30 sec at 52ºC, 1 min at 68ºC followed by 34 additional cycles of 20 sec at 95ºC, 30 sec at 52ºC, 1 min at 68ºC and a final extension of 3 min at 68ºC. The blunt ended PCR products generated were ligated in to PCR-Blunt® (Invitrogen, Carlsbad, CA) plasmid vector and transformed to DH5α max efficiency competent *E.coli* cells (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol to construct the three different 16S libraries. The transformed cells were grown overnight at 37°C on 22X22 cm Nunc® bioassay plates (Nalge Nunc International, Rochester, NY, USA) containing 2X YT with 100 µg/ml of kanamycin. Random libraries of approximately 1920 colonies were picked from each library, grown in Terrific Broth (TB) medium supplemented with 50

micrograms of kanamycin for 14 hours at 37°C with shaking at 250 rpm, and the sequencing templates were isolated by a cleared lysate-based protocol (Bodenteich et al., 1993; Roe, 2004).

Sequencing reactions were performed as previously described (Chissoe et al., 1995) using Thermus aquaticus (Taq) DNA polymerase, and either the Applied Biosystems Big Dye terminator or the Perkin-Elmer Cetus fluorescent-labeled Big Dye Taq terminator sequencing kits at a 1:16 dilution. The reactions were incubated for 60 cycles in a Perkin-Elmer Cetus DNA Thermocycler 9600 and after removal of unincorporated dye terminators by ethanol precipitation followed by a 70% ethanol wash, the fluorescent-labeled nested fragment sets were resolved by electrophoresis on an ABI 3700 Capillary DNA Sequencer. After base calling with Phred (Ewing et al., 1998), the analyzed data was transferred to a Sun Workstation Cluster, and assembled using Phrap (Ewing and Green, 1998). The overlapping sequences (contigs) were analyzed using Consed (Gordon et al., 1998). The resulting data was cleaned of vector sequences, using the software “Crossmatch” and was analyzed using analysis tools available at the Ribosomal Database Project (http://rdp.cme.msu.edu). The assembled data was blasted against the 16S RDP database (Cole et al., 2003; Cole et al., 2005) and the NCBI database to identify the bacterial populations present.

Phylogenetic and Comparative Analyses

The data was aligned using the RDP pipline available at *myRDP* space (Cole et al., 2007). The alignment generated was imported to the ARB software package

(Ludwig et al., 2004) to generate phylogenetic trees with hierarchical classification of the sequences. Phylogenetic trees were constructed using the neighbor-joining method. Furthermore, “Classifier” (Wang et al., 2007) and “Library Compare” (Cole et al., 2005) software tools available at ribosomal database project (http://rdp.cme.msu.edu) were used to further analyze the sequences and to classify the clones to taxanomic hierarchy. The “Library Compare” software tool was used to compare the libraries with each other and to identify statistically, significant changes in bacterial populations present among the libraries (Cole et al., 2003; Cole et al., 2005).

Quantitative Real-Time PCR analysis

Quantitative analysis of population changes of selected bacterial species were conducted using quantitative real-time polymerase chain reaction (qPCR). Samples collected from control animals and animals dosed with Monensin and Lasalosid were used for real-time PCR analysis. The population changes were evaluated using SYBR green I reporter assay kit available from Roche Diagnosticis (Indianapolis, IN, USA). The primers used for analyzing *Prevotella ruminicola, Prevotella bryantii, Megaspaera elsdenii, Selenomonas ruminantium*, *Streptococcus bovis, Butyrivibrio fibrosolvens,* and *Fibrobacter succinosolvens* bacterial populations were, primers previously reported by Tajima et al. (2001), Ouwerkerk et al. (2002), and Fernando et al. (2008).

A 15 µl reaction contained 400 nM forward primer, 400 nM reverse primer, 1X Master mix (Roche Diagnosticis, Indianapolis, IN, USA), and 40 ng of total rumen

DNA. Thermal cycling conditions were 95°C for 10 min, followed by 50 additional cycles of 95°C for 30 sec 50-62°C for 30 sec, depending on the annealing temperature of each primer set (see Table 1) and 72°C for 40 sec or 1 min based on the size of the amplicon followed by the melting curve. The single copy universal gene *rpoB* was used for normalization (Dahllof et al., 2000; Fernando et al., 2008; Santos and Ochman, 2004). The qRT-PCR reactions were performed in an ABI PRISM 7500® sequence detection system (Applied Biosystems, Foster City, CA). Relative quantification of bacterial population changes were performed using the comparative C_T method as previously described (Hettinger et al., 2001). The ΔC_T value was determined by subtracting the 16S C_T value of each sample from the corresponding sample *rpoB* gene C_T value. Calculation of $\Delta\Delta C_T$ was carried out using the BEFORE time point (when all animals were on wheat pasture before dosing) mean ΔC_T value as an arbitrary constant to subtract from all other ΔC_T mean values. Fold changes in bacterial populations were calculated from the $\Delta\Delta$CT values using the formula $2^{\pm\Delta\Delta Ct}$.

Results

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis

To evaluate the microbial diversity and microbial population dynamics within the rumen during ionophore dosing, T-RFLP analysis was performed on each rumen sample using 3 different restriction enzymes. T-RFLP analysis identified bacteria belonging to 255 different bacterial genera among the different treatments. This large bacterial population was further analyzed and classified by phylum for ease of presentation. The summarized microbial population fluctuation and microbial diversity plots generated based on T-RFLP analysis is shown in Figure 1. T-RFLP analysis shows significant changes in microbial population structure and diversity between the control animals on wheat pasture and animals dosed with ionophore. The “BEFORE” treatment shows similar initial bacterial population structure with a few animal to animal variations. The control animals showed a slightly higher population of *Proteobacteria* (44.5%) compared to animals dosed with monensin (28.4%), and lasalosid (30.8%). The *Bacteroidetes* population was slightly higher in animals on lasalosid (28.6%) compared control (19.8%) animals and monensin treated animals (19.3%). But, “DURING” ionophore treatment the *Bacteroidetes* populations increased in both lasalosid (65%) and monensin (35.4%) fed animals compared to before ionophore treatment, whereas, *Firmicutes* population decreased during ionophore treatment. The *Proteobacteria* populations in control and monensin treated animals were similar to the before treatment population levels, but was decreased significantly in animals treated with lasalosid. The category named “Other” which contained all other phyla that had fewer members, also showed a

decrease on ionophore treated animals when compared to “BEFORE” treatment levels. Phylum *Actinobacteria* showed constant levels of population in all three treatments. After removal of ionophores, *Bacteroidetes* populations decreased significantly in ionophore treated animals, and were below initial levels of *Bacteroidetes* detected before ionophore treatment. *Firmicutes* population increased in lasalosid treated animals after removal of the ionophore and was comparable to before treatment levels, but in monensin treated animals and control animals the *Firmicutes* population decreased and was below starting levels of bacteria. *Proteobacteria* increased in all three treatments, but was highest in lasalosid treated animals. “Other” phyla also increased after the removal of ionophore treatment and were comparable to initial levels of bacteria.

16S rRNA library and Phylogenetic analysis

T-RFLP analysis uses previously reported 16S rDNA sequences to identify bacterial species, and therefore only allow the identification of previously reported bacterial species. To determine the complete microbial population structure in animals dosed with ionophore, we constructed several 16S rDNA libraries from control animals and form animals dosed with lasalosid and monensin. 384 clones from each library was sequenced and was analyzed using the “library compare” software tool available at ribosomal database project to identify microbial population structure and diversity among each treatment. The analysis detected significant differences in rumen bacterial populations among the three treatment groups (Figure 2 and Table 2 & 3). Libraries constructed from control animals and animals dosed with monensin

showed a similar population structure of *Archeae* bacteria, but animals dosed with lasalosid showed a significantly higher number of *Archeae (*Figure 2a). The higher number of Archeae in lasalosid treated animals resulted in a significantly lower population of *Eubacteria* in these animals when compared to control and monensin treated animals (Table 2). Bacteria belonging to phylum *SR1* were significantly higher in control animals when compared to animals on ionophore treatment (Figure 2 and Table 2 & 3). The *Firmicutes* populations within all treatments were consistent and accounted for ~25% of the total population sequenced. Within phylum *Firmicutes*, the predominant class was *Clostridia* and accounted for more than 90% of the bacterial population. Within *Clostridia*, bacteria belonging to genus *Anaerobaculum* were greater in ionophore treated animals when compared to control animals, but family *Lachnospiraceae* was more abundant in control animals than ionophore treated animals. Bacterial populations belonging to Phylum *Bacteroidetes* was the most abundant in all three groups which accounted for ~50% of the total bacterial population sequenced from each treatment. The *Bacteroidetes* population in lasalosid dosed animals was lower than control and monensin dosed animals. Among the *Bacteroidetes* populations, majority of the bacteria belonged to family *Prevotellaceae*. Within *Prevotellaceae*, genus *Prevotella* was high in control animals and lasalosid treated animals, while genus *Hallella* was high in control and monensin treated animals. Also, the unclassified bacteria detected were significantly higher in animals dosed with monensin when compared to control animals and animals dosed with lasalosid.

To identify the phylogenetic relationships between the libraries sequenced, phylogenetic analysis was performed using ARB software analysis program (Ludwig et al., 2004). The three libraries constructed were used to identify a set of non-redundant Operational Taxanomic Units (OTUs) for each treatment. The Non-redundant OTUs identified were used to construct phylogenetic trees as described in materials and methods (figure 3-5). Phylogenetic analysis demonstrated great diversity among the three libraries. The libraries constructed from control, monensin and lasalosid treated animals displayed 179, 148 and 162 OTUs (Operational Taxanomic Units) respectively. The OTUs displayed a relatively low redundancy in all three libraries, but was lowest in the control animals and was highest in monensin treated animals. Majority of the OTUs detected in all three libraries, belonged to diverse *Firmicutes* and *Bacteroidetes* species present within the populations. The phylogenetic trees also show an increased *Proteobacteria* population in libraries constructed from ionophore treated animals (Figure 3).

Quantitative Real-Time PCR analysis

Quantitative realtime PCR analysis was performed to evaluate the population changes of previously reported organisms during ionophore treatment. Quantitative realtime PCR (qRT-PCR) results are summarized in Figure 6. Quantitative realtime PCR analysis displayed 3.5 and 4 fold increase in *Megasphera elsdenii* populations during monensin and lasalosid treatment. This increase was significant ($p<0.05$) when compared to before treatment population size. *Megasphera* population did not show significant changes in control animals. *Streptococcus bovis* increased 2.7 fold

during lasalosid treatment and further increased to 3.5 fold after removal of lasalosid. In monensin treated animals, *Streptococcus bovis* populations decreased by 2 fold during and after ionophore treatment, but remained constant in control animals. *Selenomonas ruminantium* decreased 1.5 and 2.7 fold in animals dosed with lasalosid and monensin during ionophore treatment, and decreased further after removal of ionophore treatment, But, *Selenomonas ruminantium* remained constant in control animals. *Prevotella sp.* displayed different patterns of population changes, where *Prevotella ruminicola* populations decreased during ionophore dosing in all three treatments. But the highest (8.1 fold) decrease was detected in control animals, where, monensin and lasalosid displayed 3.3 and 4.3 fold decrease respectively. After the removal of ionophore *Prevotella ruminicola* populations recovered and were not significantly different from populations before treatment in ionophore treated animals. But, in control animals, *Prevotella ruminicola* population remained decreased (7.6 fold). *Prevotella bryantii* population did not show significant changes in population in lasalosid treated animals during the study period. But in control animas and monensin treated animals *Prevotella bryantii* population decreased 5.3 and 7.2 fold respectively and decreased further, after removal of monensin. *Fibrobacter succinogenes* and *Butyrivibrio fibrosolvens* populations were similar throughout the study period and did not show significant changes in population.

Figure 1

Summarized, hierarchical classification of Terminal Restriction Fragment length Polymorphism (T-RFLP) data. T-RFLP analysis was performed using the Phylogenetic Analysis Tool (PAT). Population fluctuations of animals on different treatments (Control, Monensin and Lasalosid) are displayed after classification by Phylum.

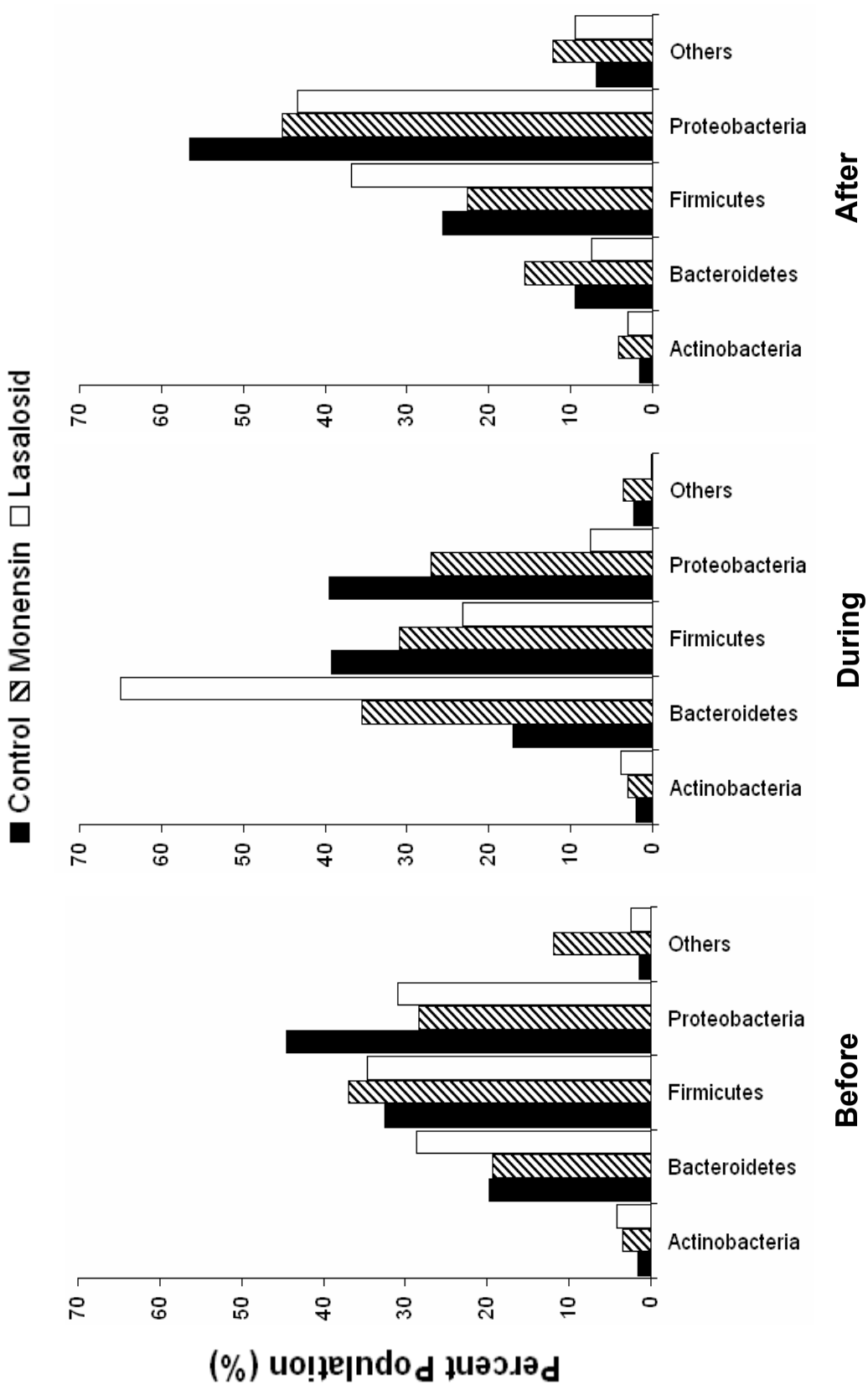

Control
Monensin
Lasalosid
Percent Population (%)
0
10
20
30
40
50
60
70
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Others
Before
During
After

Figure 2

Comparison of Libraries at the phylum level using “Library Compare” software available at the ribosomal database project. Populations that are significantly different at p> 0.01 are shown with an asterix “*”.

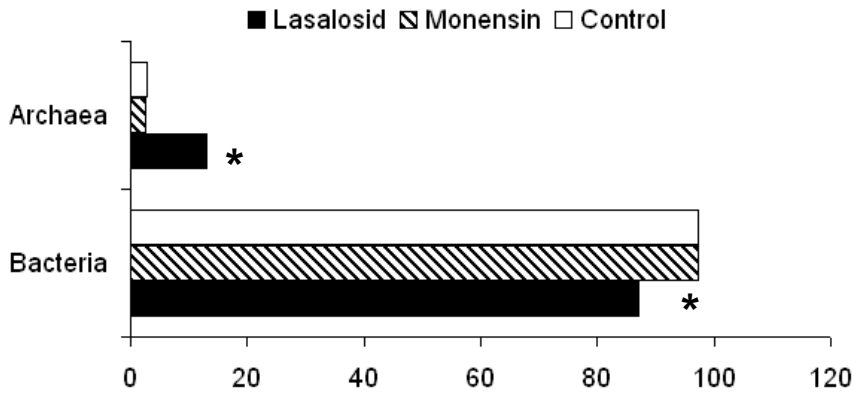
(A) Archea Vs Eubacteria

(B) Control Vs Monensin

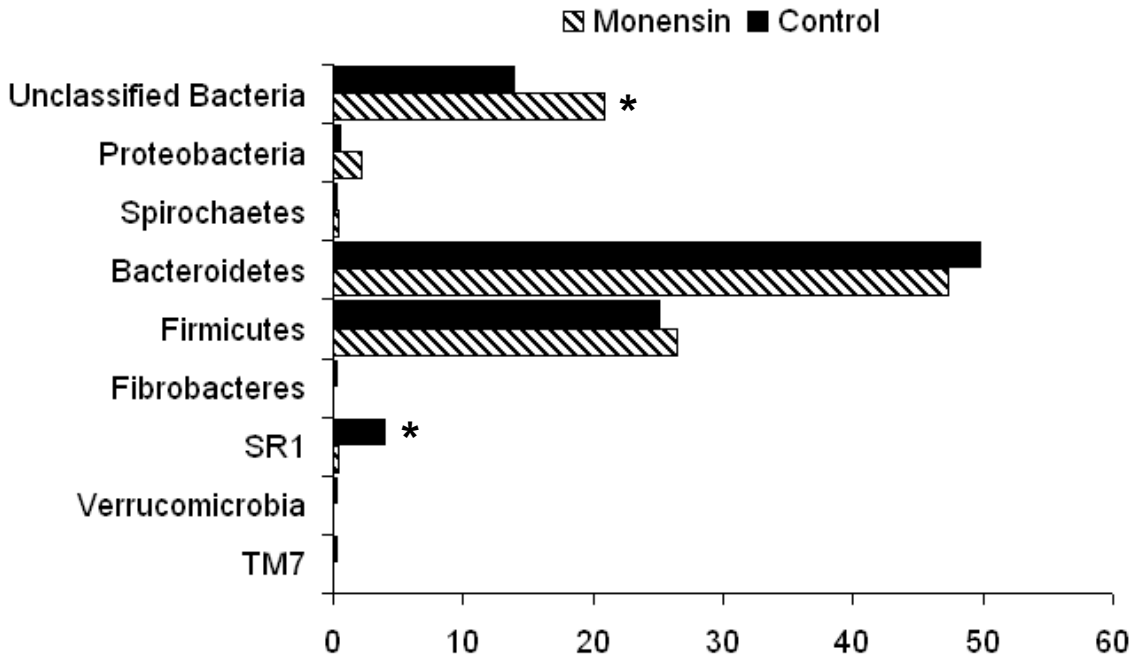
(C) Control Vs Lasalosid

(D) Lasalosid Vs Monensin

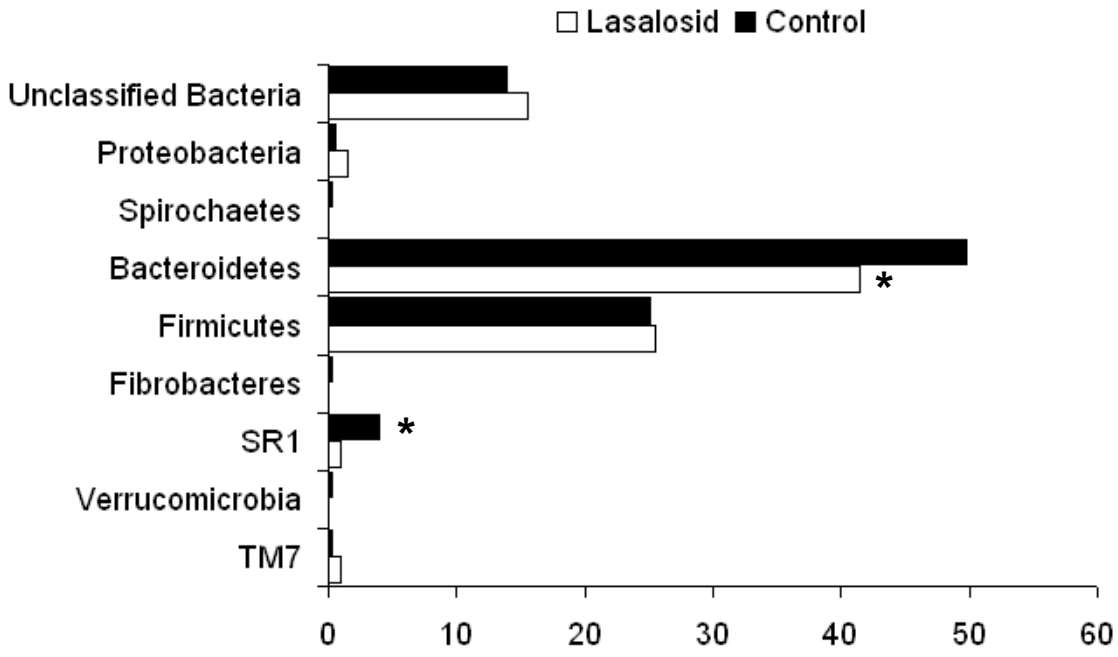
A


Lasalosid
Monensin
Control
Archaea
Bacteria
*
*
0
20
40
60
80
100
120

B


Monensin
Control
Unclassified Bacteria
Proteobacteria
Spirochaetes
Bacteroidetes
Firmicutes
Fibrobacteres
SR1
Verrucomicrobia
TM7
*
*
0
10
20
30
40
50
60

C


Lasalosid
Control
Unclassified Bacteria
Proteobacteria
Spirochaetes
Bacteroidetes
Firmicutes
Fibrobacteres
SR1
Verrucomicrobia
TM7
*
*
0
10
20
30
40
50
60

D

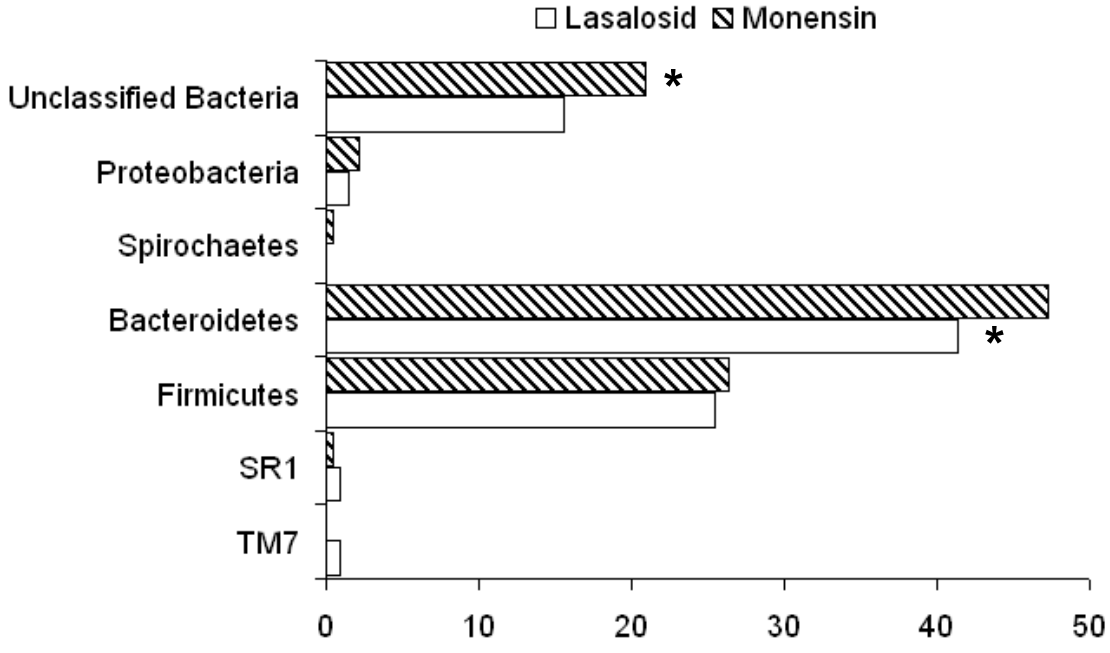

Lasalosid
Monensin
Unclassified Bacteria
Proteobacteria
Spirochaetes
Bacteroidetes
Firmicutes
SR1
TM7
*
*
0
10
20
30
40
50

Figure 3

Phylogenetic analysis of libraries constructed from control animals. Consensus phylogenetic trees were constructed using the Neighbour joining method and p-distance matrix. The scale bars indicates the length of 10 substitutions per 100 residues.

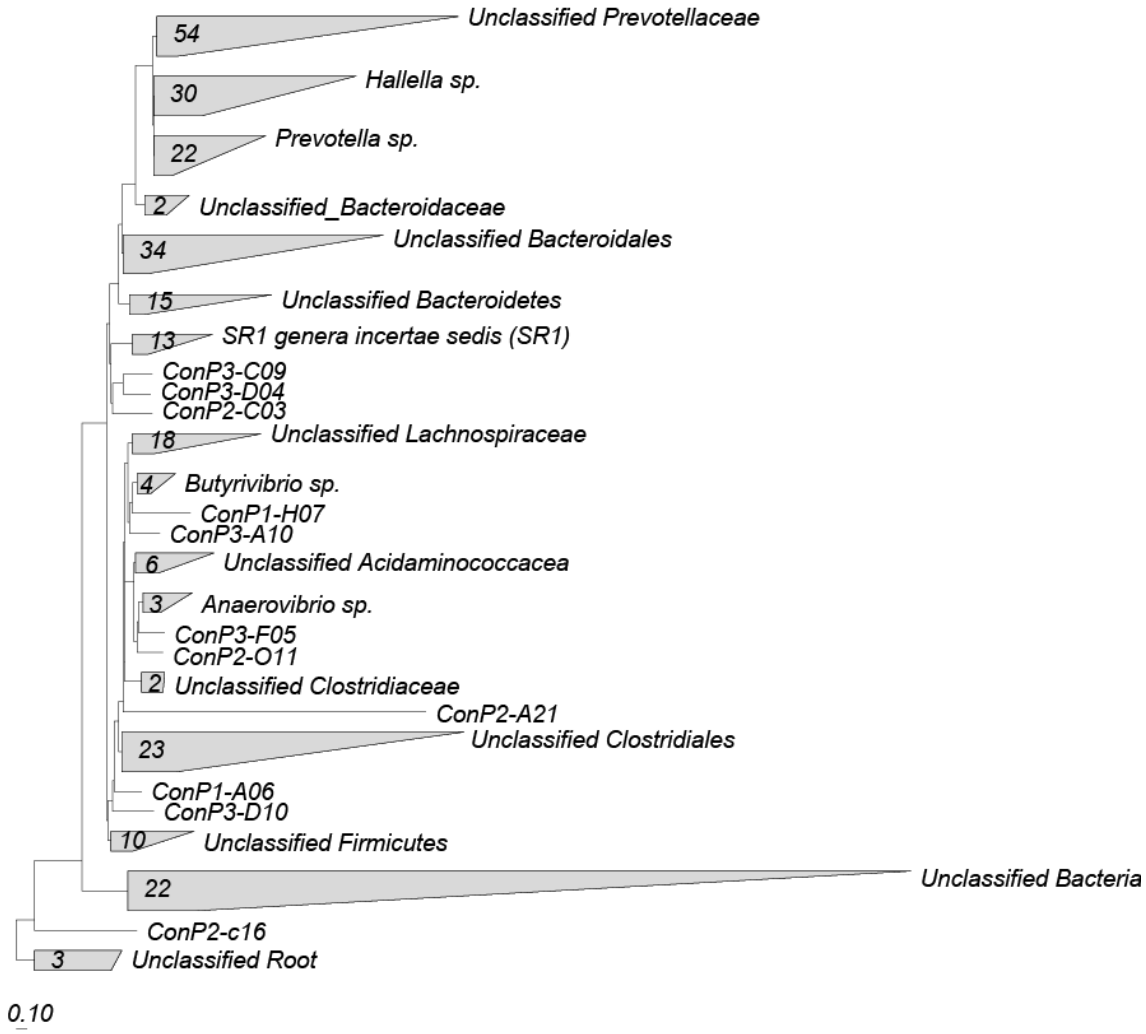

54
Unclassified Prevotellaceae
30
Hallella sp.
22
Prevotella sp.
2
Unclassified_Bacteroidaceae
34
Unclassified Bacteroidales
15
Unclassified Bacteroidetes
13
SR1 genera incertae sedis (SR1)
ConP3-C09
ConP3-D04
ConP2-C03
18
Unclassified Lachnospiraceae
4
Butyrivibrio sp.
ConP1-H07
ConP3-A10
6
Unclassified Acidaminococcacea
3
Anaerovibrio sp.
ConP3-F05
ConP2-O11
2
Unclassified Clostridiaceae
ConP2-A21
23
Unclassified Clostridiales
ConP1-A06
ConP3-D10
10
Unclassified Firmicutes
22
Unclassified Bacteria
ConP2-c16
3
Unclassified Root
0.10

Figure 4

Phylogenetic analysis of libraries constructed from animals on Monensin. Consensus phylogenetic trees were constructed using the Neighbour joining method and p-distance matrix. The scale bars indicates the length of 10 substitutions per 100 residues.

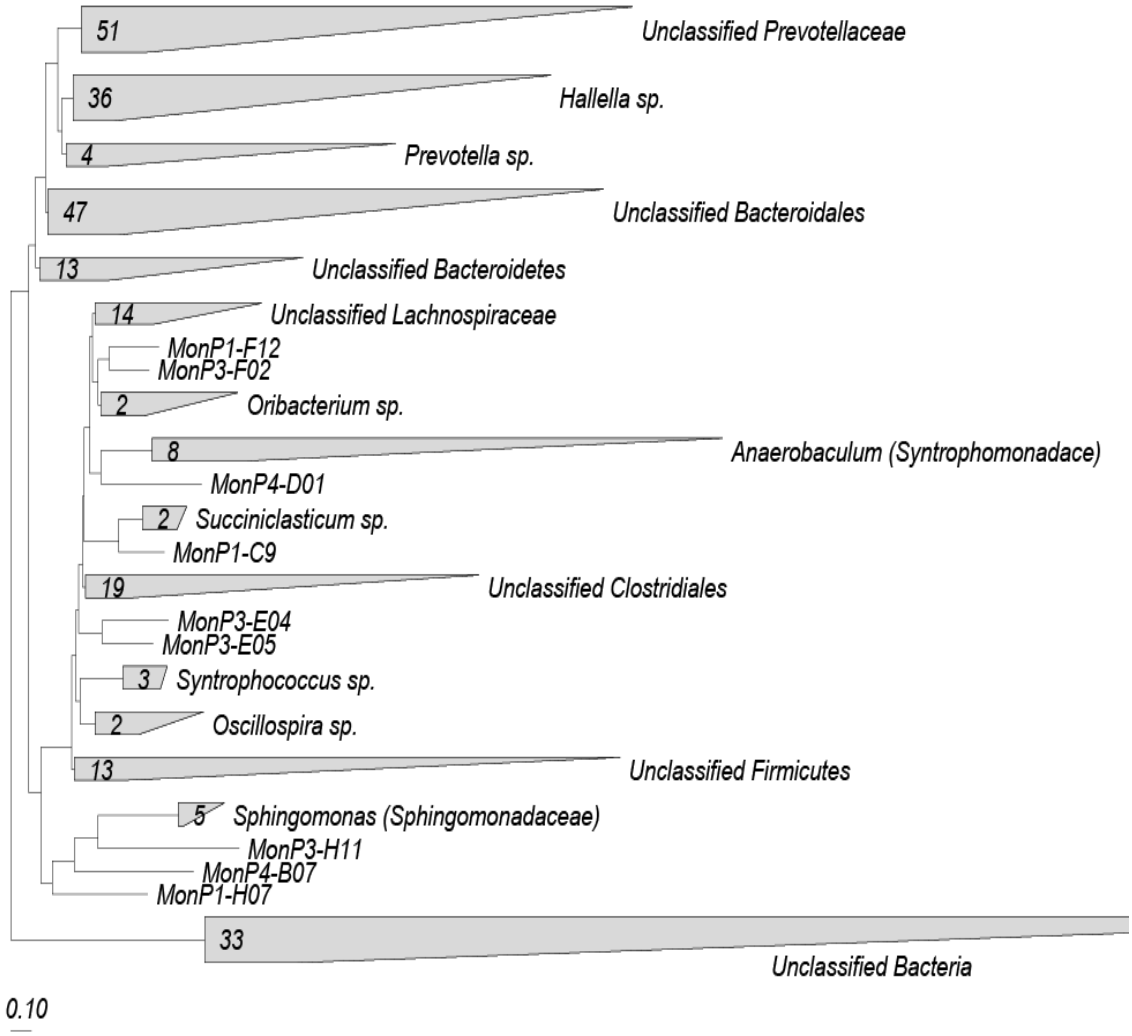

51
Unclassified Prevotellaceae
36
Hallella sp.
4
Prevotella sp.
47
Unclassified Bacteroidales
13
Unclassified Bacteroidetes
14
Unclassified Lachnospiraceae
MonP1-F12
MonP3-F02
2
Oribacterium sp.
8
Anaerobaculum (Syntrophomonadace)
MonP4-D01
2
Succiniclasticum sp.
MonP1-C9
19
Unclassified Clostridiales
MonP3-E04
MonP3-E05
3
Syntrophococcus sp.
2
Oscillospira sp.
13
Unclassified Firmicutes
5
Sphingomonas (Sphingomonadaceae)
MonP3-H11
MonP4-B07
MonP1-H07
33
Unclassified Bacteria
0.10

Figure 5

Phylogenetic analysis of libraries constructed from animals on Lasalosid. Consensus phylogenetic trees were constructed using the Neighbour joining method and p-distance matrix. The scale bars indicates the length of 10 substitutions per 100 residues.

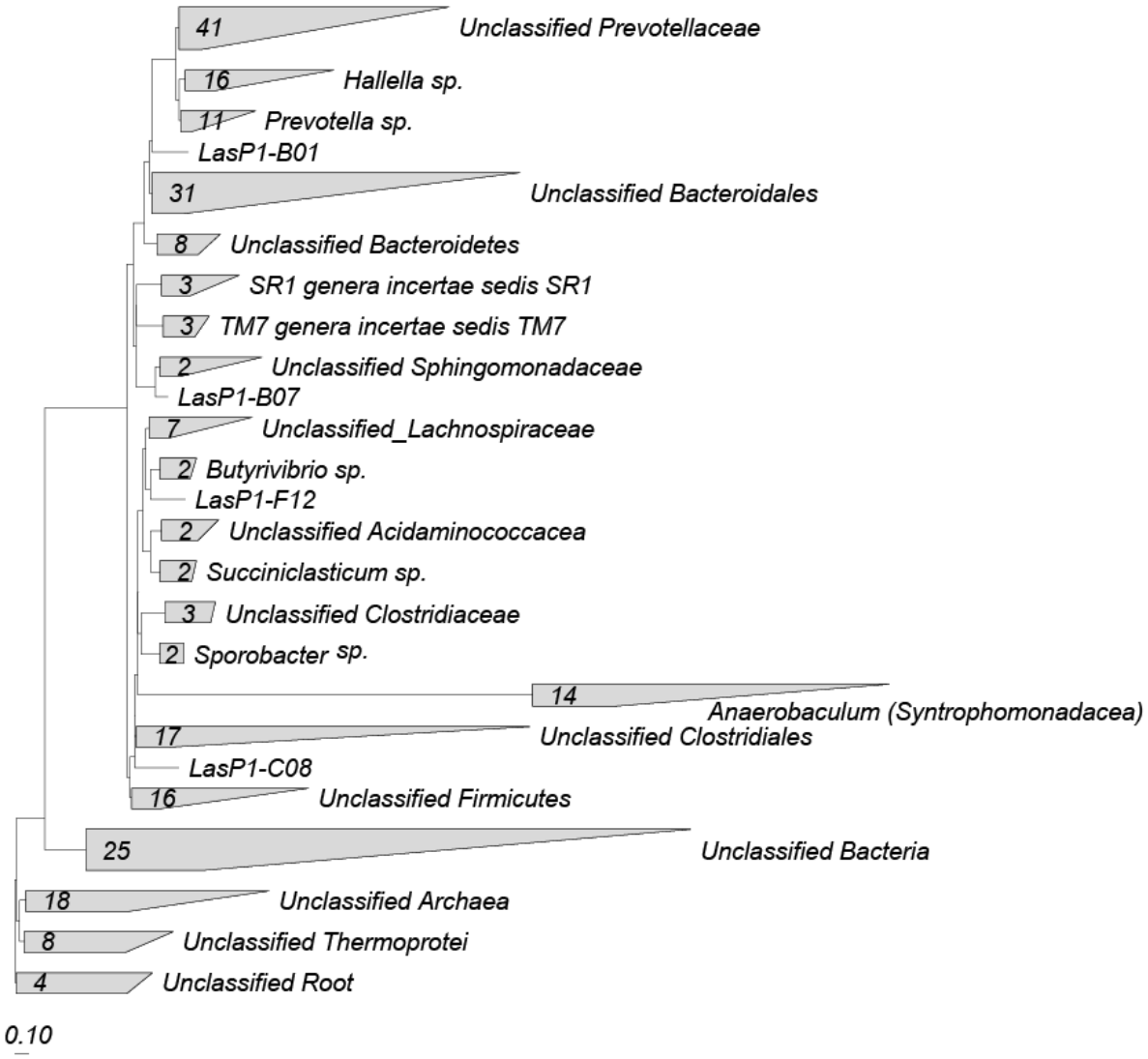
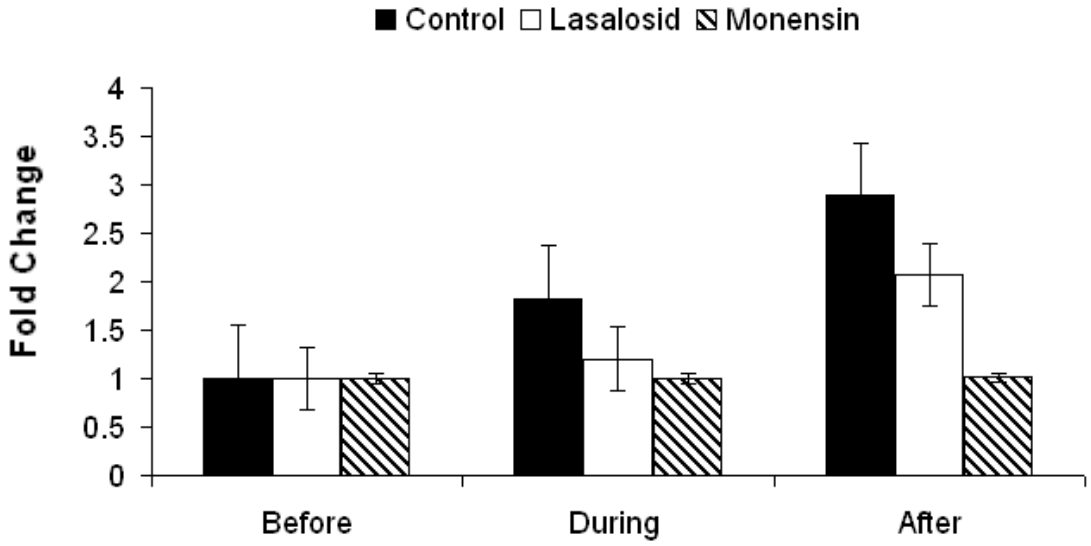

41
Unclassified Prevotellaceae
16
Hallella sp.
11
Prevotella sp.
LasP1-B01
31
Unclassified Bacteroidales
8
Unclassified Bacteroidetes
3
SR1 genera incertae sedis SR1
3
TM7 genera incertae sedis TM7
2
Unclassified Sphingomonadaceae
LasP1-B07
7
Unclassified_Lachnospiraceae
2
Butyrivibrio sp.
LasP1-F12
2
Unclassified Acidaminococcacea
2
Succiniclasticum sp.
3
Unclassified Clostridiaceae
2
Sporobacter sp.
14
Anaerobaculum (Syntrophomonadacea)
17
Unclassified Clostridiales
LasP1-C08
16
Unclassified Firmicutes
25
Unclassified Bacteria
18
Unclassified Archaea
8
Unclassified Thermoprotei
4
Unclassified Root
0.10

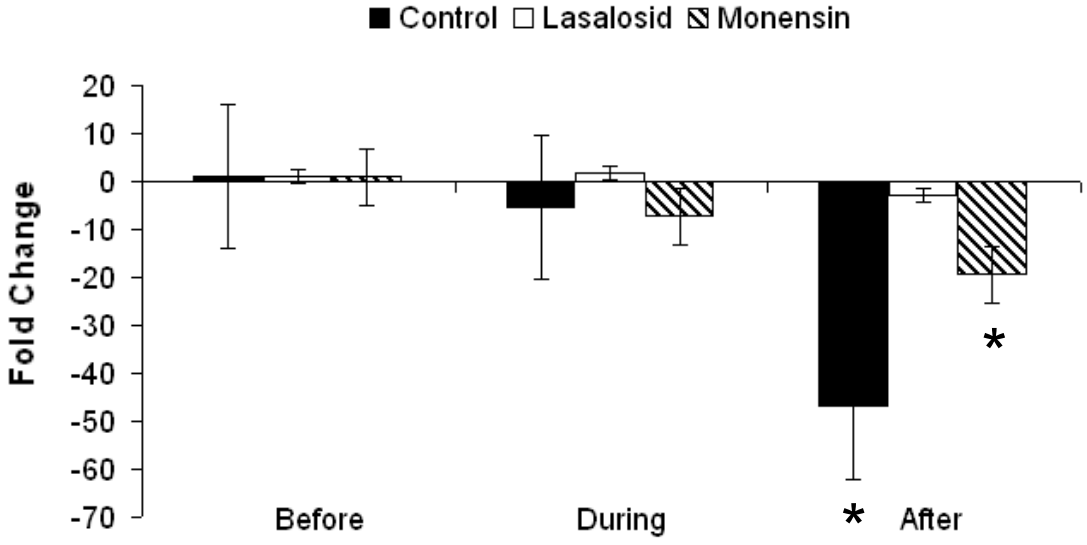
Figure 6

Population changes of selected rumen bacterial species using qRT-PCR. Microbial population changes were calculated as described in materials and methods.

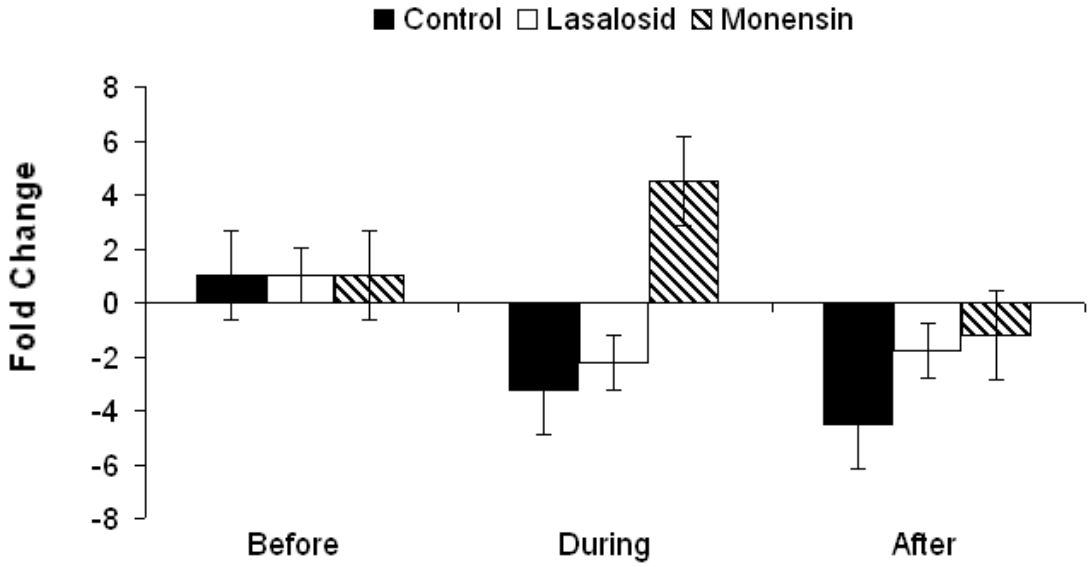
Butyrivibrio fibrosolvens



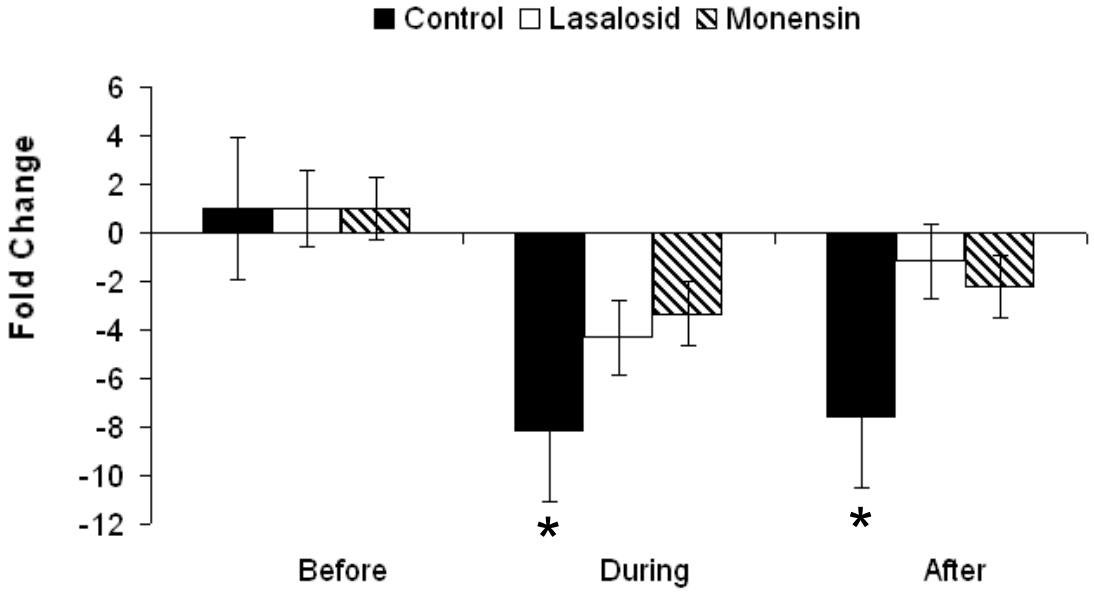
Prevotella bryanti



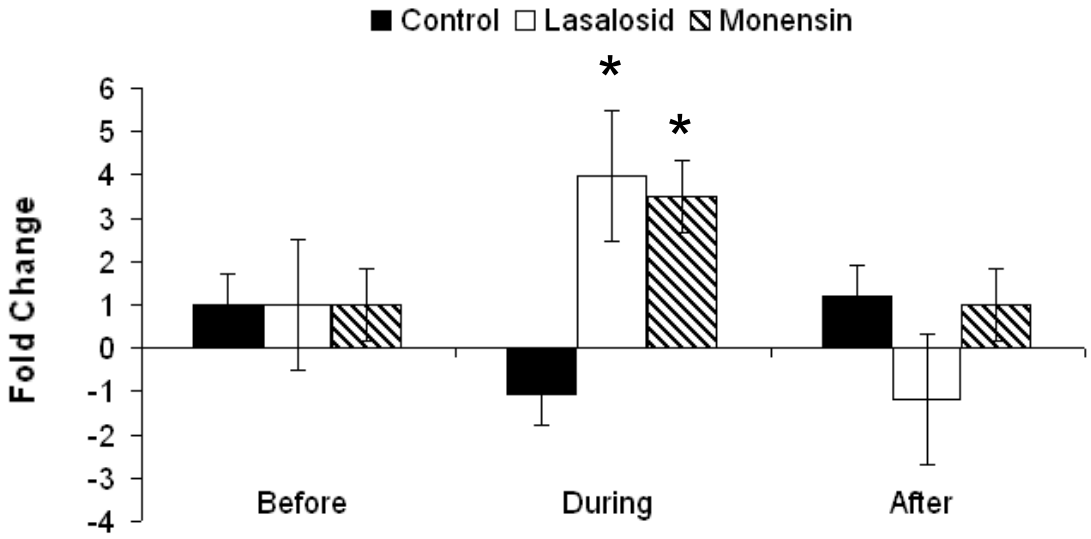
Fibrobacter succinogenes



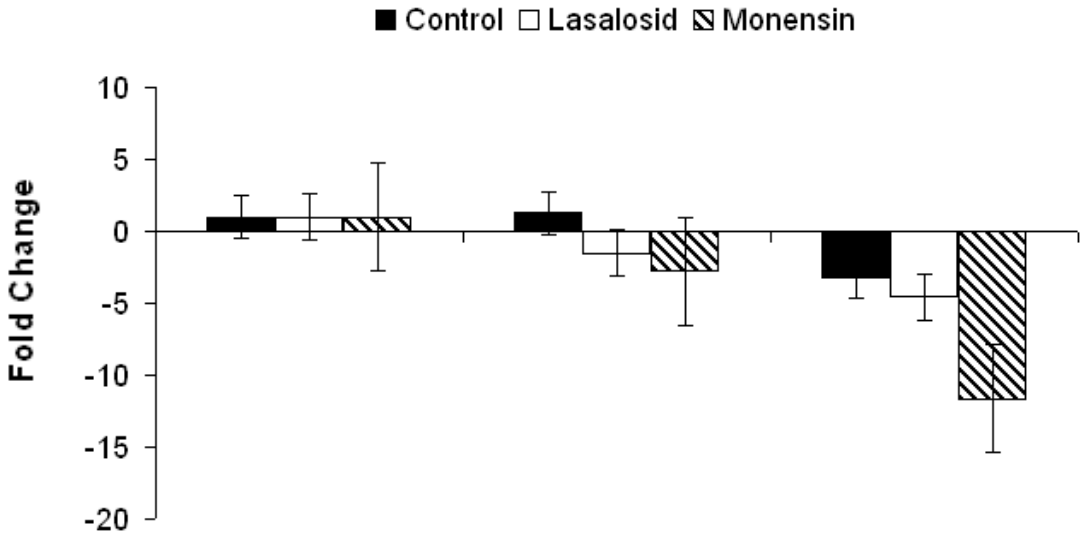
Prevotella ruminicola



Megasphera elsdenii



Selenomonas ruminantium



Streptococcus bovis

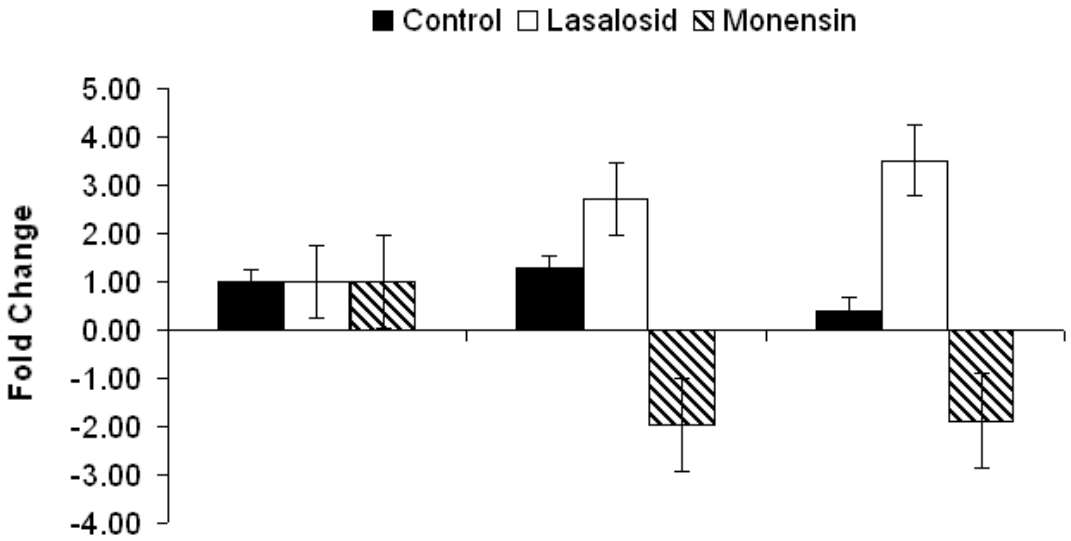


Table 1. Primer sequences used for Real Time PCR analysis.

Primer	Sequence 5'-3'	Product Size (bp)	Tm Used (°C)	Reference
Prevotella ruminicola –For	GGTTATCTTGAGTGAGTT	485	58	(Tajima et al., 2001) (47)
Prevotella ruminicola –Rev	CTGATGGCAACTAAAGAA			
Prevotella bryantii –For	ACTGCAGCGCGAACTGTCAGA	421	58	(Tajima et al., 2001)
Prevotella bryantii –Rev	ACCTTACGGTGGCAGTGTCTC			
Fibrobacter succinogenes -For	GGTATGGGATGAGCTTGC	445	62	(Tajima et al., 2001)
Fibrobacter succinogenes -Rev	GCCTGCCCCTGAACTATC			
Selenomonas ruminantium -For	TGCTAATACCGAATGTTG	513	53	(Tajima et al., 2001)
Selenomonas ruminantium -Rev	TCCTGCACTCAAGAAAGA			
Megasphaeara eslandi i-For	GACCGAAACTGCGATGCTAGA	128	60	(Ouwerkerk et al., 2002)
Megasphaeara eslandii –Rev	TCCAGAAAGCCGCTTTCGCCACT			
Streptococcus bovis –For	ATTCTTAGAGATAGGGTTTCTCTT	134	60	(Fernando et al., 2008d)
Streptococcus bovis –Rev	ACCTTATGATGGCAACTAACAATA			
Butyrivibrio fibrisolvens –For	CGCATGATGCAGTGTGAAAAGCTC	625	56	(Fernando et al., 2008d)
Butyrivibrio fibrisolvens –Rev	CCTCCCGACACCTATTATTCATCG			
rpoB –For	AACATCGGTTTGATCAAC	371-390	53.5	(Dahllof et al., 2000)
rpoB –Rev	CGTTGCATGTTGGTACCCAT			

Table 2. Classification and comparison of the 16S libraries constructed from animals dosed with Monensin, Lasalosid and Control animals. The 16S libraries constructed were analyzed using software tools available at the ribosomal database project using a confidence threshold value of 80%.

Rank	Library 1 Control	Library 2 Monensin	Library 3 Lasalosid
Domain Archaea	9	6	42
Phylum Crenarchaeota	5	2	27
Class Thermoprotei	5	2	27
Order Desulfurococcales	0	1	2
Unclassified Desulfurococcales	0	1	2
Unclassified Thermoprotei	5	1	25
Unclassified Archaea	4	4	15
Domain Bacteria	321	319	290
Phylum Planctomycetes	0	1	0
Class Planctomycetacia	0	1	0
Order Planctomycetales	0	1	0
Family Planctomycetaceae	0	1	0
Unclassified Planctomycetaceae	0	1	0
Phylum SR1	13	1	3
Genus Genera_incertae_sedis_SR1	13	1	3
Phylum Genera_incertae_sedis_TM7	1	0	4
Genus TM7	1	0	4
Phylum Verrucomicrobia	1	0	0
Class Verrucomicrobiae	1	0	0
Order Verrucomicrobiales	1	0	0
Family Subdivition 5	1	0	0
Genus Subdivition 5_incertae_sedis	1	0	0
Phylum Spirochaetes	1	1	0
Class Spirochaetes	1	1	0
Order Spirochaetales	1	1	0
Family Spirochaetaceae	1	1	0
Genus Treponema	1	1	0
Phylum Fibrobacteres	1	0	0
Class Fibrobacteres	1	0	0
Order Fibrobacterales	1	0	0
Family Fibrobacteraceae	1	0	0
Genus Fibrobacter	1	0	0
Phylum Firmicutes	87	83	92
Class Bacilli	2	6	1
Order Lactobacillales	2	6	1
Family Incertae sedis 9	2	6	1
Genus Syntrophococcus	1	4	1
Genus Oscillospira	1	2	0
Class Clostridia	78	68	76
Order Clostridiales	77	67	75

Family Acidaminococcaceae	12	4	4
Genus Schwartzia	1	0	0
Genus Mitsuokella	1	0	0
Genus Succiniclasticum	2	2	2
Genus Anaerovibrio	3	0	0
Unclassified Acidaminococcaceae	5	2	2
Family Syntrophomonadaceae	7	16	27
Genus Anaerobaculum	7	16	27
Family Lachnospiraceae	32	26	19
Genus Ruminococcus	0	0	1
Genus Pseudobutyrivibrio	0	1	2
Genus Catenibacterium	1	1	0
Genus Oribacterium	2	3	0
Genus Butyrivibrio	8	1	2
Unclassified Lachnospiraceae	21	20	14
Family Clostridiaceae	5	3	6
Genus Anaerotruncus	0	0	1
Genus Sporobacter	0	0	2
Genus Acetanaerobacterium	1	0	0
Unclassified Clostridiaceae	4	3	3
Unclassified Clostridiales	21	18	19
Unclassified Clostridia	1	1	1
Unclassified Firmicutes	7	9	15
Phylum Proteobacteria	2	6	5
Class Alphaproteobacteria	0	5	5
Order Sphingomonodalase	0	5	5
Family Sphingomonodaceae	0	5	5
Genus Sphingomonas	0	5	4
Unclassified Sphingomonodaceae	0	0	1
Class Gammaproteobacteria	2	1	0
Order Enterobacteriales	2	1	0
Family Enterobacteriaceae	2	1	0
Genus Leclercia	1	0	0
Unclassified Enterobacteriaceae	1	1	0
Phylum Bacteroidetes	165	166	139
Class Bacteroidetes	153	156	134
Order Bacteroidales	153	156	134
Family Bacteroidaceae	2	0	0
Unclassified Bacteroidaceae	2	0	0
Family Rikenellaceae	0	1	0
Unclassified Rikenellaceae	0	1	0
Family Porphyromonadaceae	3	4	8
Genus Tannerella	2	2	1
Unclassified Porphyromonadaceae	1	2	7
Family Prevotellaceae	116	103	87
Genus Prevotella	34	6	23
Genus Hallella	45	51	28

Unclassified Prevotellaceae	37	46	36
Unclassified Bacteroidales	32	48	39
Unclassified Bacteroidetes	12	10	5
Unclassified Bacteria	49	62	47

Table 3. Comparison of Libraries constructed from animals on Monensin, Lasalosid and Control animals at the phylum level using "Library Compare" software available at the ribosomal database project. Populations that are significantly different at p> 0.01 are shown with an asterix "*". Values are shown as percentage from total population

(A) Archea Vs Eubacteria

(B) Different phyla within Eubacteria

A

	Control	Monensin	Lasalosid
Archeae	2.8	2.6	12.9*
Eubacteria	97.8	97.4	87.1*

***Significantly different at 0.01**

B

Phylum	Control	Monensin	Lasalosid
TM7	0.3	0.0	0.9
Verrucomicrobia	0.3	0.0	0.0
SR1	3.9*	0.4	0.9
Fibrobacteres	0.3	0.0	0.0
Firmicutes	25.1	26.4	25.5
Bacteroidetes	49.8	47.3	41.4*
Spirochaetes	0.3	0.4	0.0
Proteobacteria	0.6	2.2	1.5
Unclassified Bacteria	13.9	20.9*	15.6

***Significantly different at 0.01**

Discussion

Ionophore supplementation is an efficient management tool that improves feed efficiency and weight gain in grazing cattle (Zinn, 1987; Zinn et al., 1994). Supplementation of ionophores has been shown to decline molar ratio of acetate and butyrate within the rumen (Chalupa, 1980; Roe, 2004) by increasing propionate production. Ionophore treatment also increase efficiency of energy and nitrogen metabolism, and help reduce feedlot disorders, such as lactic acidosis and bloating (Bergen and Bates, 1984). The decline in acetate and butyrate is associated with a reduction in methane production and improves carbon and energy retention within the rumen (Chalupa, 1980; Richardson et al., 1976). Treatment of monensin has a protein sparing effect (Chalupa, 1980), where dietary protein bypasses the rumen for digestion and uptake in the small intestine. Ionophores alter rumen microflora (Chen and Wolin, 1979) via changes in the microbial membrane's permeability to specific ions, thus altering various biological concentration gradients and selectively eliminating gram-positive bacteria (Russell, 1987). Although several studies have presented the increase of gram positive bacteria, the exact population change during ionophore treatment is poorly understood as all studies have utilized culture dependent methods to estimate microbial population structure, where a majority of the rumen microbial population is resistant to in-vitro culture. Therefore, this study is among the first to report rumen microbial population dynamics using culture independent molecular techniques during ionophore treatment in cattle.

The survey of rumen microbial species using T-RFLP analysis identified bacteria belonging to 255 different bacterial genera suggesting great diversity within the rumen. Due to the animal to animal variation among the experimental animals, the initial bacterial populations did not seem identical and had a few variations among the treatment groups, thus the comparisons during ionophore treatment was carried out within each treatment to evaluate microbial population changes (Figure 1). “DURING” ionophore treatment the *Bacteroidetes* populations increased in both lasalosid (28.6% to 65%) and monensin (19.3% to 35.4%) fed animals compared to control animals (19.8% to 17.03). A majority of the bacteria belonging to phylum *Bacteroidetes* are anaerobic, Gram-negative bacteria (Holdeman et al., 1984). Therefore, the increase in bacteria belonging to phylum *Bacteroidetes* is consistent with previous observations reported by Russell and Houlihan (2003), who reported that Gram positive bacteria are more susceptible to ionophore treatment than Gram-negative bacteria. The decrease in *Firmicutes* population during ionophore treatment is also consistent with Russell and Houlihan’s (2003) observation of Gram-positive bacteria being more susceptible to ionophore treatment, as phylum *Firmicutes* is mostly composed of low G-C Gram positive bacteria (Murray, 1989), which may have been eliminated by ionophore treatment. The decrease in *Proteobacteria* populations, in animals treated with lasalosid may be due to the presence of lasalosid sensitive Gram-negative bacteria. Thus, the model of Gram-negative bacteria being resistant to ionophore treatment is more complex (Ipharraguerre and Clark, 2003; Russell and Houlihan, 2003) than previously anticipated, as certain Gram-positive and

negative bacteria within the rumen may have variable resistance or susceptibility to ionophore treatment. The category named “Other” which contained all other phyla that had fewer bacterial densities mostly contained the high G-C Gram-positive bacterial species. Therefore, the decrease of these bacterial species may be due to their susceptibility to ionophore treatment. Removal of ionophores, decreased *Bacteroidetes* populations, as the ruminal environment which favored Gram-negative bacteria during ionophore treatment was changed by removing the ionophore treatment. This new ruminal environment provided more competition between Gram-negative and Gram-positive bacteria for nutrients which may have resulted in the increase of low G-C Gram-positive bacteria belonging to phylum *Firmicutes* in ionophore treated animals. The *Proteobacteria* are composed of predominantly Gram-negative bacteria, which have highly diverse metabolic function (Garrity et al., 2005b; Garrity et al., 2005c; Garrity, 2001). As most of the Gram-negative bacteria in phylum *Proteobacteria* may be susceptible to ionophore treatment, the removal of ionophore may have led to the increase of *Proteobacteria* in ionophore treated animals. The increase of “Other” phyla, which mainly consists of bacteria belonging to high G-C Gram-positive bacteria, may also be due to the change in ruminal environment after removal of ionophore treatment.

T-RFLP analysis is a reliable and reproducible technique that provides a glimpse of the rumen microbial populations that exist within the rumen at a given time. But, T-RFLP analysis only identifies previously characterized bacterial species. Therefore, to further evaluate and characterize the microbial fluctuations during

ionophore treatment, we constructed three 16S rDNA libraries from control animals and animals treated with ionophores and sequenced a 384 well plate from each library. Several 16S rDNA libraries have been constructed to access rumen microbial population changes in animals on high concentrate diet and prairie hay (Edwards et al., 2004; Fernando et al., 2008c; Tajima et al., 1999; Tajima et al., 2000), but no 16S rDNA libraries has been constructed from animals dosed with ionophores or from animals on wheat pasture. The 16S rDNA libraries show significant microbial population changes during ionophore treatment (Table 2 & 3 and Figure 2). An increased number of *Archaea* was detected in lasalosid treated animals. The majority of the *Archaea* detected belonged to phylum *Crenarchaeota* (Table 2), which are Gram-negative, morphologically diverse organisms (Garrity, 2001). Since, Ionophore treatment increases the growth of Gram-negative bacteria by eliminating Gram-positive bacteria (Russell and Houlihan, 2003), lasalosid treatment may have enhanced the growth of *Crenarchaeota*. But, a similar increase in *Crenarchaeota* was not detected in monensin treated animals, suggesting that the mode of action of lasalosid and monensin on rumen microbial species are different. The higher number of Archaea in lasalosid treated animals may have resulted in the significantly lower population of *Eubacteria* due to the competition within rumen populations. Bacteria belonging to phylum *SR1* were significantly higher in control animals when compared to animals on ionophore treatment (Figure 2 and Table 2 & 3). The decrease in *SR1* populations in Ionophore treated animals may be due to the susceptibility of SR1 bacteria to ionophore treatment. *Firmicutes*

populations within all treatments were consistent and accounted for ~25% of the total population sequenced. This observation is not consistent with our T-RFLP analysis, which showed a decrease in *Firmicutes* during ionophore treatment. This difference may be due to the different analysis methods used, as T-RFLP analysis was carried out using the Phylogenetic Analysis Tool that compares the restriction fragments to an electronic database, whereas, the 16S libraries were analyzed using the "Library compare" and "Classifier" tools available at ribosomal database project. Within phylum *Firmicutes*, the predominant class was *Clostridia* which accounted for more than 90% of the bacterial population. Among the families within *Clostridia*, bacteria belonging to family *Acidaminococcaceae* were lower in ionophore treated animals, suggesting their susceptibility to ionophore treatment. Bacteria belonging to family *Syntrophomonadaceae,* genus *Anaerobaculum* were higher in Ionophore treated animals (Table 2). Members of genus *Anaerobaculum* has been reported to have peptide fermenting capabilities (Menes and Muxi, 2002). During ionophore treatment, improved nitrogen metabolism has been reported (Bergen and Bates, 1984). Therefore, the increased *Anaerobaculum sp.* may play a role in nitrogen metabolism within the rumen. The decrease in Family *Lachnospiraceae* in lasalosid treated animals may be due to the susceptibility of *Lachnospiraceae* to lasalosid treatment. Bacterial populations belonging to Phylum *Bacteroidetes* was the most abundant in all three groups, which accounted for ~50% of the total bacterial population sequenced from each treatment. Majority of *Bacteroidetes sp.* are anaerobic, Gram-negative bacteria (Garrity, 2001), and are therefore, resistant to ionophore

treatment (Russell and Houlihan, 2003). Also, previous studies have reported that *Bacteroidetes* populations are among the most abundant proteolytic organisms within the rumen (Bergen and Bates, 1984). Among the *Bacteroidetes* populations, majority of the bacteria belonged to family *Prevotellaceae*, which include genus *Prevotella* and *Hallella*. Genus *Prevotella* has been shown to have great diversity in their sensitivity to monensin (Callaway and Russell, 2000), therefore the lower numbers of *Prevotella sp.* during monensin treatment (Table 2) may be due to the presence of monensin susceptible species. Genus *Hallella* is known to have only one species, which is an anaerobic, Gram-negative bacteria (Moore and Moore, 1994). But hierarchical classification of the 16S rDNA sequences could not classify theses sequences to the known *Hallella seregens*, suggesting that the 16S rDNA sequences generated may belong to new bacterial species not identified thus far. As the bacteria belonging to genus *Hallella* was decreased during lasalosid treatment, this suggests that the *Hallella sp.* present within the rumen may be susceptible to lasalosid treatment. The unclassified bacteria accounted for ~12.5% or more in each library. This suggests that at least 1/8 of the rumen bacterial population have not been characterized, in addition, the number of unclassified bacteria increase further during monensin treatment suggesting that the unclassified bacteria within the rumen may be much grater than we currently anticipate. Therefore, the rumen microbial population has a great number of unclassified organisms and may lead to the identification of new microbes of great economic importance.

The phylogenetic analysis of rumen microbial populations displayed great diversity in population structure and allowed identification of relatedness among the bacterial populations. The phylogenetic analysis was performed using arb software analysis program (Ludwig et al., 2004). The three libraries constructed were used to identify a non-redundant set of Operational Taxanomic Units (OTUs) for each treatment. The Non-redundant OTUs identified were used to construct phylogenetic trees as described in materials and methods (Figure 3-5). Although the number of OTUs identified within each library was similar, the distribution and relatedness of the 16S sequences were significantly different among the three different ruminal environments, suggesting that the microbial populations within the rumen are highly dynamic and react rapidly to changes in their environment to adapt to their new environment. The significantly large group of phylogenetically distant *Firmicutes* population observed in control and ionophore treated animals corresponded to ~25% of the total sequences and was composed of organisms belonging to the genera *Syntrophococcus, Oscillospira, Schwartzia, Mitsuokella, Succiniclasticum, Anaerovibrio, Anaerobaculum, Ruminococcus, Pseudobutyrivibrio, Catenibacterium, Oribacterium, Butyrivibrio, Anaerotruncus, Sporobacter, Acetanaerobacterium* and several unclassified *Firmicutes sp.* At first we were surprised to identify any *Archaea* at all, as the primers we used to amplify the 16S rRNA gene was believed to be specific to all Eubacterial species. But, previously we have been able to amplify *Archaea, Methanobrevibacter* species (Fernando et al., 2008c) in prairie hay fed animals, suggesting that this primer set may be able to detect certain groups of *Archaea*

bacteria that have high similarity to *Eubacterial* sequences. The phylogenetically diverse *Bacteroidetes* population detected belonged to genera *Tannerella, Prevotella, Hallella,* and a large number of unclassified *Bacteroidetes sp. (*Table 2). The *Bacteriodetes* population was similar in control and monensin treated animals but was lower in lasalosid treated animals. Although the *Proteobacterial* populations are small in all three libraries, the immerging *Proteobacteria* are visible in the ionophore treated libraries. Sequence and phylogenetic analysis of the 16S rDNA libraries constructed, reveals the presence of numerous uncultured rumen bacterial species with unknown function. Thus, the classification based on conserved regions of the 16S rDNA sequence will enable prediction of the genus the bacteria belong to and will help to predict the functional role of unclassified bacteria within the rumen. Recent studies have evaluated and quantified rumen microbial population changes during adaptation to a high concentrate diet, acidosis, and prairie hay using culture independent techniques such as quantitative realtime PCR and 16S probes (qRT-PCR) (Attwood et al., 1988; Fernando et al., 2008c; Fernando et al., 2008; Stahl et al., 1988; Tajima et al., 2001; Tajima et al., 2000) as culture dependent techniques have failed to provide accurate estimates of microbial population changes due to the bias towards overrepresentation of easy to culture bacterial species (Tajima et al., 2001). But, qRT-PCR has not been employed to investigate microbial population changes during ionophore treatment. Quantitative realtime PCR is a powerful technique which allows the identification of susceptibility or resistance of an individual bacterial species towards ionophore treatment. Therefore, to

identify the microbial changes and the susceptibility of selected bacteria to ionophore treatment, we quantified rumen microbial population changes of functionally important groups of bacteria using qRT-PCR (Figure 6). Quantitative realtime PCR analysis displayed 3.5 and 4 fold increase in *Megasphera elsdenii* populations during monensin and lasalosid treatment. This increase in *Megasphera elsdenii* populations during ionophore treatment is consistent with pure culture experiment performed by Henderson et al. (1981). Where they reported that monensin favored growth of propionate produces such as *Megasphera elsdenii* is likely to occur through selective inhibition of organisms not producing propionate (Henderson et al., 1981). Therefore the increase in *Megasphera elsdenii* populations during ionophore treatment may help decrease acetate:propionate ratio and to increase the energy balance in the animal (Ipharraguerre and Clark, 2003). *Megasphera elsdenii* has also been shown to utilize lactic acid synthesized within the rumen (Russell and Dombrowski, 1980) and to help maintain ruminal pH against lactic acid accumulation and acidosis (Counotte et al., 1981). Therefore, the increasing *Megasphera elsdenii* population during ionophore treatment may be the reason that ionophore treatment in beneficial against acidosis. *Streptococcus bovis* is a Gram-positive model organism that is susceptible for monensin treatment (Russell and Houlihan, 2003; Russell and Strobel, 1989). During monensin treatment *Streptococcus bovis* decreased 2 fold. This is consistent with previous reports by Henderson et al. (1981), who evaluated the effect of monensin on pure cultures of *Streptococcus bovis*. But, during lasalosid treatment, *Streptococcus bovis*

populations increased. Although this increase was not statistically significant, this was surprising as we expected *Streptococcus bovis* populations to decrease during ionophore treatment. This resistance to lasalosid treatment may be due to a resistance strain *Streptococcus bovis*. *Selenomonas ruminantium* decreased 1.5 and 2.7 fold in animals dosed with lasalosid and monensin during ionophore treatment. This observation is contradictory to the observation reported (Henderson et al., 1981) using pure cultures. The reason for the decrease in *Selenomonas ruminantium* population may be due to the competition with *Megasphera elsdenii*, as both these organisms are lactate utilizes and have similar functions within the rumen. *Prevotella sp.* displayed different patterns of population changes during ionophore treatment. This may be due to the variations in the ability of *Prevotella sp.* to resist ionophore treatment (Callaway et al., 1997). However, since *Prevotella ruminicola* populations decreased in all 3 treatment groups including the control animals, we can not make any assumptions on the effect of ionophore treatment on *Prevotella ruminicola* populations. But, the statistically insignificant decrease of *Prevotella ruminicola* in ionophore treated animals compared to control animals, suggests that *Prevotella* populations are stable during ionophore treatment (Henderson et al., 1981). *Prevotella bryantii* population did not show significant changes in lasalosid treated animals during the study period. But in control animas and monensin treated animals, during ionophore treatment, *Prevotella bryantii* population decreased, but was insignificant. However, after removal of ionophore treatment the populations decreased significantly. This decrease in *Prevotella ruminicola*

and *Prevotella bryantii* populations may be due to an environmental factor as the largest decrease in population was seen in control animals. *Fibrobacter succinogenes* and *Butyrivibrio fibrosolvens* populations were similar throughout the study period and did not show significant changes in population. *Butyrivibrio fibrosolvens* has been shown to have proteolytic activity (Bergen and Bates, 1984) and may be involved in nitrogen metabolism during ionophore treatment. But, previous studies have reported that *Butyrivibrio fibrosolvens* is a monensin sensitive Gram-negative bacterium that can tolerate low monensin concentrations (Dinius et al., 1976). Therefore, the reason we did not observe a significant decrease in *Butyrivibrio fibrosolvens* may be due to a resistant strain or low ionophore treatment.

This preliminary study of rumen microbial population changes during ionophore treatment in wheat pasture cattle using T-RFLP analysis, 16S rRNA sequencing and quantitative real-time PCR shows significant changes in rumen microbial population structure and diversity during ionophore treatment, and reports the presence large number of previously unreported organisms as well as previously unidentified changes within the rumen. This investigation of rumen microbial population structure and diversity using T-RFLP analysis, 16S rDNA libraries, and real-time PCR is among the first from ionophore treated animals and will provide valuable information towards identifying new rumen bacterial species. Hence, this study provides new insight into the role and changes of rumen bacteria during ionophore treatment in wheat pasture cattle. But, ionophore treatment has been reported to toxic not only to bacteria but also to protozoa,

fungi that reside within the rumen (Russell and Strobel, 1989). Therefore, we believe that further studies need to be carried out to evaluate the effect of ionophores on rumen fungi and protozoa populations to identify the total microbial populations during ionophore treatment.

Reference List

Attwood,G.T., Lockington,R.A., Xue,G.P., and Brooker,J.D. (1988) Use of A Unique Gene Sequence As A Probe to Enumerate A Strain of Bacteroides-Ruminicola Introduced Into the Rumen. *Applied and Environmental Microbiology* **54:** 534-539.

Bergen,W.G. and Bates,D.B. (1984) Ionophores - Their Effect on Production Efficiency and Mode of Action. *Journal of Animal Science* **58:** 1465-1483.

Bodenteich,A., Chissoe,S., Wang,Y.F., and Roe,B.A. (1993) Shotgun Cloning as the Strategy of Choice to Generate Templates for High-throughput Dideoxynucleotide Sequencing. In *Automated DNA Sequencing and Analysis Techniques*. Venter,J.C. (ed). Academic Press, London.

Callaway,T.R., Demelo,A.M.S., and Russell,J.B. (1997) The effect of nisin and monensin on ruminal fermentations in vitro. *Current Microbiology* **35:** 90-96.

Callaway,T.R. and Russell,J.B. (2000) Variations in the ability of ruminal Gram-negative Prevotella species to resist monensin. *Current Microbiology* **40:** 185-189.

Chalupa,W. (1980) Chemical control of rumen microbial metabolism. In *Digestive Physiology and Metabolism in Ruminants*. Ruckebush,Y. and Thivend,P. (eds). Westport, CT: AVI Publishing Co., Inc..

Chen,M. and Wolin,M.J. (1979) Effect of Monensin and Lasalocid-Sodium on the Growth of Methanogenic and Rumen Saccharolytic Bacteria. *Applied and Environmental Microbiology* **38:** 72-77.

Chissoe,S.L., Bodenteich,A., Wang,Y.F., Wang,Y.P., Burian,D., Clifton,S.W. et al. (1995) Sequence and Analysis of the Human Abl Gene, the Bcr Gene, and Regions Involved in the Philadelphia Chromosomal Translocation. *Genomics* **27:** 67-82.

Chow,J.M., Vankessel,J.A.S., and Russell,J.B. (1994) Binding of Radiolabeled Monensin and Lasalocid to Ruminal Microorganisms and Feed. *Journal of Animal Science* **72:** 1630-1635.

Cole,J.R., Chai,B., Farris,R.J., Wang,Q., Kulam,S.A., McGarrell,D.M. et al. (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Research* **33:** D294-D296.

Cole,J.R., Chai,B., Farris,R.J., Wang,Q., Kulam-Syed-Mohideen,A.S., McGarrell,D.M. et al. (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Research* **35:** D169-D172.

Cole,J.R., Chai,B., Marsh,T.L., Farris,R.J., Wang,Q., Kulam,S.A. et al. (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows

regular updates and the new prokaryotic taxonomy. *Nucleic Acids Research* **31:** 442-443.

Counotte,G.H.M., Prins,R.A., Janssen,R.H.A.M., and Debie,M.J.A. (1981) Role of Megasphaera-Elsdenii in the Fermentation of Lactate-2-C-13 in the Rumen of Dairy-Cattle. *Applied and Environmental Microbiology* **42:** 649-655.

Dahllof,I., Baillie,H., and Kjelleberg,S. (2000) rpoB-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. *Applied and Environmental Microbiology* **66:** 3376-3380.

Dinius,D.A., Simpson,M.E., and Marsh,P.B. (1976) Effect of Monensin Fed with Forage on Digestion and Ruminal Ecosystem of Steers. *Journal of Animal Science* **42:** 229-234.

Edwards,J.E., Mcewan,N.R., Travis,A.J., and Wallace,R.J. (2004) 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **86:** 263-281.

Ewing,B. and Green,P. (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Research* **8:** 186-194.

Ewing,B., Hillier,L., Wendl,M.C., and Green,P. (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Research* **8:** 175-185.

Fernando,S.C., Purvis II,H.T., Najar,F.Z., Sukharnikov,L., Nagaraja,T.G., Krehbiel,C.R. et al. Rumen microbial changes during adaptation to high concentrate diet. 2008a.
Ref Type: Unpublished Work

Fernando,S.C., Purvis II,H.T., Najar,F.Z., Sukharnikov,L., Nagaraja,T.G., Krehbiel,C.R. et al. Rumen microbial population dynamics during sub acute acidosis. 2008.
Ref Type: Unpublished Work

Garrity,G.M., Brenner,D.J., Krieg,N.R., and Staley,J.T. (2005b) *Bergey's Manual of Systematic Bacteriology: The Proteobacteria; The Gammaproteobacteria*. Springer.

Garrity,G.M., Brenner,D.J., Krieg,N.R., and Staley,J.T. (2005a) *Bergey's Systematic Bacteriology: The Proteobacteria; The Alpha-, Beta-, Delta-, and Epsilonproteobacteria.* Springer.

Garrity,G.M. (2001) *Bergey's Manual of Systematic Bacteriology: The Archaea and the deeply branching and phototrophic bacteria.* Springer.

Gordon,D., Abajian,C., and Green,P. (1998) Consed: A graphical tool for sequence finishing. *Genome Research* **8:** 195-202.

Henderson,C., Stewart,C.S., and Nekrep,F.V. (1981) The Effect of Monensin on Pure and Mixed Cultures of Rumen Bacteria. *Journal of Applied Bacteriology* **51:** 159-169.

Hettinger,A.M., Allen,M.R., Zhang,B.R., Goad,D.W., Malayer,J.R., and Geisert,R.D. (2001) Presence of the acute phase protein, bikunin, in the endometrium of gilts during estrous cycle and early pregnancy. *Biology of Reproduction* **65:** 507-513.

Holdeman,L.V., Kelley,R.W., and Moore,W.E.C. (1984) Anaerobic gram-negative straight, curved and helical rods. In *Bergey'S Manual of Systematic Bacteriology*. Wiliams & Wilkins, pp. 602-662.

Ipharraguerre,I.R. and Clark,J.H. (2003) Usefulness of ionophores for lactating dairy cows: a review. *Animal Feed Science and Technology* **106:** 39-57.

Kent,A.D., Smith,D.J., Benson,B.J., and Triplett,E.W. (2003) Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. *Applied and Environmental Microbiology* **69:** 6768-6776.

Ludwig,W., Strunk,O., Westram,R., Richter,L., Meier,H., Yadhukumar et al. (2004) ARB: a software environment for sequence data. *Nucleic Acids Research* **32:** 1363-1371.

Menes,R.J. and Muxi,L. (2002) Anaerobaculum mobile sp nov., a novel anaerobic, moderately thermophilic, peptide-fermenting bacterium that uses crotonate as an electron acceptor, and emended description of the genus Anaerobaculum. *International Journal of Systematic and Evolutionary Microbiology* **52:** 157-164.

Moore,L.V.H. and Moore,W.E.C. (1994) Oribaculum Catoniae Gen-Nov, Sp-Nov - Catonella Morbi Gen-Nov, Sp-Nov - Hallella Seregens Gen-Nov, Sp-Nov - Johnsonella Ignava Gen-Nov, Sp-Nov - and Dialister Pneumosintes Gen-Nov, Comb-Nov, Nom Rev - Anaerobic Gram-Negative Bacilli from the Human Gingival Crevice. *International Journal of Systematic Bacteriology* **44:** 187-192.

Murray,R.G.E. (1989) The higher taxa, or, a place for everything...? In *Bergey's Manual of Systematic Bacteriology*. Staley,J.T., Bryant,M.P., and Pfenning,N. (eds). pp. 1631-1634.

Ouwerkerk,D., Klieve,A.V., and Forster,R.J. (2002) Enumeration of Megasphaera elsdenii in rumen contents by real-time Taq nuclease assay. *Journal of Applied Microbiology* **92:** 753-758.

Richardson,L.F., Raun,A.P., Potter,E.L., Cooley,C.O., and Rathmacher,R.P. (1976) Effect of Monensin on Rumen Fermentation Invitro and Invivo. *Journal of Animal Science* **43:** 657-664.

Roe,B.A. (2004) Shotgun Library Construction for DNA Sequencing. In *Methods in Molecular Biology*. Zhao,S. and Stodolsky,M. (eds). Human Press Inc, Totowa, New Jersey, pp. 171-187.

Russell,J.B. (1987) A Proposed Mechanism of Monensin Action in Inhibiting Ruminal Bacterial-Growth - Effects on Ion Flux and Protonmotive Force. *Journal of Animal Science* **64:** 1519-1525.

Russell,J.B. and Dombrowski,D.B. (1980) Effect of Ph on the Efficiency of Growth by Pure Cultures of Rumen Bacteria in Continuous Culture. *Applied and Environmental Microbiology* **39:** 604-610.

Russell,J.B. and Houlihan,A.J. (2003) Ionophore resistance of ruminal bacteria and its potential impact on human health. *Fems Microbiology Reviews* **27:** 65-74.

Russell,J.B. and Strobel,H.J. (1989) Effect of Ionophores on Ruminal Fermentation. *Applied and Environmental Microbiology* **55:** 1-6.

Santos,S.R. and Ochman,H. (2004) Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. *Environmental Microbiology* **6:** 754-759.

Stahl,D.A., Flesher,B., Mansfield,H.R., and Montgomery,L. (1988) Use of Phylogenetically Based Hybridization Probes for Studies of Ruminal Microbial Ecology. *Applied and Environmental Microbiology* **54:** 1079-1084.

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

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

Tajima,K., Arai,S., Ogata,K., Nagamine,T., Matsui,H., Nakamura,M. et al. (2000) Rumen bacterial community transition during adaptation to high-grain diet. *Anaerobe* **6:** 273-284.

Wallace,R.J., Czerkawski,J.W., and Breckenridge,G. (1981) Effect of Monensin on the Fermentation of Basal Rations in the Rumen-Simulation-Technique (Rusitec). *British Journal of Nutrition* **46:** 131-148.

Wang,Q., Garrity,G.M., Tiedje,J.M., and Cole,J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73:** 5261-5267.

Zinn,R.A. (1987) Influence of Lasalocid and Monensin Plus Tylosin on Comparative Feeding Value of Steam-Flaked Versus Dry-Rolled Corn in Diets for Feedlot Cattle. *Journal of Animal Science* **65:** 256-266.

Zinn,R.A., Plascencia,A., and Barajas,R. (1994) Interaction of Forage Level and Monensin in Diets for Feedlot Cattle on Growth-Performance and Digestive Function. *Journal of Animal Science* **72:** 2209-2215.

CHAPTER V

MICROBIAL POPULATION DIVERSITY AND DISTRIBUTION THROUGHOUT THE GASTROINTESTINAL TRACT IN CATTLE

Introduction

Microbial ecosystem within the rumen is composed of an immense variety of bacteria, protozoa, and fungi (Ozutsumi et al., 2006). Among these microbial populations, the bacterial population predominates and has a significant effect on the animal's performance and productivity. The synergistic relationship between the microbial flora and the animal provides the animal with nutrients that are not available to monogastric animals (Church, 1993). Recently, increased attention has been placed on manipulating the ruminal microbial ecosystem to increase the animal's performance and efficiency (Martin, 1998). Traditionally, characterization of microbial populations from the gastrointestinal tract has relied on culture dependent approaches (Krause and Russell, 1996), where an estimated 90-99.9% of the organisms present resist *in-vitro* culture (Theron and Cloete, 2000). Therefore, these culture dependent techniques have failed to identify the total microbial population present within the rumen and gastrointestinal tract. However, in the last few decades molecular techniques, such as PCR and rapid sequence determination systems have emerged that enable the identification and characterization of bacteria that are unable to culture (Tajima et al., 1999; Tajima et al., 2001; Tajima et al., 2000). Although extensive research have been reported on microbial changes in the rumen during adaptation to different diets (Fernando et al., 2008c; Tajima et al., 2001; Tajima et al., 2000) and during metabolic disorders such as acidosis (Fernando et al., 2008d; Kleen et al., 2003; Nocek, 1997; Osborne et al., 2004; Owens et al., 1998) using traditional culture dependent and culture independent molecular techniques, little is known about the microbial population diversity and distribution

with respect to locality within the rumen and gastrointestinal tract. Previous studies that have evaluated microbial population structure within the gastrointestinal tract have shown that out of the total microbes detected, 91% in the rumen, 88% in the cecum and 96% in the lower gastrointestinal tract belong to bacteria (Lin et al., 1997), suggesting that the bacterial populations within the cecum and lower gastrointestinal tract also plays an important role within the animal. The bacterial populations in the mammalian midgut has been reported to be predominantly anaerobes and enterobacteria (Savage, 1986; Stevens and Hume, 1998), which include pathogenic enterohemorrhagic, *Escherichia coli 0157:H7* (Callaway et al., 2003). Similar studies have also reported that the microbial population found in the hindgut is distinctly different form populations found in the rumen, with the exception of *Bacteroides* which is found in both locations (Wolin, 1981). Furthermore, studies that have evaluated bacterial diversity within feedlot manure have reported that spore forming; gram positive bacteria are more abundant within the fecal samples than intestinal gram negative bacteria (Ouwerkerk and Klieve, 2001), suggesting that analyzing fecal samples to estimate intestinal bacterial population diversity may be bias towards gram positive bacteria (Ouwerkerk and Klieve, 2001).

Many of these studies have either utilized culture based techniques or have used indirect methods such as molecular probes to evaluate microbial population distribution within the bovine rumen and gastrointestinal tract. Therefore, as a first step towards discovering the microbial composition and the different microbial species present throughout the gastrointestinal tract in cattle fed on a high energy

diet, we have evaluated microbial population changes using T-RFLP analysis and quantitative real-time PCR.

Experimental Procedures

Animals and Sampling

Three beef steers (837$\pm$ 60 lbs) were fed a high energy diet that contained 2.7 Mcal of ME/Kg of dry matter with a fiber to concentrate ratio of 40:60. The diet was formulated to meet the animal's requirements as described in NRC-1996. After the animals have been adapted to the high energy diet, the animals were sacrificed and samples were collected from the reticulum, ventral sac, ventral blind sac, dorsal sac, dorsal blind sac, omasum, abomasum, proximal small intestine, distal small intestines, cecum, large intestine, and rectum to document the resident microbial populations in the rumen and gastrointestinal tract. The rumen and gastrointestinal contents were collected from all 3 steers and were snap frozen in liquid nitrogen until DNA extraction.

DNA isolation

Frozen rumen and gastrointestinal contents samples were ground in liquid nitrogen and were homogenized in a generic coffee grinder. 0.5 g of the homogenized sample was used to extract total bacterial DNA. Total bacterial DNA was extracted using the QIAamp® DNA mini stool kit (Qiagen, Valencia, CA, USA) according to manufacturers protocol with a few modifications described by Fernando et. al (2008b). Briefly, 1.4 ml of buffer ASL was added to 0.5g of each sample and was

vortexed for 15 sec. Following vortexing the samples were incubated at 95°C for 10 min. Next, the samples were vortexed for 15 sec and were centrifuged at 20,000g for 1 min. The supernatant was aspirated to a fresh 2 ml tube and an inhibitorEX tablet was added and vortexed immediately for 1 min, until the tablet was completely dissolved. The sample was then incubated at room temperature for 3 min, and was centrifuged at 20,000g for 3 min. The solution was aspirated and re-centrifuged for an additional 3 min. To 400 µl of the supernatant, 20 µl of proteinase K and 400 µl of AL buffer was added and incubated at 70°C for 30 min. 400 µl of 100% ethanol was added to the proteinase K digested sample. This sample was then added to the QIAamp spin column and centrifuged at 20,000g for 1 min. The column was washed with 500 µl of buffer AW1 and AW2 respectively. The spin columns were centrifuged at 20,000g to remove residual buffer AW2 and the DNA was eluted using 20 µl of distilled water. The concentration of the extracted DNA was measured using a UV spectrophotometer, and the quality of the DNA was evaluated by agarose gel-electrophoresis using a 1.2% gel.

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis

100 ng of total bacterial genomic DNA isolated from rumen and gastrointestinal tract contents were used for Terminal-Rescticton Fragment Length Polymorphisum (T-RFLP) analysis. 100 ng of total bacterial DNA was PCR amplified in a MJ dyad thermocycler (MJ research, Watertown, MA) using FAMBacT0008F 5'-AGAGTTTGATCCTGGCTCAG-3' and BacT0805R 5'-GGACTACCAGGGTATCTAATCCC-3' (Fernando et al., 2008d) to amplify a 809 bp

fragment (in *E. coli*) of the 16S rRNA gene in all Eubacteria. The FAMBacT008F primer is labeled at the 5' end with the fluorophore 6-FAM. T-RFLP analysis was performed as described by Fernando et al. (2008). Briefly, a ~ 805 bp fragment was PCR amplified as follows. The 50 µl PCR reaction contained, 100 ng of DNA, 1X PCR buffer (Promega, Madison, WI, USA) 1.5 mM $MgCl_2$, 400 nM of each primer, 200 µM dNTPs, 100 ng/µl BSA and 3 U Taq DNA Polymerase (Promega, Madison, WI, USA). Cycling conditions were: one cycle of 1 min at 95ºC, 30 sec at 52ºC, 1 min at 72ºC followed by 34 additional cycles of 30 sec at 95ºC, 30 sec at 52ºC, 1 min at 72ºC and a final extension of 3 min at 72ºC.

The amplified PCR products were ethanol precipitated, and were digested separately with RSA I *(*Invitrogen, Carlsbad, CA,USA), HAE III *(*Invitrogen, Carlsbad, CA,USA) and MSP I *(*Promega, Madison, WI, USA) according to Fernando et al. (2008b). A 10 µl reaction contained 1X buffer, 2.5 U Enzyme and 5 µl of the PCR product. 250 ng/µl of BSA was added to the MSP I digestion. The digestion reactions were incubated at 37°C for 4 hours, followed by 65°C for 20 min. 2 µl of the digested PCR product was mixed with 0.5 µl of GeneScan ROX 1000 size standard (Applied Biosystems, Foster City, CA) and 3.5 µl of loading dye (Applied Biosystems, Foster City, CA) and was electrophoresed for 8 hours in an ABI 377 sequence analyzer (Applied Biosystems, Foster City, CA). The data collected by the ABI 377 sequence analyzer was analyzed using GeneScan 3.1 analysis software (Applied Biosystems, Foster City, CA). The T-RFLP profiles generated were analyzed using Phylogenetic Assignment Tool (PAT) (Kent et al., 2003) to assign species for each restriction fragment generated. The bacterial species assignment was based on the restriction

digestion patterns obtained for all 3 enzymes of each sample. The peak area for each set of restriction fragments was used to construct microbial diversity plots. The peak areas for the 3 different digests were averaged and were used as an estimate of bacterial population. The representation of each bacterial species within the total bacterial population was calculated as the percentage of peak area of a bacterial species relative to the total peak area of the sample. Therefore, the bacterial populations and microbial diversity plots show the bacterial diversity and the relative abundance of each bacterial species identified within the population.

Quantitative Real-Time PCR analysis

Quantitative analysis was performed on selected bacterial species belonging to different Phyla to validate the bacterial fluctuation detected using T-RFLP analysis. Quantitative analysis of population changes of selected bacterial species were evaluated using real-time polymerase chain reaction (qPCR) using the SYBR I green reporter assay kit available from Roche Diagnosticis (Indianapolis, IN, USA). The primers used for analyzing *Prevotella ruminicola,* and *Treponema bryantii* were previously reported by Tajima et al. (2001). Primers for *Bifidobacterium ruminantium,* and *Butyrivibrio fibrosolvens* were previously reported by Fernando et al. (2008b) and the primers used for detection of *Escherichia coli 0157:H7* was previously reported by Sharma & Dean-Nystrom (Sharma and an-Nystrom, 2003). For all assays the PCR products generated were sequenced to confirm the amplification of the correct bacterial species.

A 15 µl reaction contained 400 nM forward primer, 400 nM reverse primer, 1X Master mix (Roche Diagnosticis, Indianapolis, IN, USA), and 40 ng of total rumen DNA. Thermal cycling conditions were 95°C for 10 min, followed by 50 additional cycles of 95°C for 30 sec 50-62°C for 30 sec, depending on the annealing temperature of each primer set (see Table 1) and 72°C for 40 sec or 1 min based on the size of the amplicon followed by the melting curve. The single copy universal gene *rpoB* was used for normalization (Dahllof et al., 2000; Santos and Ochman, 2004). The qRT-PCR reactions were performed in an ABI PRISM 7500® sequence detection system (Applied Biosystems, Foster City, CA).

Relative quantification of bacterial population changes was performed using the comparative C_T method as previously described (Hettinger et al., 2001). The ΔC_T value was determined by subtracting the 16S C_T value of each sample from the corresponding sample *rpoB* gene C_T value. Calculation of $\Delta\Delta C_T$ was carried out by using the highest normalized ΔC_T value as an arbitrary constant to subtract from all other ΔC_T mean values. Fold changes in bacterial populations were calculated from the $\Delta\Delta$CT values using the formula $2^{\pm\Delta\Delta Ct}$.

Table 1. Primer sequences used for Real Time PCR analysis.

Primer	Sequence 5'-3'	Product Size (bp)	Tm Used (°C)	Phylum
Prevotella ruminicola –For	GGTTATCTTGAGTGAGTT	485	58	Bacteroidetes
Prevotella ruminicola –Rev	CTGATGGCAACTAAAGAA			
Bifidobacterium ruminantium -For	TCCATCGCTTAACGGTGGATCAGC	662	62	Actinobacteria
Bifidobacterium ruminantium -Rev	CTCACGGGGTCGCATCCCGTTGTA			
Butyrivibrio fibrisolvens -For	CGCATGATGCAGTGTGAAAAGCTC	625	56	Firmicutes
Butyrivibrio fibrisolvens -Rev	CCTCCCGACACCTATTATTCATCG			
Treponema bryantii -For	AGTCGAGCGGTAAGATTG	421	58	Spirocheates
Treponema bryantii -Rev	CAAAGCGTTTCTCTCACT			
Escherichia coli –For	GTAAGTTACACTATAAAAGCACCGTCG	106	55	Proteobacteria
Escherichia coli –Rev	TCTGTGTGGATGGTAATAAATTTTTG			
rpoB –For	AACATCGGTTTGATCAAC	371-390	53.5	Universal
rpoB –Rev	CGTTGCATGTTGGTACCCAT			

Results

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis

To identify the microbial diversity and population distribution within the rumen and in the gastrointestinal tract in animals fed high energy diets, T-RFLP analysis was performed on ruminal and gastrointestinal contents. T-RFLP analysis shows significant changes in the microbial population structure and distribution within the rumen and in the gastrointestinal tract. Due to the great diversity and the vast number of different bacteria detected in the rumen and gastrointestinal tract, we have further analyzed the microbial populations and have classified the microbes using analysis tools available at the ribosomal database project (http://rdp.cme.msu.edu) (Cole et al., 2003; Cole et al., 2005). Microbial population distribution and microbial diversity plots generated based on T-RFLP analysis at phylum level is shown in figures 1&2. Bacteria belonging to phylum *Actinobacteria* accounted for a higher percentage in the reticulum (12%) than in the different compartments of the rumen. The *Actinobacterial* population seemed to be consistent in the dorsal sac (7.5%), dorsal blind sac (6.0%) and ventral blind sac (9.0%), but displayed a significantly lower percentage in the ventral sac (4.5%). Percentage of *Actinobacteria* increased again in the omasum (11.9%) and abomasum (12.5%) and was similar to the percentage detected within the reticulum. But, the percentage of *Actinobacteria* decreased significantly in the proximal region of the small intestines (3.4%) and was the lowest among all locations. From the distal small intestines (12.2%) onwards, cecum (16.0%), large intestine (17.3%) and the rectum (12.0%) had an increased percentage of A*ctinobacterial* populations. The distribution of

phylum *Bacteroidetes* was similar to the distribution pattern of phylum *Actinobacteria* within the reticulum and rumen. Where, the percentage of *Bacteroidetes* in the reticulum (18.5%) was higher than the dorsal sac (10.5%), dorsal blind sac (13.0%), ventral sac (9.7%) and the ventral blind sac (14.5%). The percentage of *Bacteroidetes* populations within the reticulum and rumen was higher than the percentage of *Actinobacterial* populations. The percentage of *Bacteroidetes* populations in the omasum (12.5%) and abomasum (9.1%) was similar to the percentage of *Actinobacteria* present in the omasum and abomasum. The *Bacteroidetes* population did not show a decrease in percentage, at the proximal small intestine (12.0%) as seen with *Actinobacteria* and was similar to the levels in the omasum and abomasum. From the distal small intestines (9.7%) onwards, cecum (10.4%), large intestine (8.7%) and the rectum (12.2%) had a lower percentage of *Bacteroidetes* populations when compared to *Actinobacterial* populations. This observation was opposed to the observation detected in the reticulum and rumen, where the *Bacteroidetes* populations were higher than the *Actinobacterial* populations.

The *Firmicutes* population within the reticulum (17.2%) was similar to the *Bacteroidetes* population. The *Firmicutes* within the ruminal compartments, dorsal sac (22.8%), dorsal blind sac (30.4%), ventral sac (16.0%) and ventral blind sac (25.6%) was higher than the *Bacteroidetes* and *Actinobacteria* detected within the rumen. From omasum to the rectum, *Firmicutes* population remained constant but was greater than the *Actinobacteria* and *Bacteroidetes* populations detected. The *Proteobacteria* populations were among the largest detected throughout the rumen

and gastrointestinal tract and accounted for 30%-50% of the total bacterial population. The *Proteobacteria* within the ventral sac (59.0%) was among the highest detected within the rumen. This was contradictory to the observation with *Actinobacteria, Bacteroidetes and Firmicutes*, where the ventral sac displayed the lowest numbers. T-RFLP analysis also detected bacteria belonging to phylum *Aquificae, Chloroflexi, Cyanobacteria, Dictyoglomi, Fusobacteria, Nitrospira, Planctomycetes* and *Thermotogae.* T-RFLP analysis also detected several other phyla at very low levels. These phyla have been combined and are presented under "Other Phyla". Furthermore, several unclassified bacteria were detected and are presented as unclassified phyla.

Quantitative Real-Time PCR analysis

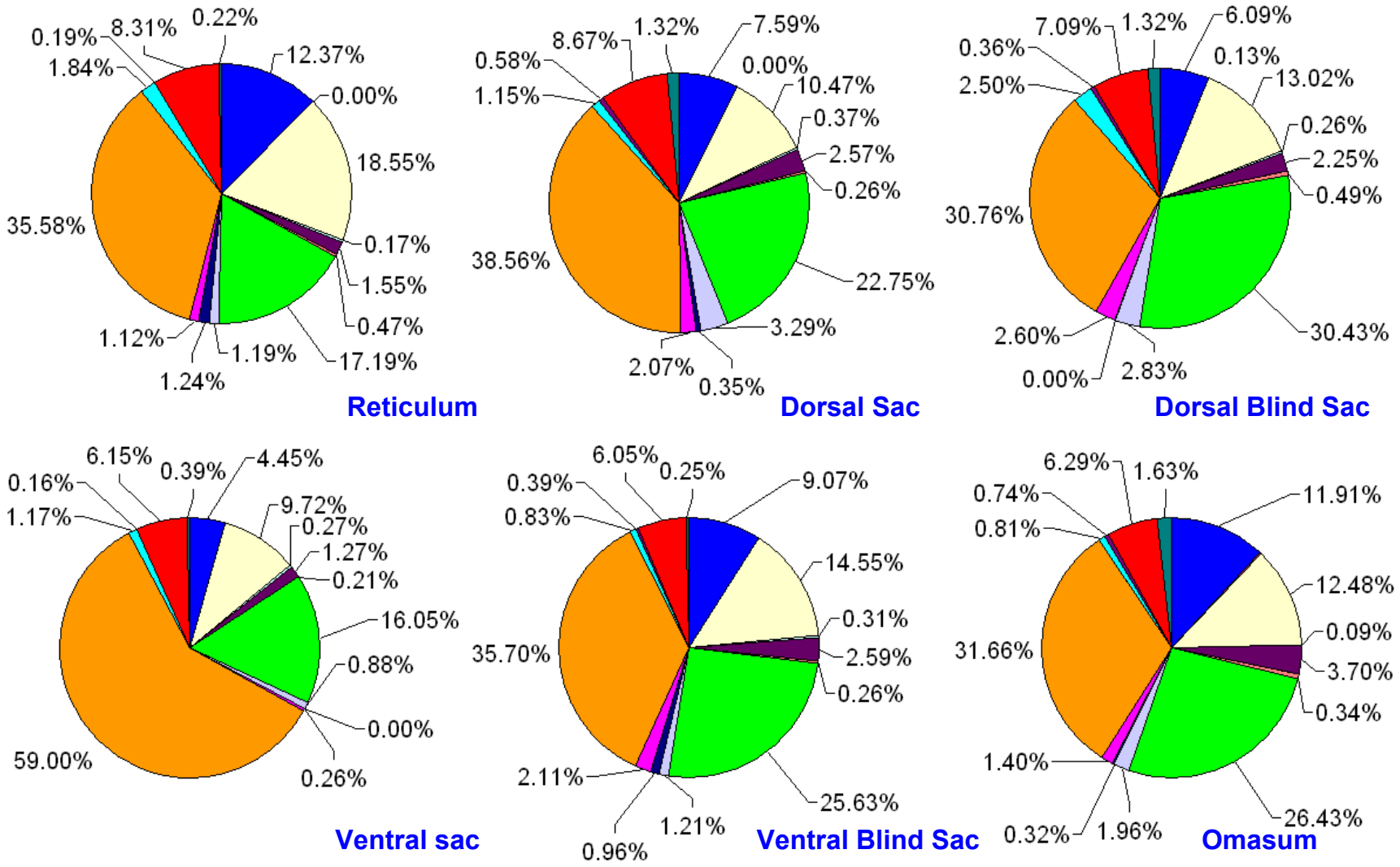
Quantitative real-time PCR analysis was performed to validate the T-RFLP analysis performed and to evaluate the populations of previously reported rumen and gut bacteria. Quantitative real-time PCR (qRT-PCR) results are summarized in Figure 3. Quantitative real-time PCR analysis was performed using a representative bacterial species from each phylum. *Bifidobacterium ruminantium, Treponema bryantii, Butyrivibrio fibrosolvens, Prevotella bryantii,* and *Escherichia coli 0157:H7* were selected to represent bacterial populations in phylum, *Actinobacteria, Spirocheates, Firmicutes, Bacteroidetes,* and *Proteobacteria* respectively. *Bifidobacterium ruminantium* displayed a similar trend to the one observed during T-RFLP analysis for *Actinobacteria*. As with T-RFLP analysis, real-time PCR analysis of *Bifidobacterium ruminantium* displayed the lowest level in the ventral sac out of the

four ruminal compartments, but increased by 5 fold in the omasum and abomasum. In the proximal region of the small intestine, *Bifidobacterium ruminantium* population decreased by 4 fold and was consistent with the observation of *Actinobacteria* by T-RFLP analysis. Form the distal small intestine to the rectum, *Bifidobacterium ruminantium* population was low and remained consistent. *Butyrivibrio fibrosolvens* displayed a similar trend to the T-RFLP profile generated for *Firmicutes*. The lowest levels of *Butyrivibrio fibrosolvens* were detected in the ventral sac, proximal small intestines and the distal small intestines, which is similar to the t-RFLP profile of *Firmicutes*. The highest levels of *Butyrivibrio fibrosolvens* (>12 fold) was detected in the reticulum, cecum, large intestine and rectum. *Prevotella ruminicola* populations were more widely dispersed than *Butyrivibrio fibrosolvens,* and *Bifidobacterium ruminantium*. *Prevotella ruminicola* population was highest in the reticulum (350-fold), but was significantly lower in the dorsal sac (3.2-fold), dorsal blind sac (1.7-fold), and the ventral sac (13-fold). *Prevotella ruminicola* population increased significantly in the ventral blind sac (291-fold), but decreased again, and was lower in the omasum (1-fold) and abomasum (32.8-fold). The proximal small intestine (2.3-fold) had a decreased level of *Prevotella ruminicola*, but increased to 17.3-fold at the distal small intestine. The *Prevotella ruminicola* population was constant at ~16-fold in the cecum and large intestine, but increased to 37-fold in the rectum. The *Treponema bryantii* population showed a similar trend to the *Spirosheates* population detected by T-RFLP analysis, but displayed several differences. *Treponema bryantii* was higher in the reticulum (7.5-fold) than the ruminal compartments (dorsal sac (1-fold), dorsal blind sac (3.6-fold), ventral sac (3.8-fold),

and ventral blind (6.5-fold). *Treponema bryantii* population dropped in the omasum (1.5-fold), but recovered to 7.3-fold in the abomasum. In the proximal small intestines *Treponema bryantii* population decreased to 4.8-fold. Within the rest of the gastrointestinal tract, *Treponema bryantii* population fluctuated up and down (distal small intestine (7.5-fold), cecum (4.8-fold), Large intestine (14.0-fold), and rectum (8.2-fold)). To validate the distribution of *Proteobacteria* populations throughout the rumen and gastrointestinal tract we evaluated the population distribution of *Escherichia coli 0157:H7*. *Escherichia coli 0157:H7* population was highest in the ventral sac (23.1-fold) and in the proximal small intestine (51.4%), this was similar to the T-RFLP analysis which revealed the highest level of *Proteobacteria* in the ventral sac and the proximal small intestine. *Escherichia coli 0157:H7* population levels were higher in the reticulum and rumen than in the gastrointestinal tract. Within the gastrointestinal tract *Escherichia coli 0157:H7* levels were 3-4 fold higher in the large intestine and rectum compared to the distal small intestine and cecum.

Figure 1

Distribution of microbial diversity at each location of the ruminal and gastrointestinal tract content based on T-RFLP analysis. T-RFLP results displayed are analyzed at phylum level due to the large number of bacteria and diversity present within the samples.


0.19%
8.31%
0.22%
1.84%
12.37%
0.00%
18.55%
35.58%
0.17%
1.55%
0.47%
1.12%
1.19%
17.19%
1.24%
Reticulum
0.58%
8.67%
1.32%
7.59%
1.15%
0.00%
10.47%
0.37%
2.57%
0.26%
38.56%
22.75%
3.29%
2.07%
0.35%
Dorsal Sac
0.36%
7.09%
1.32%
6.09%
2.50%
0.13%
13.02%
0.26%
2.25%
0.49%
30.76%
30.43%
2.60%
0.00%
2.83%
Dorsal Blind Sac
0.16%
6.15%
0.39%
4.45%
1.17%
9.72%
0.27%
1.27%
0.21%
16.05%
0.88%
0.00%
59.00%
0.26%
Ventral sac
0.39%
6.05%
0.25%
9.07%
0.83%
14.55%
0.31%
35.70%
2.59%
0.26%
2.11%
25.63%
0.96%
1.21%
Ventral Blind Sac
0.74%
6.29%
1.63%
11.91%
0.81%
12.48%
0.09%
31.66%
3.70%
0.34%
1.40%
26.43%
0.32%
1.96%
Omasum
Phylum: Actinobacteria
Phylum: Aquificae
Phylum: Bacteroidetes
Phylum: Chloroflexi
Phylum: Cyanobacteria
Phylum: Dictyoglomi
Phylum: Firmicutes
Phylum: Fusobacteria
Phylum: Nitrospira
Phylum: Planctomycetes
Phylum: Proteobacteria
Phylum: Spirochaetes
Phylum: Thermotogae
Unclassified Phyla
Other Phyla

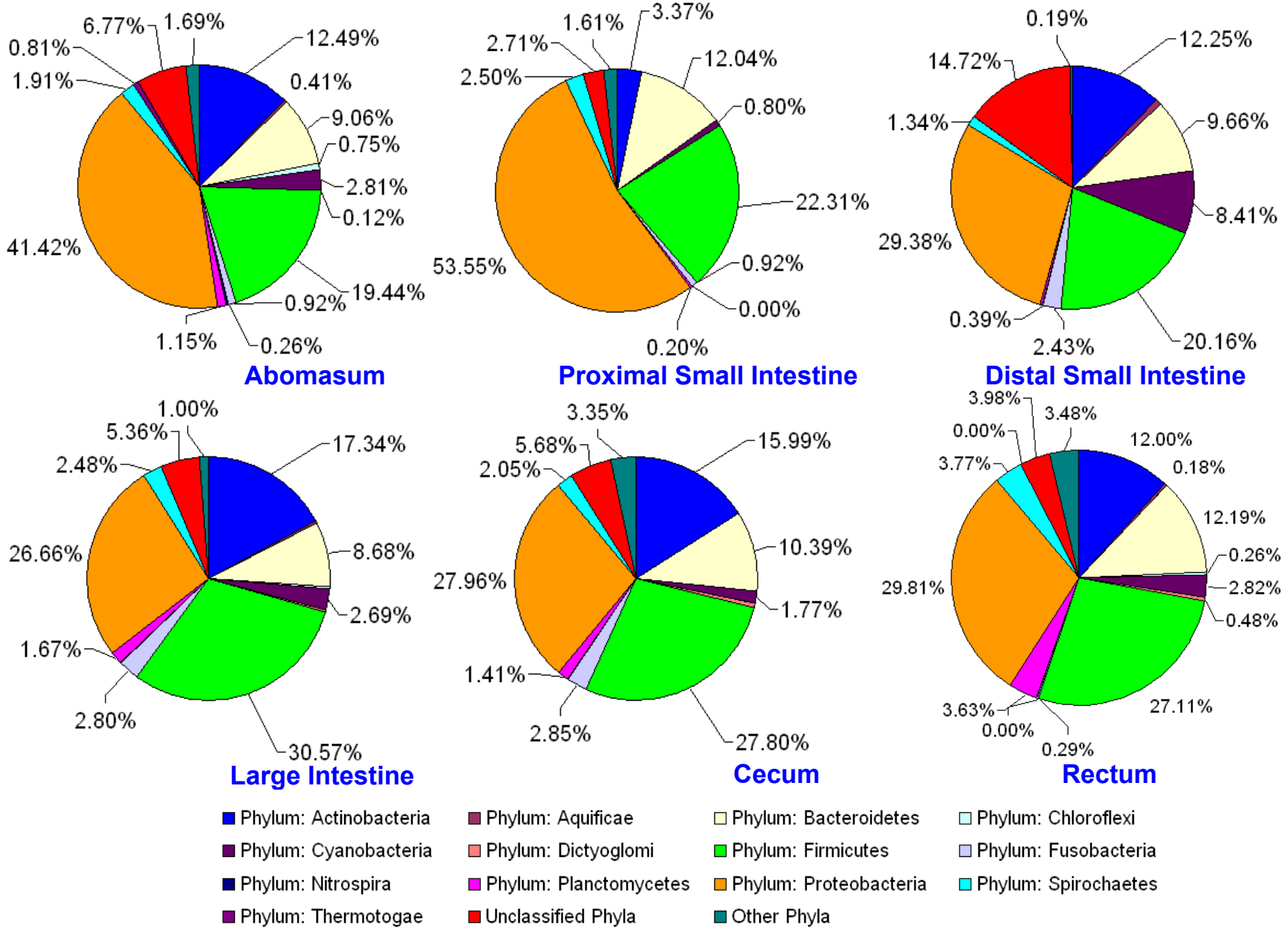
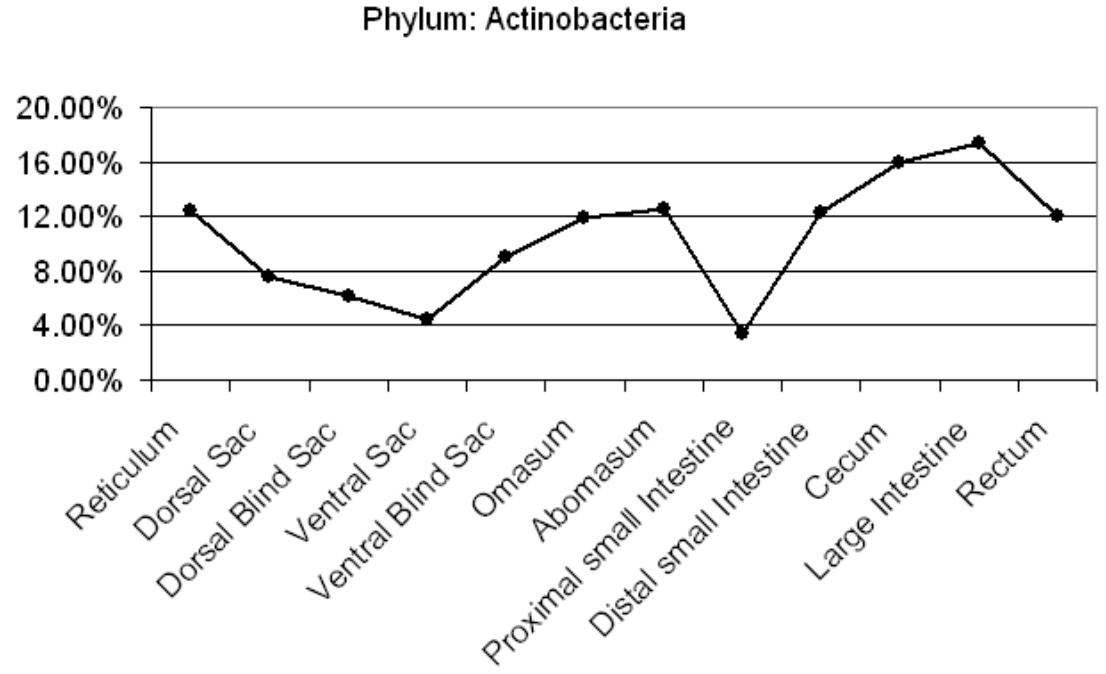
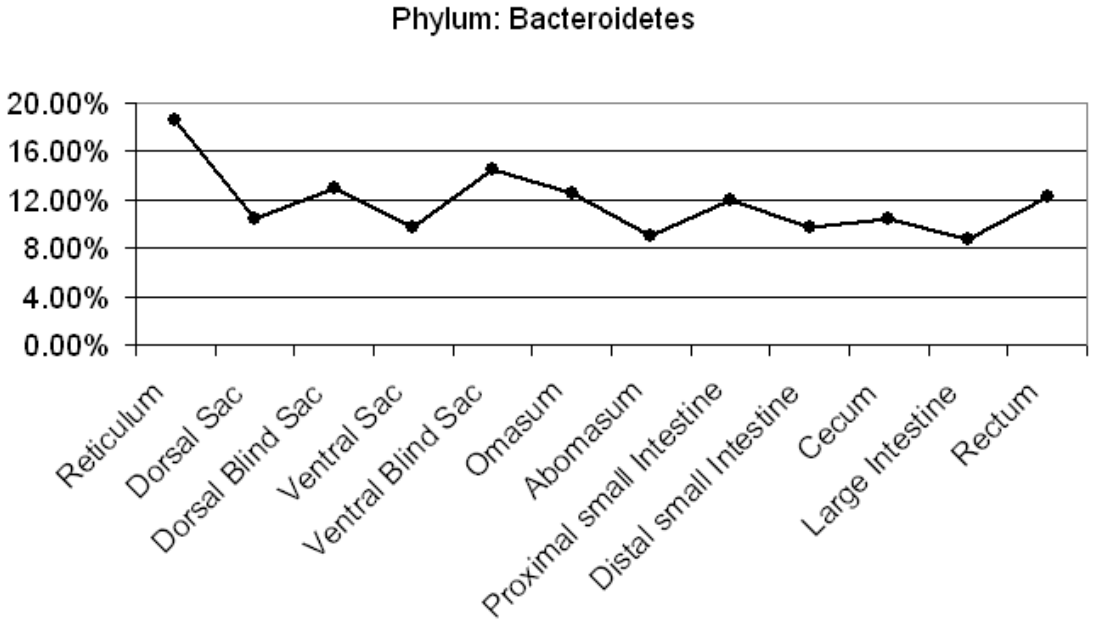
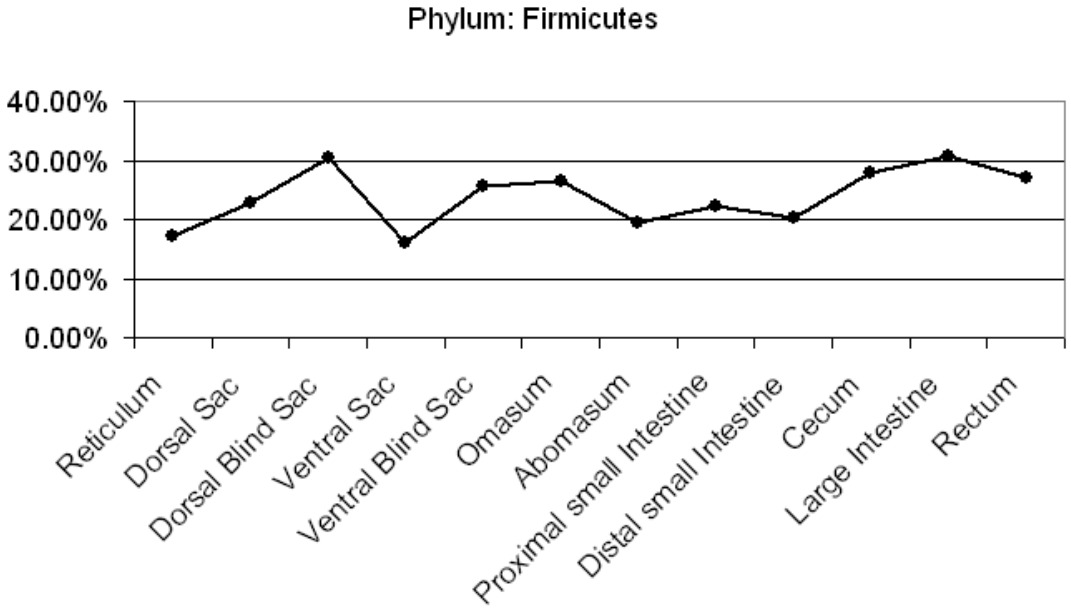

6.77%
1.69%
0.81%
12.49%
1.91%
0.41%
9.06%
0.75%
2.81%
0.12%
41.42%
19.44%
0.92%
1.15%
0.26%
Abomasum
1.61%
3.37%
2.71%
12.04%
2.50%
0.80%
22.31%
53.55%
0.92%
0.00%
0.20%
Proximal Small Intestine
0.19%
12.25%
14.72%
9.66%
1.34%
8.41%
29.38%
0.39%
2.43%
20.16%
Distal Small Intestine
1.00%
5.36%
17.34%
2.48%
8.68%
26.66%
2.69%
1.67%
2.80%
30.57%
Large Intestine
3.35%
5.68%
15.99%
2.05%
10.39%
27.96%
1.77%
1.41%
2.85%
27.80%
Cecum
3.98%
3.48%
0.00%
12.00%
3.77%
0.18%
12.19%
0.26%
29.81%
2.82%
0.48%
3.63%
27.11%
0.00%
0.29%
Rectum
Phylum: Actinobacteria
Phylum: Aquificae
Phylum: Bacteroidetes
Phylum: Chloroflexi
Phylum: Cyanobacteria
Phylum: Dictyoglomi
Phylum: Firmicutes
Phylum: Fusobacteria
Phylum: Nitrospira
Phylum: Planctomycetes
Phylum: Proteobacteria
Phylum: Spirochaetes
Phylum: Thermotogae
Unclassified Phyla
Other Phyla

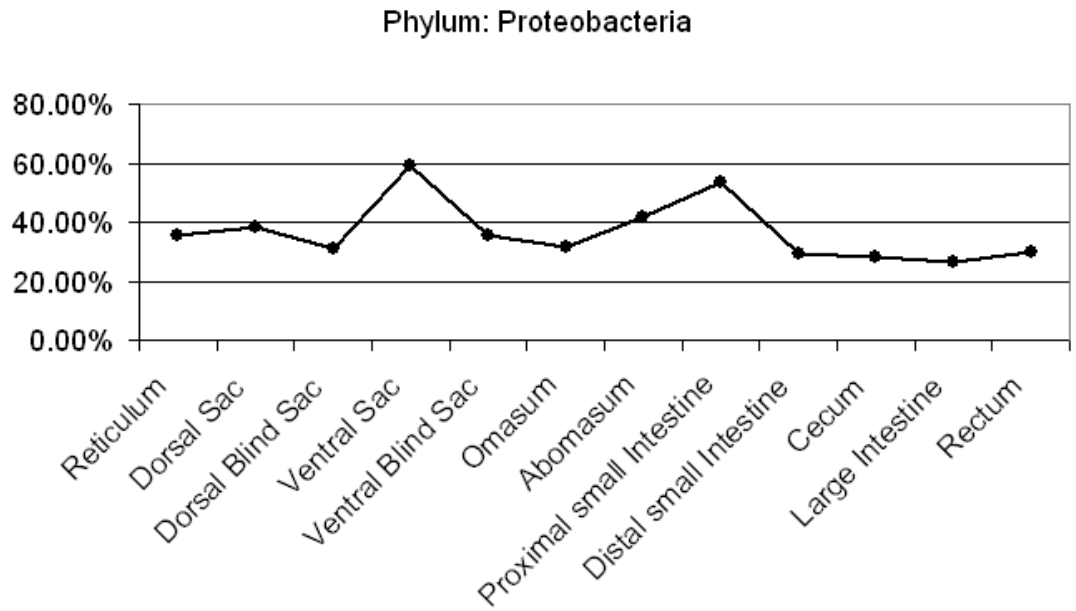
Figure 2

Distribution of each bacterial phyla throughout the rumen and gastrointestinal tract based on T-RFLP analysis.

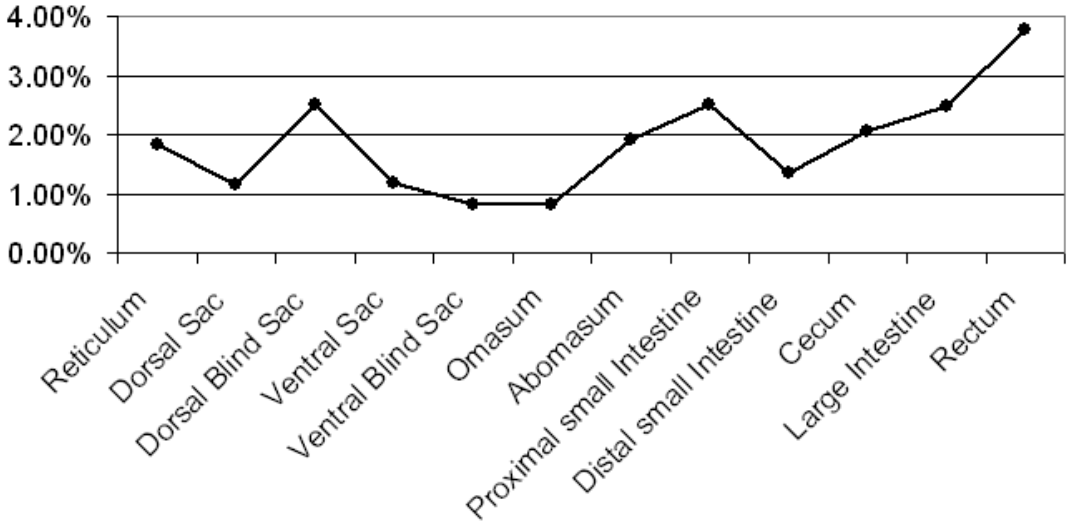

Phylum: Actinobacteria
20.00%
16.00%
12.00%
8.00%
4.00%
0.00%
Reticulum
Dorsal Sac
Dorsal Blind Sac
Ventral Sac
Ventral Blind Sac
Omasum
Abomasum
Proximal small Intestine
Distal small Intestine
Cecum
Large Intestine
Rectum


Phylum: Bacteroidetes
20.00%
16.00%
12.00%
8.00%
4.00%
0.00%
Reticulum
Dorsal Sac
Dorsal Blind Sac
Ventral Sac
Ventral Blind Sac
Omasum
Abomasum
Proximal small Intestine
Distal small Intestine
Cecum
Large Intestine
Rectum

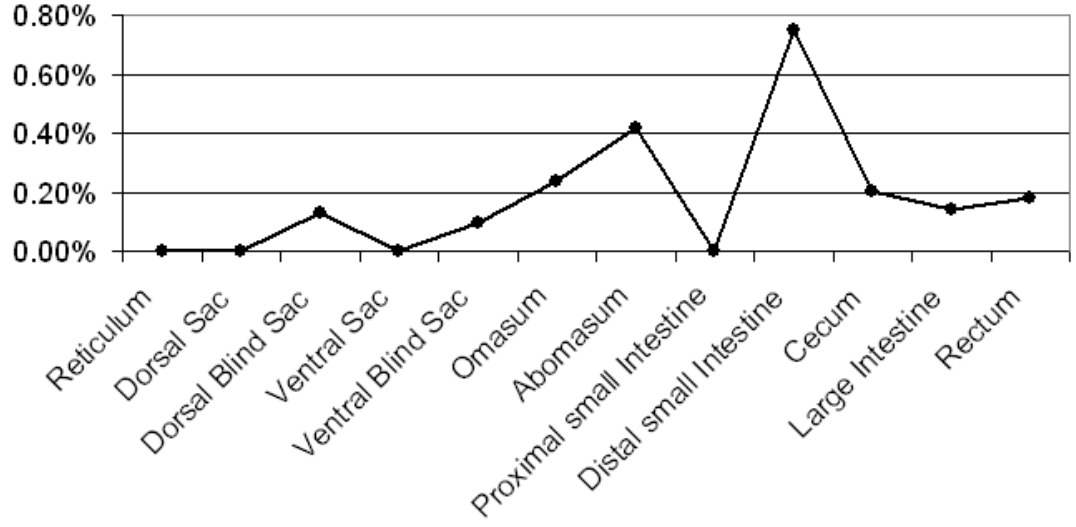

Phylum: Firmicutes
40.00%
30.00%
20.00%
10.00%
0.00%
Reticulum
Dorsal Sac
Dorsal Blind Sac
Ventral Sac
Ventral Blind Sac
Omasum
Abomasum
Proximal small Intestine
Distal small Intestine
Cecum
Large Intestine
Rectum


Phylum: Proteobacteria
80.00%
60.00%
40.00%
20.00%
0.00%
Reticulum
Dorsal Sac
Dorsal Blind Sac
Ventral Sac
Ventral Blind Sac
Omasum
Abomasum
Proximal small Intestine
Distal small Intestine
Cecum
Large Intestine
Rectum

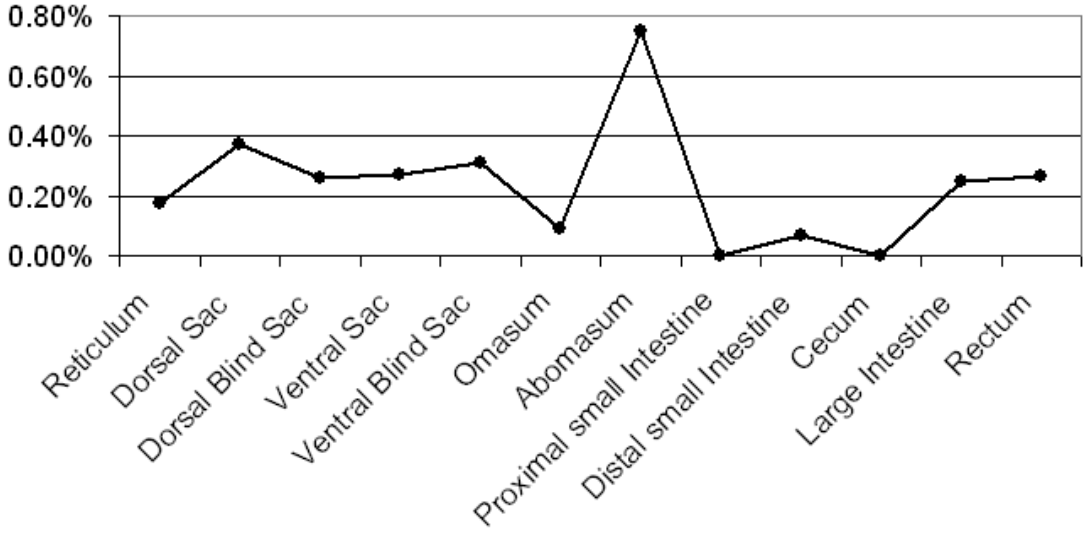
Phylum: Spirochaetes



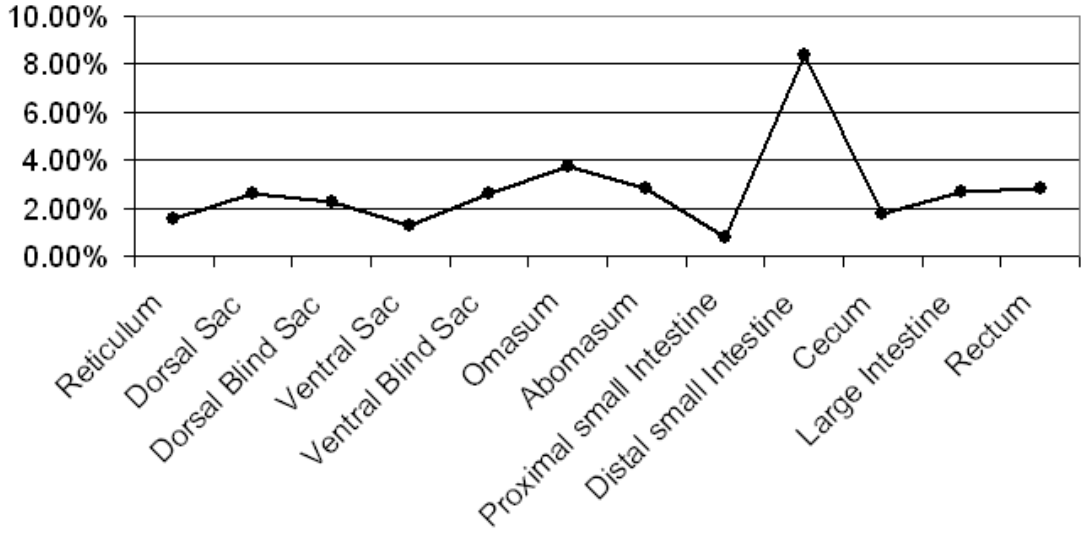
Phylum: Aquificae



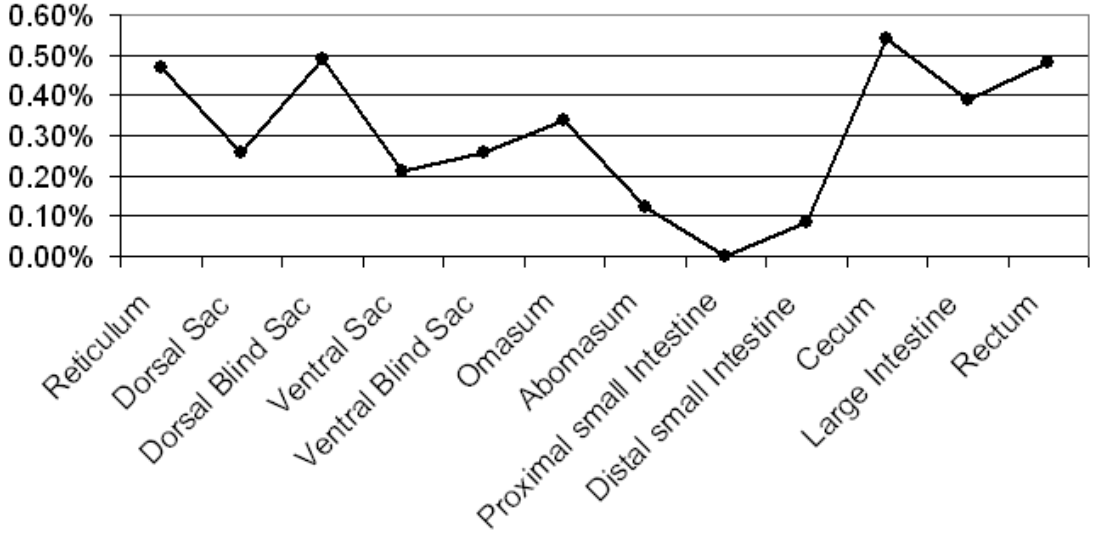
Phylum: Chloroflexi



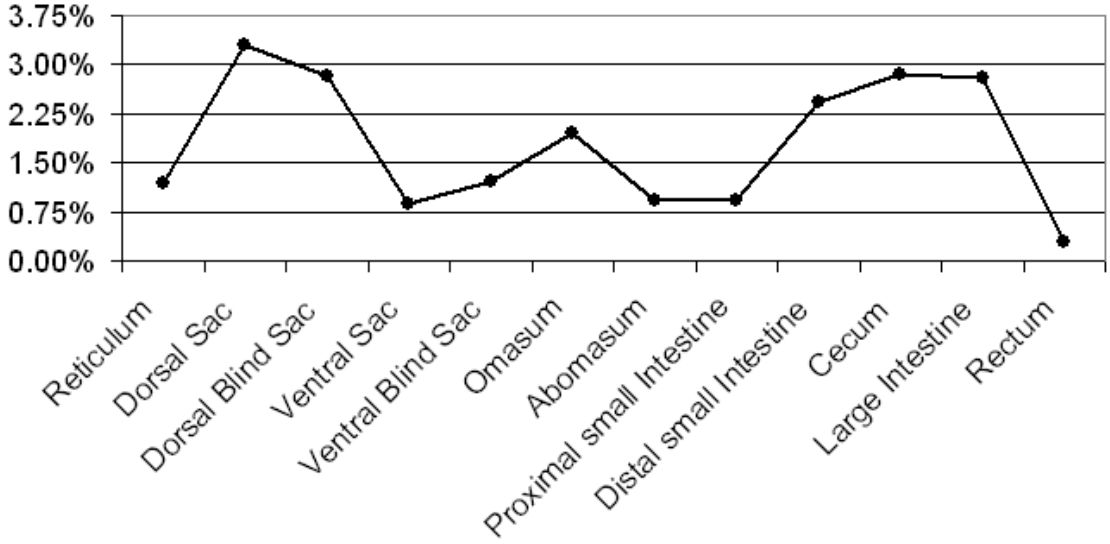
Phylum: Cyanobacteria



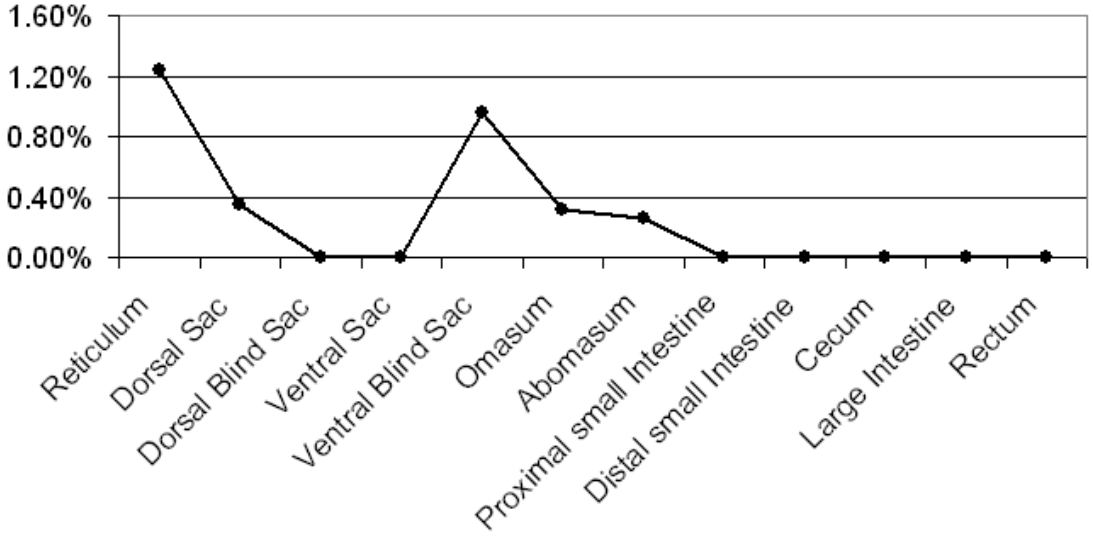
Phylum: Dictyoglomi



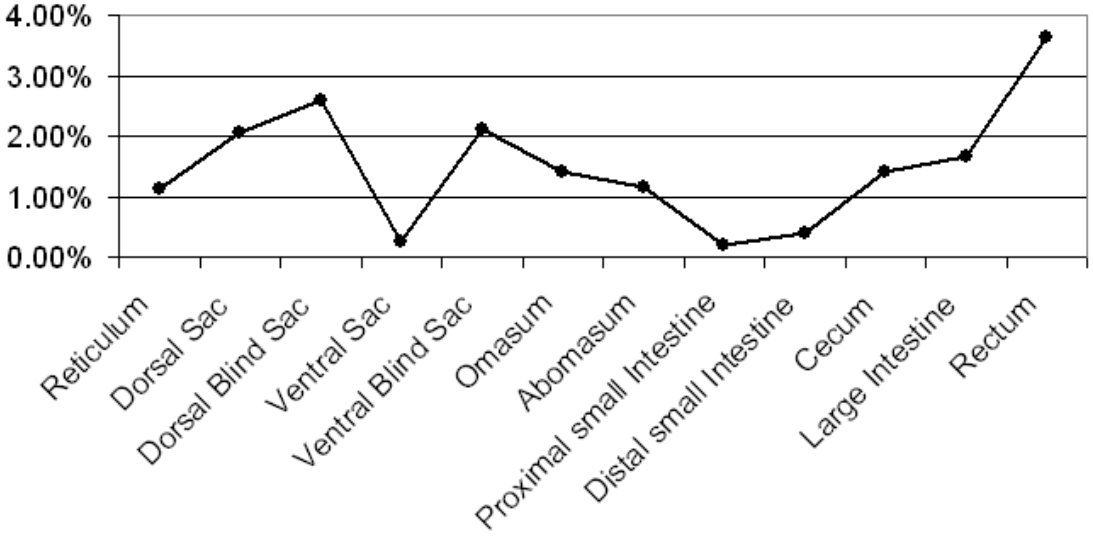
Phylum: Fusobacteria



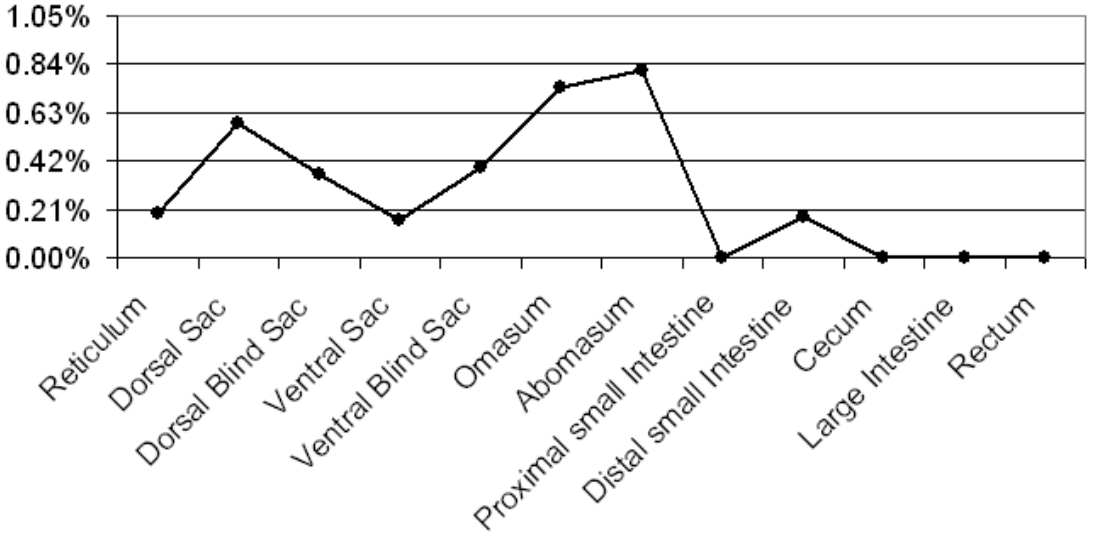
Phylum: Nitrospira



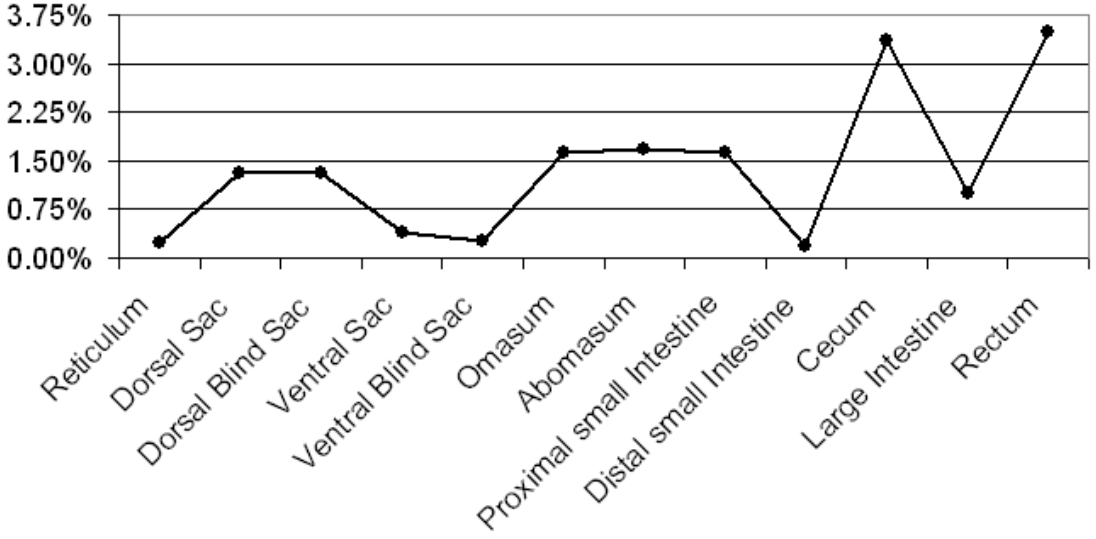
Phylum: Planctomycetes



Phylum: Thermotogae



Other Phyla



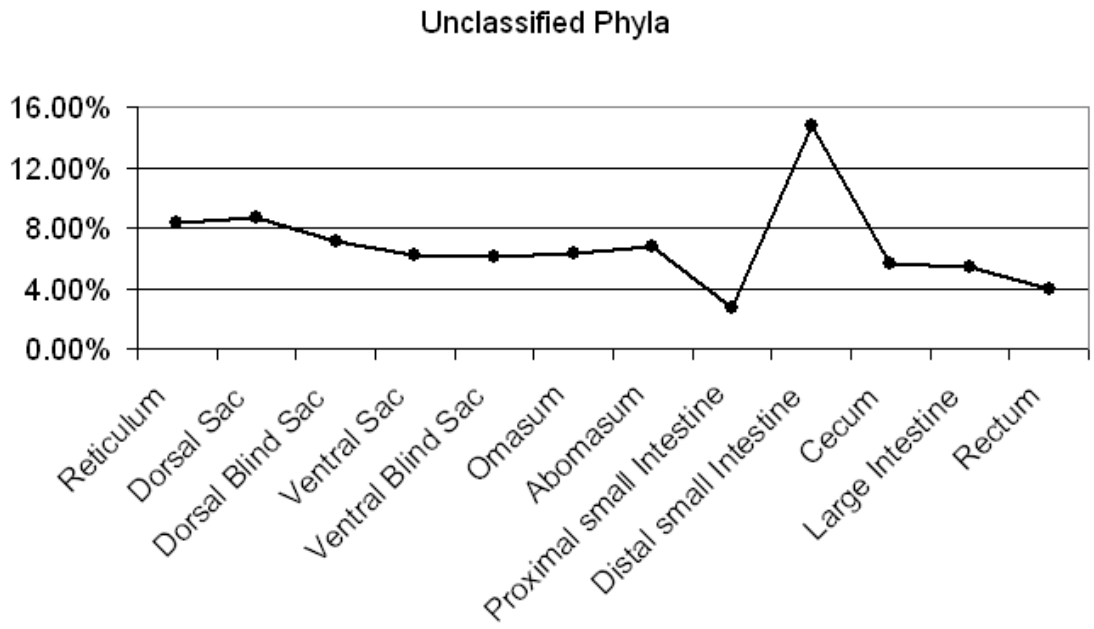
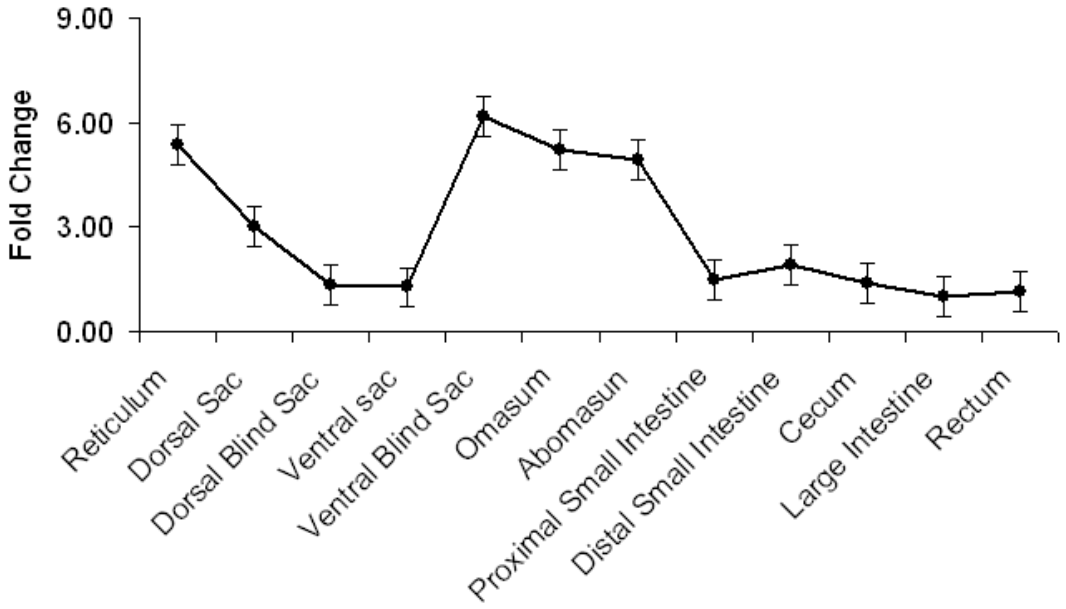

Unclassified Phyla
16.00%
12.00%
8.00%
4.00%
0.00%
Reticulum
Dorsal Sac
Dorsal Blind Sac
Ventral Sac
Ventral Blind Sac
Omasum
Abomasum
Proximal small Intestine
Distal small Intestine
Cecum
Large Intestine
Rectum

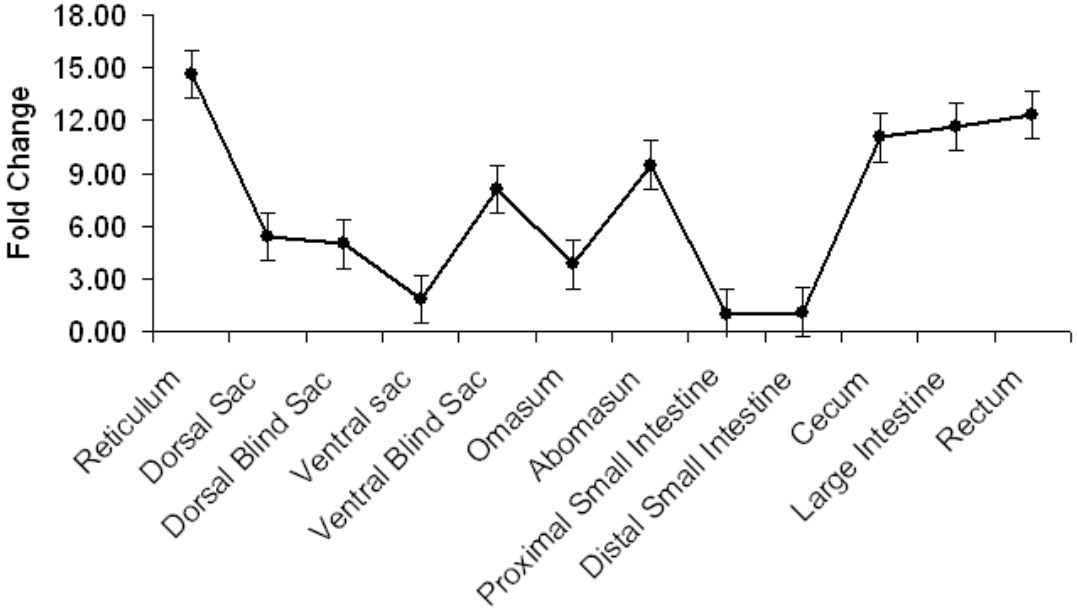
Figure 3

Population changes of selected rumen and gastrointestinal bacterial species using qRT-PCR in animals fed high concentrate diet. Microbial population changes were calculated using the comparative $\Delta\Delta$Ct method as described in materials and methods.

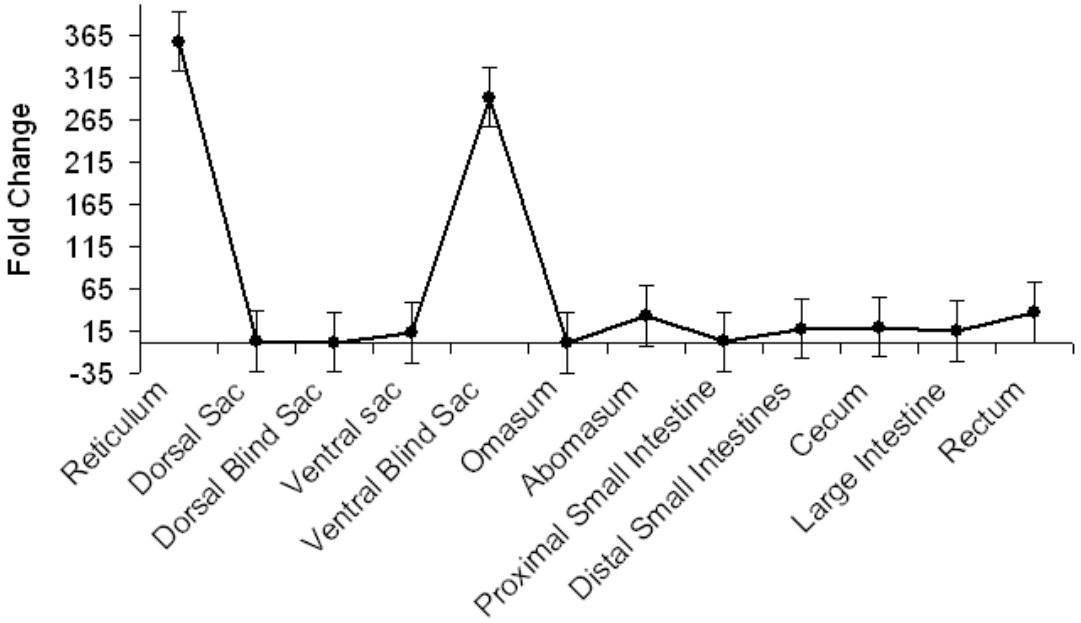
Bifidobacterium ruminantium



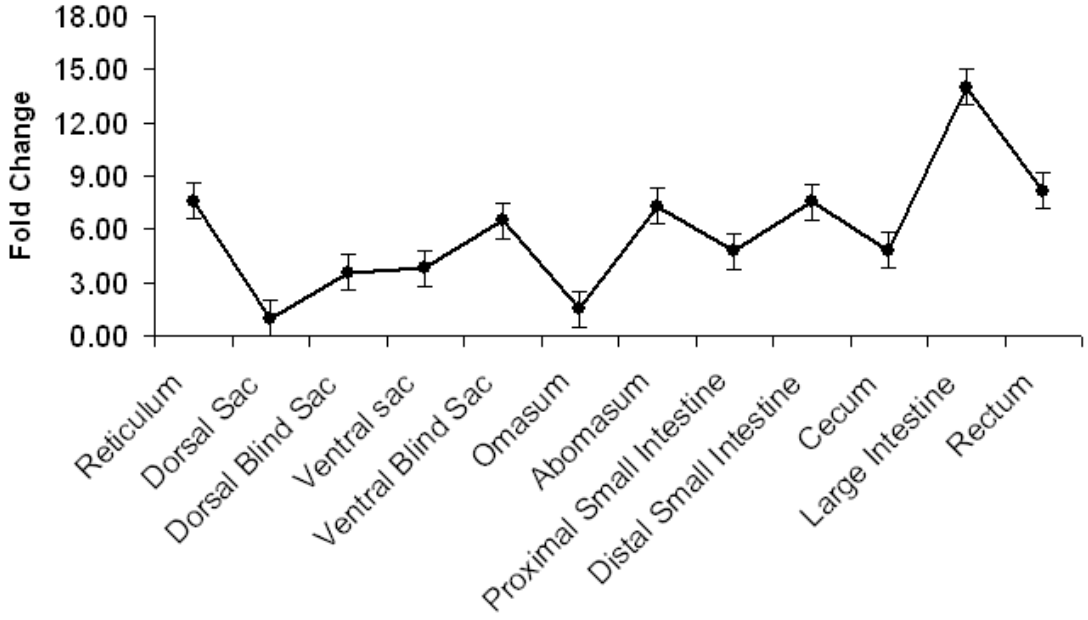
Butyrivibrio fibrosolvens



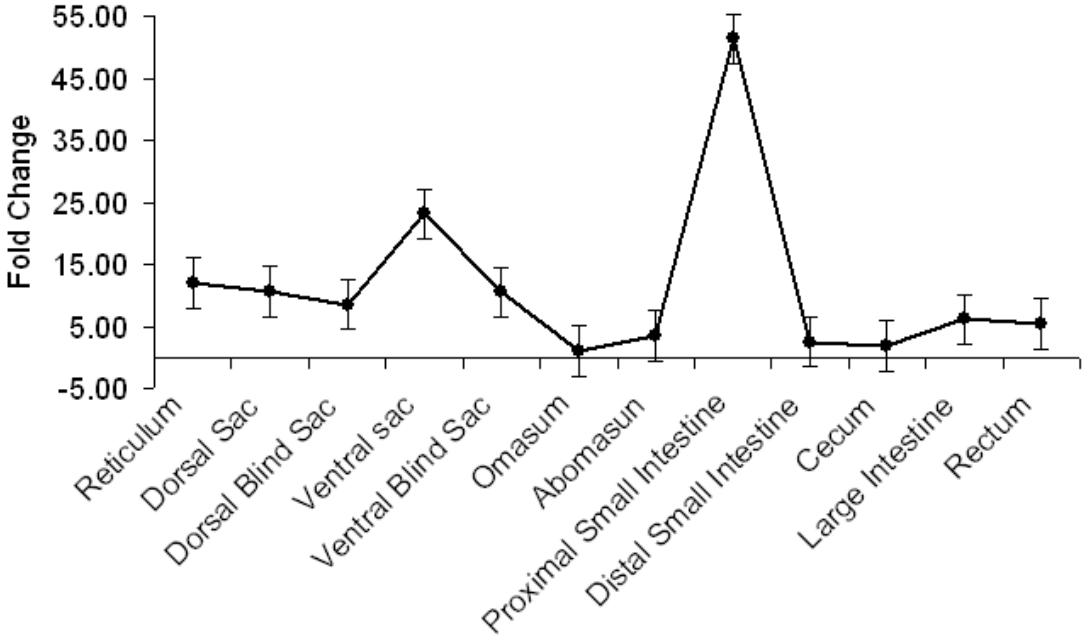
Prevotella ruminicola



Treponema bryantii



Escherichia coli 0157:H7



Discussion

In the early 1950s to 1970s it was widely accepted that only 22 predominant ruminal bacterial species existed in the rumen (Krause and Russell, 1996). However, in the last few decades molecular techniques, such as PCR and rapid sequence determination systems have emerged that enable the identification and characterization of bacteria that are unable to culture. These new PCR based molecular techniques have spurred re-evaluation of ruminal bacteria, which have led to the discovery of a more complex bacterial community within the rumen (Fernando et al., 2008c; Fernando et al., 2008d; Tajima et al., 2001; Tajima et al., 2000). Many of these recent studies have evaluated rumen microbial population fluctuations due to changes in diet, and metabolic disorders (Fernando et al., 2008c; Fernando et al., 2008d; Tajima et al., 2001; Tajima et al., 2000) but none have evaluated the distribution of microbial populations with respect to locality within the different compartments of the rumen and the gastrointestinal tract. Therefore, this study presents the first survey of the microbial diversity and distribution within the rumen and gastrointestinal tract in cattle.

T-RFLP analysis displayed significant changes in microbial population structure and distribution within the rumen and the gastrointestinal tract. The T-RFLP profiles detected over 280 different bacterial species within the rumen and gastrointestinal tract. These microbial species were further analyzed at phylum level using analysis tools available at the ribosomal database project (http://rdp.cme.msu.edu) (Cole et al., 2003; Cole et al., 2005) (see figure 1&2). Reticulum being the first compartment of the rumen is the point of contact where the microbes meet with the feed particles.

Therefore, the microbes within the reticulum are expected to carryout the initial digestion of the feed. T-RFLP analysis detected greater number of *Actinobacteria* and *Bacteroidetes* species within the reticulum than in the rumen. *Actinobateria*, are characterized as high C+G, gram positive bacteria and are known to play important roles in cellulose and chitin digestion, carbon recycling, and secondary metabolite production (Chin et al., 1999; Stackebrandt et al., 1997). Therefore, the increased *Actinobateria* within the reticulum may help cellulose digestion to gain access into the feed particles, and to provide secondary metabolites for ruminal bacteria for digestion. Similarly, *Bacteroidetes* populations were grater in the reticulum than in the rumen. *Bacteroidetes,* mostly being gram negative bacteria (Holdeman et al., 1984); help starch digestion within the rumen (Stevenson and Weimer, 2007). Therefore, *Bacteroidetes* populations may have increased within the reticulum as the animals were fed a high starch diets. The *Firmicutes,* population in the reticulum was lower than the population within the rumen. *Firmicutes,* which include *Megasphera sp.* and *Selenomonas sp.* are low G+C gram positive bacteria that increase in high starch diets and can tolerate relatively low pH values (5.8-6.2) (Fernando et al., 2008a; Fernando et al., 2008c). Therefore, as majority of the digestion occurs within the rumen, and as the feed stays the longest within the rumen, *Firmicutes,* populations may have increased in the rumen. The *Proteobacterial* populations accounted for a majority of the bacteria detected in the reticulum and rumen. Previously, we have reported a significant increase in *Proteobacterial* populations in animals fed high starch diets and in animals with acidosis (Fernando et al., 2008c; Fernando et al., 2008d). Hence, the increase in *Proteobacterial* populations within

the reticulum and rumen are consistent with the microbial populations observed in animals on high energy diets. Within the rumen, the distribution of microbial populations was similar in the dorsal sac, dorsal blind sac, and ventral blind sac. But, the ventral sac displayed decreased *Actinobacteria, Bacteroidetes,* and *Firmicutes* populations, and an increased *Proteobacteria* population. This change in the distribution of ventral sac bacterial populations may be due to the fact that large feed particles within the rumen settle at the bottom of the ventral sac causing increased fermentation and lower pH within the ventral sac leading to favorable conditions for *Proteobacteria*. The omasum displayed increased *Actinobacteria* and *Firmicutes* populations, and a decrease *Proteobacterial* population. The omasal bacterial population changes are dictated by the flow through bacteria leaving the rumen, therefore this suggests that the majority of the bacteria leaving the rumen may be of phylum *Actinobacteria* and *Firmicutes* origin. The abomasum displayed decreased *Bacteroidetes* and *Firmicutes* populations, and an increased *Proteobacteria* population, suggesting that *Bacteroidetes* and *Firmicutes* may reside within the "*Laminae omasi*" to help further digestion within the omasum or that the pepsinogen and the HCl released from the abomasum (Church, 1993) may have lead to the digestion of *Bacteroidetes* and *Firmicutes* species within the abomasum. The bacterial population within the proximal small intestine was very interesting as most bacterial populations decreased when compared to other locations in the gastrointestinal tract. This decrease in bacterial populations is expected as the microbial cells that flow out of the rumen is known to help provide the animal with its protein requirement in the form of microbial cell protein (Church, 1993). Therefore,

this drop in microbial diversity within the proximal small intestine provides support to the notion that, the rumen microbes play an important role in supplying the animal with its protein requirement. *Proteobacteria* was predominant in the proximal small intestine and accounted for more than 50% of the total population, suggesting that *Proteobacteria* may be more resistant to digestion in the proximal small intestine. *Bacteroidetes, Firmicutes,* and *Actinobacteria* populations increased significantly in the distal small intestines and were maintained at similar levels in the cecum, large intestine, and rectum. Whereas, the Proteobacterial population decreased significantly at the distal small intestine and was maintained at similar levels thereafter. This change in bacterial population in the distal small intestine may be due to change in the available nutrients, removal of gastric enzymes and the increased pH due to removal of HCl, leading to favorable conditions for other bacterial species. A gradual increase of most bacterial phyla in the cecum, large intestines and rumen was detected during T-RFLP analysis. This increase in bacterial populations in the hind gut may be due to the starch escaping rumen microbial degradation which passes to the hind gut for fermentation (Callaway et al., 2003). Although, no previous studies have been reported on the diversity and distribution of microbial populations in the gastrointestinal tract of cattle, several studies have been reported on the bacterial diversity of the human gastrointestinal tract. These reports show that in an adult, a majority of the bacterial populations belong to phylum *Firmicutes* (~60%) and *Bacteroidetes* (~12%) (Holdeman et al., 1984). The populations belonging to *Proteobacteria* and *Actinobacteria* are very minute in the human gastrointestinal tract and contribute only ~10% and ~5%

respectively (Holdeman et al., 1984). This distribution of bacteria within the human gastrointestinal tract is contradictory to the distribution of bacteria within the gastrointestinal tract of cattle. This difference in microbial populations may be due to diet (herbivorous Vs omnivorous) and pre-gastric Vs post-gastric fermentation (Dethlefsen et al., 2006). But, a recent study of human infant intestinal microbial populations show a similar bacterial population diversity to the ruminant animal (*Firmicutes 32.0%, Bacteroidetes 20.0%, Proteobacteria 46.0%,* and *Actinobacteria 1.2%*) (Palmer et al., 2007). As, this study utilized stool samples from new born infants and from infants during their first year, where the infants were breast fed and was presumably on a mostly herbivorous diet, this suggests that diet and feeding patterns may play a pivotal role in determining microbial diversity within an individual.

The real-time PCR analysis of selected bacterial species from different phyla displayed a similar trend to the bacterial distribution detected by T-RFLP analysis (Figure 3). *Bifidobacterium ruminantium* displayed a similar trend to the *Actinobacteria* population detected by T-RFLP analysis. *Bifidobacterium sp.* has been detected in animals on high starch diets (Biavati and Mattarelli, 1991; Scardovi et al., 1969). Where, *Bifidobacterium sp.* are known to metabolize maltose and glucose which are intermediates or end products of starch digestion (Biavati and Mattarelli, 1991). Similar to the T-RFLP analysis, real-time PCR analysis of *Bifidobacterium ruminantium* displayed the lowest level in the ventral sac, suggesting that the increased fermentation and decreased pH within the ventral sac may be inhibitory to the growth of *Bifidobacterium sp.*. The 5-fold increase in

Bifidobacterium ruminantium within the omasum and abomasum is also consistent with the T-RFLP analysis of *Actinobacteria* populations and further suggests that *Actinobacteria* may provide a significant contribution towards the microbial cell protein entering the small intestine. The decrease of *Bifidobacterium ruminantium* in the proximal region of the small intestine also suggests that *Actinobacteria* may be a significant source of microbial cell protein to the animal. Real-time analysis of *Butyrivibrio fibrosolvens* displayed a similar trend to the T-RFLP profile generated for *Firmicutes*. *Butyrivibrio sp.* are fibrolytic organisms that have high preference towards maltose and sucrose utilization (Russell and Baldwin, 1978), hence, possess the capability to digest cellulose and starch. Similar to *Bifidobacterium ruminantium*, *Butyrivibrio fibrosolvens* population also decreased in the ventral sac suggesting that *Butyrivibrio sp.* may also be susceptible to the low pH generated by increased fermentation within the ventral sac. *Butyrivibrio fibrosolvens* population was also decreased in the proximal and distal small intestines suggesting that *Butyrivibrio fibrosolvens* population may also contribute towards providing the animal's protein requirement. But, as the total *Firmicutes* population detected by T-RFLP analysis did not decrease in the small intestine, this suggests that *Firmicutes* populations may have variable susceptibility towards digestion in the small intestine. *Prevotella ruminicola* populations were more widely dispersed than *Butyrivibrio fibrosolvens,* and *Bifidobacterium ruminantium*. *Prevotella ruminicola* population displayed a similar trend to *Bacteroidetes* population detected by T-RFLP analysis, but, displayed grater distribution among the rumen and gastrointestinal tract. We were surprised to see increased *Prevotella ruminicola* population within the reticulum

and ventral blind sac, and can not explain the reason for this increase of *Prevotella ruminicola* population. *Treponema bryantii* population displayed a similar trend to the *Spirosheates* population detected by T-RFLP analysis. As detected by T-RFLP analysis, *Treponema bryantii* population did not significantly change in the proximal small intestine suggesting that *Treponema bryantii* and other *Spirocheates* may be resistant to digestion by gastric enzymes within the small intestines. To evaluate the distribution of *Proteobacterial* populations throughout the rumen and gastrointestinal tract we evaluated the population distribution of the pathogenic *Escherichia coli* strain *0157:H7*. *Escherichia coli 0157:H7* was highest in the ventral sac and the proximal small intestine. This was similar to the trend observed by T-RFLP analysis for *Proteobacteria.* The increase in *Escherichia coli 0157:H7* in the ventral sac and the proximal small intestines suggests that *Escherichia coli 0157:H7* and other *Proteobacterial* populations are more resistant to low pH and gastric enzymes than other bacterial species.

This preliminary study of microbial population distribution in the rumen and gastrointestinal tract using T-RFLP analysis, and quantitative real-time PCR shows significant changes in microbial population structure and diversity, and reports the distribution of microbial populations within the gastrointestinal tract. This study is among the first to survey the rumen and the gastrointestinal tract of cattle using culture independent molecular techniques and will provide new insight into the bacterial distribution and role of bacteria within the gastrointestinal tract.

Reference List

Biavati,B. and Mattarelli,P. (1991) Bifidobacterium-Ruminantium Sp-Nov and Bifidobacterium-Merycicum Sp-Nov from the Rumens of Cattle. *International Journal of Systematic Bacteriology* **41:** 163-168.

Callaway,T.R., Elder,R.O., Keen,J.E., Anderson,R.C., and Nisbet,D.J. (2003) Forage feeding to reduce preharvest Escherichia coli populations in cattle, a review. *Journal of Dairy Science* **86:** 852-860.

Chin,K.J., Hahn,D., Hengstmann,U., Liesack,W., and Janssen,P.H. (1999) Characterization and identification of numerically abundant culturable bacteria from the anoxic bulk soil of rice paddy microcosms. *Applied and Environmental Microbiology* **65:** 5042-5049.

Church,D.C. (1993) *Ruminant Animal: Digestive Physiology and Nutrition*. New Jersey: Prentice Hall.

Cole,J.R., Chai,B., Farris,R.J., Wang,Q., Kulam,S.A., McGarrell,D.M. et al. (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Research* **33:** D294-D296.

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

Dahllof,I., Baillie,H., and Kjelleberg,S. (2000) rpoB-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. *Applied and Environmental Microbiology* **66:** 3376-3380.

Dethlefsen,L., Eckburg,P.B., Bik,E.M., and Relman,D.A. (2006) Assembly of the human intestinal microbiota. *Trends in Ecology & Evolution* **21:** 517-523.

Fernando,S.C., Horn,G.W., Purvis II,H.T., Najar,F.Z., Sukharnikov,L., Nagaraja,T.G. et al. Bacterial population dynamics during ionophore treatment. 2008a.
Ref Type: Unpublished Work

Fernando,S.C., Purvis II,H.T., Najar,F.Z., Sukharnikov,L., Nagaraja,T.G., Krehbiel,C.R. et al. Rumen microbial changes during adaptation to high concentrate diet. 2008d.
Ref Type: Unpublished Work

Fernando,S.C., Purvis II,H.T., Najar,F.Z., Sukharnikov,L., Nagaraja,T.G., Krehbiel,C.R. et al. Rumen microbial population dynamics during sub acute acidosis. 2008c.
Ref Type: Unpublished Work

Hettinger,A.M., Allen,M.R., Zhang,B.R., Goad,D.W., Malayer,J.R., and Geisert,R.D. (2001) Presence of the acute phase protein, bikunin, in the endometrium of gilts during estrous cycle and early pregnancy. *Biology of Reproduction* **65:** 507-513.

Holdeman,L.V., Kelley,R.W., and Moore,W.E.C. (1984) Anaerobic gram-negative straight, curved and helical rods. In *Bergey'S Manual of Systematic Bacteriology*. Wiliams & Wilkins, pp. 602-662.

Kent,A.D., Smith,D.J., Benson,B.J., and Triplett,E.W. (2003) Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. *Applied and Environmental Microbiology* **69:** 6768-6776.

Kleen,J.L., Hooijer,G.A., Rehage,J., and Noordhuizen,J.P.T.M. (2003) Subacute ruminal acidosis (SARA): a review. *Journal of Veterinary Medicine Series A-Physiology Pathology Clinical Medicine* **50:** 406-414.

Krause,D.O. and Russell,J.B. (1996) How many ruminal bacteria are there? *Journal of Dairy Science* **79:** 1467-1475.

Lin,C.Z., Raskin,L., and Stahl,D.A. (1997) Microbial community structure in gastrointestinal tracts of domestic animals: Comparative analyses using rRNA-targeted oligonucleotide probes. *Fems Microbiology Ecology* **22:** 281-294.

Martin,S.A. (1998) Manipulation of ruminal fermentation with organic acids: A review. *Journal of Animal Science* **76:** 3123-3132.

Nocek,J.E. (1997) Bovine acidosis: Implications on laminitis. *Journal of Dairy Science* **80:** 1005-1028.

Osborne,J.K., Mutsvangwa,T., Alzahal,O., Duffield,T.F., Bagg,R., Dick,P. et al. (2004) Effects of monensin on ruminal forage degradability and total tract diet digestibility in lactating dairy cows during grain-induced subacute ruminal acidosis. *Journal of Dairy Science* **87:** 1840-1847.

Ouwerkerk,D. and Klieve,A.V. (2001) Bacterial diversity within feedlot manure. *Anaerobe* **7:** 59-66.

Owens,F.N., Secrist,D.S., Hill,W.J., and Gill,D.R. (1998) Acidosis in cattle: A review. *Journal of Animal Science* **76:** 275-286.

Ozutsumi,Y., Tajima,K., Takenaka,A., and Itabashi,H. (2006) Real-time PCR detection of the effects of protozoa on rumen bacteria in cattle. *Current Microbiology* **52:** 158-162.

Palmer,C., Bik,E.M., DiGiulio,D.B., Relman,D.A., and Brown,P.O. (2007) Development of the human infant intestinal microbiota. *Plos Biology* **5:** 1556-1573.

Russell,J.B. and Baldwin,R.L. (1978) Substrate Preferences in Rumen Bacteria - Evidence of Catabolite Regulatory Mechanisms. *Applied and Environmental Microbiology* **36:** 319-329.

Santos,S.R. and Ochman,H. (2004) Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. *Environmental Microbiology* **6:** 754-759.

Savage,D.C. (1986) Gastrointestinal Microflora in Mammalian Nutrition. *Annual Review of Nutrition* **6:** 155-178.

Scardovi,V., Trovatte,L.D., Crociani,F., and Sgorbati,B. (1969) Bifid Bacteria in Bovine Rumen - New Species of Genus Bifidobacterium - B Globosum N.Sp. Andb Ruminale N.Sp. *Archiv fur Mikrobiologie* **68:** 278-&.

Sharma,V.K. and an-Nystrom,E.A. (2003) Detection of enterohemorrhagic Escherichia coli O157 : H7 by using a multiplex real-time PCR assay for genes encoding intimin and Shiga toxins. *Veterinary Microbiology* **93:** 247-260.

Stackebrandt,E., Rainey,F.A., and WardRainey,N.L. (1997) Proposal for a new hierarchic classification system, Actinobacteria classis nov. *International Journal of Systematic Bacteriology* **47:** 479-491.

Stevens,C.E. and Hume,I.D. (1998) Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. *Physiological Reviews* **78:** 393-427.

Stevenson,D.M. and Weimer,P.J. (2007) Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Applied Microbiology and Biotechnology* **75:** 165-174.

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

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

Tajima,K., Arai,S., Ogata,K., Nagamine,T., Matsui,H., Nakamura,M. et al. (2000) Rumen bacterial community transition during adaptation to high-grain diet. *Anaerobe* **6:** 273-284.

Theron,J. and Cloete,T.E. (2000) Molecular techniques for determining microbial diversity and community structure in natural environments. *Critical Reviews in Microbiology* **26:** 37-57.

Wolin,M.J. (1981) Fermentation in the Rumen and Human Large-Intestine. *Science* **213:** 1463-1468.

CHAPTER VI

META-FUNCTIONAL GENOMICS OF THE RUMEN

BACTERIAL POPULATION

Introduction

Microbes are resourceful organisms that have dominated the biosphere. The overwhelming numbers of microbes surpass all other organisms on earth put together. They are major contributors in material recycling in the biosphere (Girguis et al., 2005; Hallam et al., 2003). They also play an important role in nutrient digestion in animal systems (Fernando et al., 2008c; Fernando et al., 2008d) and are causative factors in many plant (Lebeau et al., 2008), animal (Ewers et al., 2004) and human (Agarwal and Agarwal, 2008) diseases. Apart from several pathogenic bacterial species, our knowledge of the microbial world is in its infancy. The complex microbial communities and the difficulty in culturing isolates from these environments have further hindered the investigation of such microbial ecosystems (Amann et al., 1990; Zengler et al., 2002). Recently, researchers have begun to understand the importance of microbial community analysis, and have initiated many studies to investigate microbial diversity in different environments using T-RFLP (Terminal Restriction Fragment Length Polymorphism) analysis, 16S rDNA libraries and metagenomic approaches (Abulencia et al., 2006; Fernando et al., 2008c; Fernando et al., 2008d; Tyson et al., 2004; Venter et al., 2004; Woyke et al., 2006). These 16S rDNA sequencing projects and large and small insert metagenomic sequencing projects have provided a glimpse into the phylogenetic diversity of the microbial populations (Abulencia et al., 2006; Edwards et al., 2004; Goo et al., 2004; Schloss and Handelsman, 2005; Skillman et al., 2006; Tyson et al., 2004; Venter et al., 2004; Woyke et al., 2006). A majority of these phylogenetically diverse bacterial populations detected through sequencing projects are resistant to in-vitro culture.

Therefore currently, high-throughput shotgun sequencing projects have been implemented to study the metabolic capabilities of complex microbial communities and to understand the lifestyles of theses organisms (Tringe et al., 2005). Although, high-throughput shotgun sequencing has recovered genes from the microbial communities displaying the capabilities of the microbial population, these genes that have been identified may only be active under certain conditions and therefore does not completely describe the activity of the microbial population. As a result, analyzing microbial proteins produced within the community has been proposed to identify microbial communities based on function, which bypasses the need of identifying bacterial species (Tringe et al., 2005). However, using the proteins synthesized to identify the metabolic role of microbial populations hinder the opportunity to identify the bacterial species that govern theses reaction. Therefore, we propose a community transcriptomics approach which allows identification of the functional role and the bacterial diversity of a microbial community based on the express sequence tags (ESTs).

In the present study we investigate the functional role of the rumen microbial populations on a prairie hay diet and on a high concentrate diet as a proof of concept using a community transcriptomics approach.

The microbial population within the rumen plays an important role in nutrient digestion, where the microbes provide the animal with >60% of its protein requirement (Church, 1993). The synergistic association between the animal and the microbial flora provides the animal with nutrients that are not available to the monogastric animal. Therefore, the interaction between the complex microbial

population and the animal is an important relationship which directly impacts the efficiency and performance of the animal. Hence, understanding this relationship between the rumen microbial community and the host animal with respect to microbial diversity and the functional role of bacteria within the rumen will provide an opportunity to make effective management decisions to enhance microbial and animal production. Therefore, community level metabolic and physiological profiling was carried out on rumen microbial populations from prairie hay and high concentrate diet using a community transcriptomics approach to identify the functional roles of microbial communities, and their changes within the ruminal environment.

Experimental approach

RNA Extraction

Two ruminally cannulated beef steers (837± 60 lbs) were fed prairie hay or high concentrate diet for a week to adapt the ruminal bacterial populations within the animal to their respective diets. Following the adaptation phase, rumen samples were collected from the two animals via the ruminal cannula. The samples collected were snap frozen in liquid nitrogen and were stored at -80°C until used for RNA extraction.

Total RNA from the rumen samples were extracted using the TRIzol™ method. Briefly, 3g of rumen sample was ground in liquid nitrogen using a mortar and pestle. 12 ml of TRIzol™ (Invitrogen, Carlsbad, CA, USA) was added to the grounded sample and was further ground vigorously. The sample was incubated at room

temperature for 5 min. 3 ml of chloroform was added to the sample and incubated at room temperature for a further 3 min. After incubation the sample was centrifuged at 4ºC for 30 min at 12000 rpm. The aqueous phase was transferred into a fresh tube and 7.5 ml of isopropyl alcohol was added. This was incubated at -80ºC for 20 min and was centrifuged at 4ºC for 15 min at 12,000 rpm. The supernatant was discarded and the pellet was washed with 3 ml of 75 % ethanol. The ethanol was removed and the pellet was air-dried for 5 min. The resulting pellet was resuspended in 500 µl of DEPC treated water. This RNA sample was subjected to phenol chloroform extraction (Sambrook and Russel, 2001). The purified total RNA was electrophoresed in denatration gel to evaluate the quality of the RNA.

Bacterial mRNA Isolation and cDNA Synthesis

The isolated total RNA was further purified to isolate poly A mRNA and bacterial mRNA. First the MEGAclear™ kit (Ambion, Austin, TX) was used according to manufacturer's protocol to remove majority of the 5S rRNA and tRNA. Next, the partially purified RNA was used to remove eukaryotic 28S,18S and poly A RNA using the MICROBE*enrich*™ kit (Ambion, Austin, TX). The microbial RNA enrichment was carried out according to manufacturer's protocol. The enriched microbial RNA was further purified using the MICROBE*express*™ kit (Ambion, Austin, TX) according to manufacturer's protocols to remove 16S and 23S rRNA. The resulting RNA was used for a second round of purification using the MICROBE*express*™ kit. Finally, the remaining 5S fraction of the ribosomal RNA was removed from the bacterial mRNA fraction using the MEGAclear™ kit (Ambion,

Austin, TX). The resulting purified bacterial mRNA population was electrophoresed in denatration gel to evaluate the quality of the mRNA and to ensure complete removal of rRNA.

5 µg of purified bacterial mRNA was used for double stranded cDNA synthesis. The first strand cDNA synthesis was carried out using random pentadecamers (NNNNNNNNNNNNNNN) (Stangegaard et al., 2006). A 20 µl reaction contained 5 µg of bacterial mRNA, 2 µg of random pentadecamers, 1 X first strand buffer, 10mM DTT, 1mM dNTP, and 1000U of Superscript II (Invitrogen, Carlsbad, CA). The reaction was initially heated at 70°C for 10 min (5 µg of RNA and the random pentadecamers), followed by a quick chill on ice. Next, 1 X first strand buffer, 10mM DTT, and 1mM dNTP was added to the solution and heated at 37°C for 2 min. Superscript II was added to this solution and was incubated at 37°C for 1 hour, followed by 42°C for an additional 30 min.

The first strand cDNA synthesized was directly used for second strand cDNA synthesis. The 150 µl second strand synthesis reaction contained, 1X Second strand synthesis buffer (Invitrogen, Carlsbad, CA), 200nM dNTP, 10U *E.coli* DNA ligase (Invitrogen, Carlsbad, CA), 40U *E.coli* DNA polymerase I (Invitrogen, Carlsbad, CA), 2U *E.coli* RNAse H (Invitrogen, Carlsbad, CA). The reaction was incubated at 16°C for 2 hrs. 10U of T4 DNA polymerase (Invitrogen, Carlsbad, CA) was added to the reaction and was incubated at 16°C for 5 min. 750nM of EDTA was added to stop the reaction. 10 µl of the synthesized ds cDNA was electrophoresed in a 1.5% gel to evaluate the quality and size of the cDNA synthesized.

The ds cDNA synthesized was phenol chloroform extracted and ethanol precipitated to concentrate the sample. Briefly, 150 µl of phonol: chloroform: isoamyl alcohol (25:24:1) was added to the sample and was vortexed. The sample was then centrifuged at 14,000 rpm for 10 min. 140 µl of the upper aqueous layer was transferred to a new tube and 70 µl of 7.5 M ammonium acetate and 500 µl of 100% ethanol was added to the sample. This sample was incubated at -20°C for 1 hour and was centrifuged at 14000 rpm for 20 min. The supernatant was discarded and the pellet was washed with 1 ml of 75 % ethanol. The ethanol was removed and the pellet was air-dried for 5 min. The resulting pellet was resuspended in 10 µl of DEPC treated water. This ds cDNA sample was subjected electrophoresis to evaluate the quality of the cDNA. The ds cDNA synthesized was used to identify the ESTs (Express Sequnce Tags) present within the rumen bacterial populations by sequence analysis using 454 pyro-sequencing.

454 Pyro-Sequencing

454-Pyro-Sequencing was performed as previously described (Margulies et al., 2005) with a few modifications. Briefly, The ds cDNA was sheared to the size of ~2 Kbp using nebulization at -20°C using a pressure of 30 psi for 2.5 minutes. The sheared DNA was then washed three times using MinElute columns (Qiagen, Valencia, CA) and was centrifuged for 1 minute at 12,000 rpm. The DNA was eluted from the column by rotating it 180° and incubating for 1 min with EB buffer. The column was then centrifuged at 13,000 rpm for one minute. The smaller fragments were then removed using SPRI beads (Agencourt, Beverly, MA). SPRI (Solid Phase

Reversible Immobilization) beads were prepared by vortexing 35 µl of beads and placing them on magnetic Particle Collector (MPC). After removing the supernatant, the beads were then washed twice with 500 µl of 70% ethanol. Finally, the SPRI beds were air dried. The dried SPRI beads were rehydrated in the cleaned DNA sample and was vortexed. The mixture is then placed on the MPC again and the supernatant was removed to a fresh microfuge tube.

The DNA fragments generated were end-repaired by treating them with 20U of DNA polymerase and 20U of T4 polynucleotide kinase after adding 5 µl of 10X end repair buffer (500 mM Tris-HCl, pH7.6, 100 mM $MgCl_2$, 10 mM DTT, and 50 µg/ml BSA), 5 µl of 10 mM rATP, 2 µl of 0.25 mM dNTPs, and 5 µl of bovine serum albumin (BSA)). The reaction mixture was incubated at 12°C for 15 minutes followed by an additional 15 minutes at 25°C. The cleaned DNA sample was further purified on SPRI beads as described above.

Adaptors were then ligated to the cleaned DNA sample by mixing 5 µl of the DNA samples with 1 µl of 454 A and B adaptors, 20U of DNA ligase, and 1X ligation buffer. The ligation reaction was incubated at 25°C for 15 minutes. The ligated sample was further purified using SPRI beads as described above.

The adaptor-ligated ds cDNA were filled-in by treating the sample with 15U of DNA polymerase, 800 nM dNTP mix, and 1X polymerase buffer. The reaction mixture was incubated at 37°C for 20 minuts. Purification was perofrmed on SPRI beads as described above.

The purified DNA was then immobilized on magnetic, strepavidin-coated beads to isolate single stranded cDNA. During the immobilization process, the DNA was

mixed with immobilization beads and shaken for 20 minutes at room temperature. The immobilized cDNA library was then washed with 100 µl of Library Wash Buffer. The Library Wash Buffer (supernatant) was removed by incubating the immobilized library on the Magnetic Particle Collector (MPC).

The ssDNA was then captured using DNA Capture Beads. The capture beads were prepared by washing the beads in capture beads wash buffer and by centrifuging to collect the beads. This process was repeated and the DNA was added to the beads and the mixture was vortexed for 10 seconds. The annealing of capture oligo beads to the DNA was performed on the thermocycler by incubating the mixture at 80° for 5 minutes, 70°for 1 minute, 60° 1 minute, 50° 1 minute, and 20°C 1 minute at a rate of temperature decrease of 0.1°C per second.

The single-stranded DNA was then added to emulsion oil containing 181.62 µl of amplification reaction buffer, 10 µl of $MgSo_4$, 2.08 µl of primer mix, 0.3 µl of pyrophosphatase. The mixture was shaken at 15 rps. The emulsified amplification mix was thermocycled in a Perkin-Elmer Cetus DNA Thermocycler 9600. The emulsified amplification mix was PCR amplified as follows, the reaction mix was incubated at 94°C for 4 minutes, followed by, 40 cycles of 94°C for 30 seconds, 58°C for 60 seconds, and 68°C for 90 seconds. Finally, the amplified samples underwent hybridization extension, where the samples underwent 13 cycles of 94°C for 30 seconds, and 58°C for 6 minutes. The samples were held at 10°C until ready for further processing. Finally the emulsion was broken and the beads were recovered.

Flows from the 454 runs were assembeled using Newbler, the 454 assembly

software (Margulies et al., 2005). Three different trimming lengths were used from the 454 to reduce the number of artificial contigs produced due to poor qualities at the end of the contigs. The results from such triple assembly were then utilized to assemble using Phrap (Ewing et al., 1998; Ewing and Green, 1998).

Figure 1 below illustrates the analysis steps. Metabolic reconstruction was achieved using BlastX against KEGG and COG databases at an e-value of 10^{-5}. Expression profile was estimated using the blast results for each metabolic pathway.

Sequence Analysis

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Figure 1 below illustrates the analysis steps. Metabolic reconstruction was achieved using BlastX against KEGG and COG databases at an e-value of 10^{-5}. Expression profile was estimated using the BLAST results for each metabolic pathway. The contigs and singletons generated for each library was further analyzed using KAAS (KEGG Automatic Annotation Server) (Moriya et al., 2007). The results generated from KAAS were compiled and was clustered using “Genesis” cluster analysis software (Sturn et al., 2002).

EST sequences generated were BLASTed against the protein database using BLASTX algorithm. The BLASTX results generated were further analyzed using

MEGAN (Meta Genomic Analysis) software (Huson et al., 2007). The sequences were analyzed to identify the bacterial diversity within the two samples and to identify the abundant microbial populations present on each diet. MEGAN analysis was also performed to tag function to bacterial species.

Quantitative Real-Time PCR analysis

Quantitative real-time PCR analysis was performed on selected genes to evaluate gene expression changes in metabolic pathways within the rumen. Since, it was difficult to design universal primers that would amplify the complete complement of the population; conserved regions of the selected genes were used to design primers that would lead to the amplification of as many species as possible. The primers were developed using PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest) primer design software. The primers developed were electronically tested using BLAST to identify the different bacterial species that can be amplified using each assay. The primer assays developed are shown in Table 1. As no know universally expressed gene, which has equal expression has been identified from microbial populations, real-time PCR analysis was performed using absolute quantification. Gene expression differences in the two microbial populations were evaluated using SYBR green I reporter assay kit available from Roche Diagnosticis (Indianapolis, IN, USA) using two-step reverse transcriptase-polymerase chain reactions (RT-PCR). cDNA synthesis was performed using 1 µg of total RNA using the QuantiTec reverse transcription kit (Qiagen, Valencia, CA, USA) which includes a DNA wipe out step to

eliminate genomic contamination. Manufacturer's protocol was followed with several modifications where the cDNA synthesis reaction was incubated for 1 hr at 42°C instead of the 15 min described in the protocol and random pentadecamers were used instead of the primer mix provided in the kit. The quantitative gene expression analysis was performed using the FastStart SYBR green master kit (Roche Applied Science, Indianapolis, IN, USA) in a BIORAD MyiQ detection system (BIORAD, Hercules, CA, USA). A total reaction volume of 15 µl contained 400 nM forward primer, 400 nM reverse primer, 1X master mix, and 300 ng of cDNA. Thermal cycling conditions were 95°C for 10 min, followed by 45 additional cycles of 95°C for 30 sec, 52-59°C for 30 sec, depending on the annealing temperature of each primer set (see Table 1) and 72°C for 1 min followed by a melting curve. The PCR products generated were sequenced to ensure the amplification of the correct gene. Standard curves for each assay was run using PCR products generated for each assay. The dilutions for each assay ranged from 10^{3}-10^{-3} ng of PCR template and had efficiencies between (98-105)%. Absolute quantification of gene expression in the two bacterial populations were performed using the equations derived for each standard curve. The equation y=mx+c, where y=amount of gene expression, m=slope of the curve, x= cycle threshold, and c=intercept was used to calculate the absolute amount of gene expression in each population and is presented in nanograms. The efficiency for each curve was also calculated using the formula; Efficiencey= $10^{-1/\text{Slope}}$.

Table 1. Primer sequences used for Real Time PCR analysis.

Primer	Sequence 5'-3'	Tm Used (°C)
Phosphofructokinase –For	**ATCGGTGGTGACGGTTCTTAT**	52
Phosphofructokinase –Rev	**GATATCWCCAGCRTKACGTCCCAT**	
Lactate Dehydrogenase –For	**CWCAAAARCCAGGTGAAACWCG**	54
Lactate Dehydrogenase –Rev	**CCAGCAACGTTAGCRTGTGACCAAAC**	
PEP-Carboxykinase –For	**AAGGKATGTTCTCWATSATGAACTAC**	56
PEP-Carboxykinase –Rev	**TAGATMGGRTAAGAAACACGAGT**	
Con-Lin-Acid-Reductase-For	**CATTCGCACTTGGTACATCTCAGC**	59
Con-Lin-Acid-Reductase-Rev	**ACGTACACGTGGTACTTCCTCAAG**	
Guanine Amino Hydrolase-For	**ATTGCYTTCTGYCCGACYTCCAACCT**	59
Guanine Amino Hydrolase-Rev	**TTGTAKGCYTCGTTSAGCGTYTGCAG**	
Mehtyl Melonyl CoA -For	**GGCSATYGGCAYSAACTTCTWCATGGA**	59
Mehtyl Melonyl CoA -Rev	**GTCGGTSGGCAGMGCGATSGCCTCGTC**	

Results

The total RNA isolated was compared against eukaryotic total RNA which suggested that a majority of the RNA is of prokaryotic origin (Figure 2). The mRNA extracted from the total RNA was visually free of any ribosomal rRNA (28S, 23S, 18S, 16S and 5S) and seem to range from about 200bp - 5kb (Figure 2). The ds cDNA synthesized from the mRNA was sequenced using Genome 20 sequencer system (Roche diagnostics, Indianapolis, IN, USA). The 454 pyro-sequencing generated 17357 reads from the bacterial population on high concentrate diet and 40772 reads from the bacterial population on prairie hay. The average read length of the sequences were ~150 bp. Out of the 17357 reads on high concentrate, 579 contigs and 11970 singleton reads were identified. The reads from the animal on prairie hay generated 2135 contigs and 23479 singlet reads (Table 2).

EST analyses

EST analysis was performed using KEGG (oki-Kinoshita and Kanehisa, 2007) to annotate the EST sequences and to identify the metabolic pathways that these sequence tags are involved in. EST analysis only annotated ~25% of the total EST sequences generated (Figure 3). Among the ~25% of annotated genes, 15.08% of the genes had no metabolic hits. The distribution of the annotated genes among metabolic pathways with respect to metabolic function is shown in Figure 3 and Table 3.

KEGG cluster analysis performed on the ESTs shows a core set of genes that are expressed in both populations. Interestingly, a larger set of genes expressed in the

two bacterial populations were unique to each of the two populations. These unique genes found in each population displayed core metabolic functions such as information storage and processing, cellular transport, cellular processing and signaling, and Metabolism (Figure 4). A majority of the reads generated from both populations had no significant KEGG ontology. KEGG pathway analysis was also performed using the KEGG annotation server (Moriya et al., 2007). KEGG pathway analysis displayed the presence of multiple metabolic pathways within the rumen in high concentrate and in prairie hay.

Similar to KEGG results, COG analysis (Tatusov et al., 2003; Tatusov et al., 1997) of the EST reads shows a large number of unique genes which have no significant hit (Figure 5). This un-annotated portion of the EST reads accounted for 65%-85% of the total reads. Among the annotated portion of the sequences, more reads belonging to the bacterial population on high concentrate diet could be annotated compared to the bacterial population on prairie hay. Based on COG analysis, 8.9% and 3.65% of the genes expressed were involved in information storage and processing in high concentrate and prairie hay treated bacterial populations respectively. Among these, genes belonging to Translation, ribosomal structure and biogenesis, transcription, replication, recombination and repair were detected (Figure 6a). Genes involved in translation, ribosomal structure and biogenesis, and transcription were higher in the bacterial population on high concentrate while, genes involved in replication, recombination and repair were higher in the population on prairie hay (Figure 6a). 4.22% of the genes expressed in the bacterial population on high concentrate diet were involved in cellular processors and cell signaling

whereas, the bacterial population on prairie hay only contained 1.75%. Genes identified to have functions related to cellular processors and cell signaling included genes involved in the cell cycle, signal transduction, cellular biogenesis, cell motility, and posttranslational events where, genes involved in cellular biogenesis and posttranslational events were higher in bacterial populations on prairie hay while genes involved in signal transduction was significantly higher in populations on high concentrate (Figure 6b). Among the annotated genes, a majority of the genes were characterized as genes involved in metabolism (16.89% in the bacterial population on high concentrate diet and 6.32% in the bacterial population on prairie hay). These genes were distributed throughout different metabolic pathways which ranged from energy production and conversion, carbohydrate transport and metabolism, amino acid transport and metabolism, nucleotide transport and metabolism, coenzyme transport and metabolism, lipid transport and metabolism, inorganic ion transport and metabolism, and secondary metabolite biosynthesis, transport and catabolism (Figure 6c). Genes detected in all metabolic pathways were expressed in similar levels except for genes involved in carbohydrate transport and metabolism where the bacterial population on high concentrate displayed higher levels of gene expression (Figure 6c). Although, the ESTs expressed in all other metabolic pathways displayed similar levels of expression in the two bacterial populations, the genes expressed in each population was different with respect to gene identity.

To identify bacterial diversity of the population and to identify species based on the ESTs generated, “MEGAN” (MEta Genome ANalysis) was performed using BLASTX results (Huson et al., 2007). MEGAN analysis at Phylum level displayed a larger

diversity of bacterial species in prairie hay compared to high concentrate diet. A majority of the ESTs had no significant hits suggesting that these EST signatures have never been seen before. Among the ESTs that had BLASTX results, the phylogenetic analysis using "MEGAN" identified bacteria belonging to phyla *Proteobacteria, Bacteroidetes, Chlorobi, Planctomycetes, Spirochaetes, Firmicutes, Deinococcus-Thermus, Fusobacteria, Aquificae, Chloroflexi, Cyanobacteria, Actinobacteria, Acidobacteria, Chlamydiae, Lentisphaerae,* and *Euryarchaeota* from the ESTs on prairie hay while a greater part of the ESTs from high concentrate belonged to *Proteobacteria, Bacteroidetes, Firmicutes,* and *Actinobacteria (*Figure 7). Further analysis of the EST sequences at genus level displayed a large diversity among the different bacterial Phyla (Appendix 1).

Realtime PCR Analyses

Real-time analysis was performed using absolute quantification. The genes were selected based on different metabolic pathways previously identified within the rumen (Russell, 2002). The gene expression detected is shown in figure 8. The amount of Guanine aminohydrolase gene expression in bacterial populations on prairie hay and high concentrate diet were similar and were 0.02ng and 0.03ng respectively. Phosphofructokinase gene expression in the two bacterial populations were different where, the amount of phosphofructokinase detected on prairie hay was 0.06ng while the amount on high concentrate diet was 0.0019ng. The bacterial population on high concentrate diet displayed larger amounts of conjugated linoleic acid reductase expression (0.09ng) when compared to the bacterial population on

prairie hay (0.03ng). Lactate dehydrogenase expression was not detected in the bacterial population on high concentrate diet, but the population on prairie hay displayed 0.24ng of gene expression. The bacterial population on prairie hay displayed higher levels of phosphoenolpyruvate carboxykinase (0.35ng) when compared to the bacterial population on high concentrate diet (0.06ng). The expression of methyl melonyl CoA in the bacterial population on prairie hay was greater than the population on high concentrate, where amounts of 0.68ng and 0.39ng were detected respectively.

Figure 1

Overview of the EST analysis strategy used to annotate the sequences. Metabolic construction was carried out using BlastX on the KEGG (oki-Kinoshita and Kanehisa, 2007) and COG (Tatusov et al., 2003; Tatusov et al., 1997) databases.

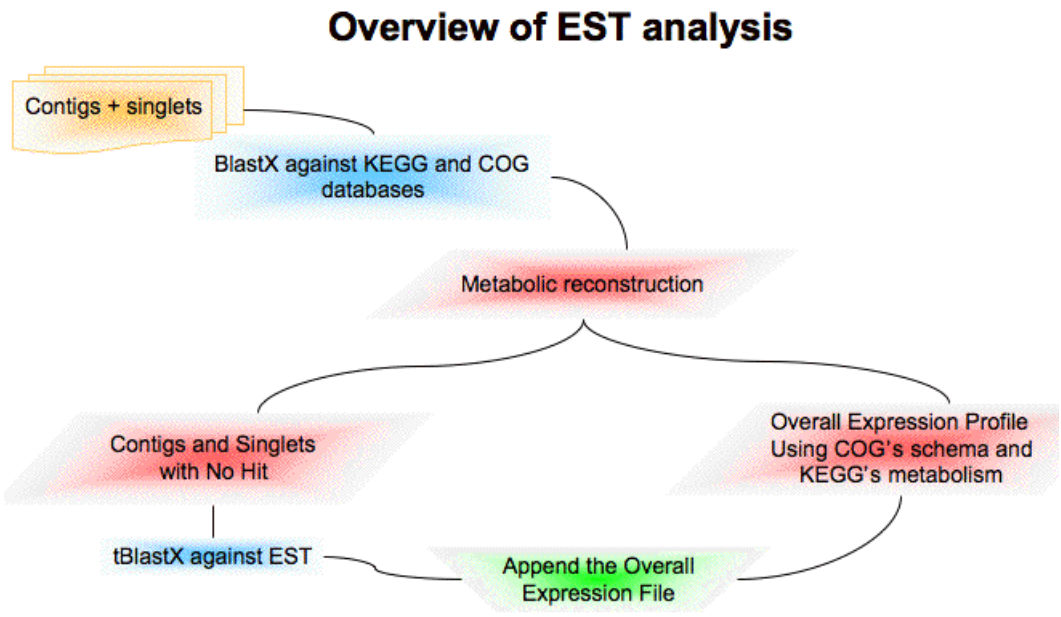

Overview of EST analysis
Contigs + singlets
BlastX against KEGG and COG databases
Metabolic reconstruction
Contigs and Singlets with No Hit
Overall Expression Profile Using COG's schema and KEGG's metabolism
tBlastX against EST
Append the Overall Expression File

Figure 2

Total RNA and mRNA extracted from the rumen bacterial populations. Total RNA was extracted using the TRIZol™ method and was further purified using the MICROBE*enrich*™, MICROBE*express*™ and MEGAclear™ kits. Marker (M), eukaryotic total RNA (1), total RNA from animals on High concentrate diet (2&4), total RNA from animals on prairie hay (3&5), mRNA from animals on high concentrate diet (6), mRNA from animals on prairie hay diet (7).

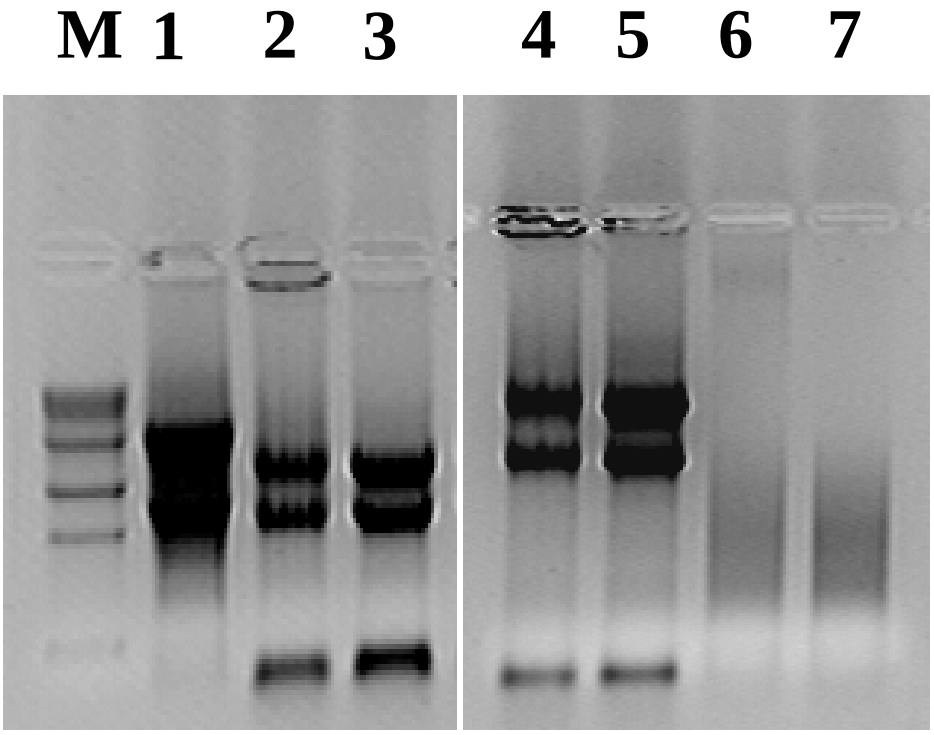

M 1 2 3 4 5 6 7

Figure 3

Summerized EST analysis results generated through KEGG (oki-Kinoshita and Kanehisa, 2007) and COG (Tatusov et al., 2003; Tatusov et al., 1997) analysis.

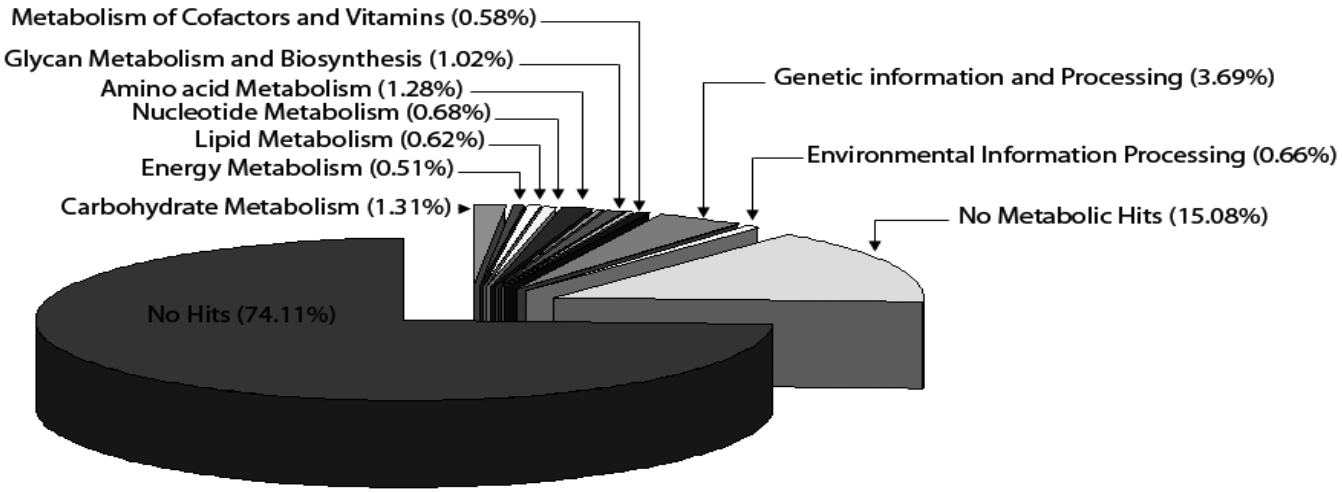

Metabolism of Cofactors and Vitamins (0.58%)
Glycan Metabolism and Biosynthesis (1.02%)
Amino acid Metabolism (1.28%)
Nucleotide Metabolism (0.68%)
Lipid Metabolism (0.62%)
Energy Metabolism (0.51%)
Carbohydrate Metabolism (1.31%)
Genetic information and Processing (3.69%)
Environmental Information Processing (0.66%)
No Metabolic Hits (15.08%)
No Hits (74.11%)

Figure 4

KEGG cluster analysis of a selected set of genes generated from the contigs. The cluster analysis was performed as described in materials and methods.

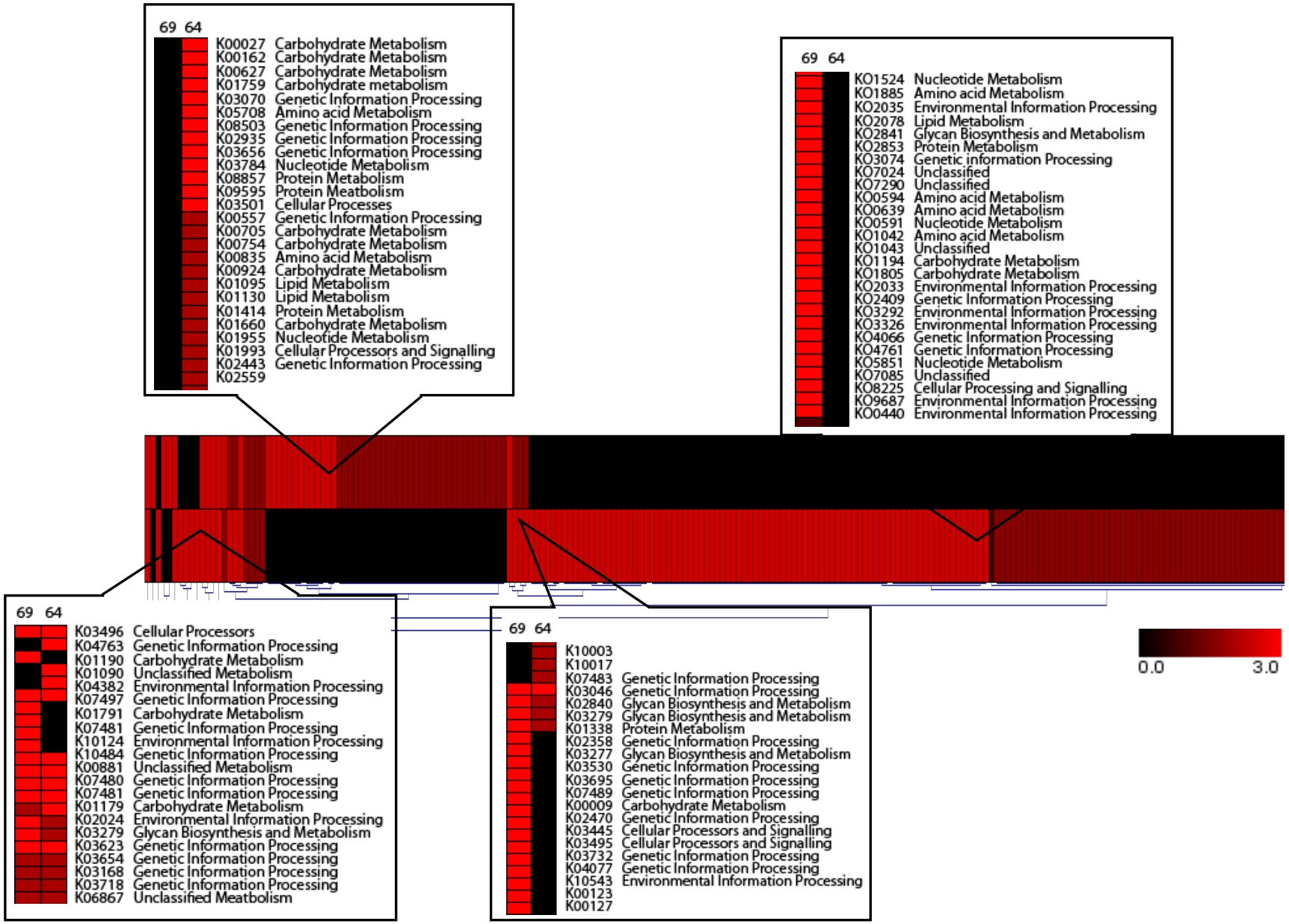

69 64
K00027 Carbohydrate Metabolism
K00162 Carbohydrate Metabolism
K00627 Carbohydrate Metabolism
K01759 Carbohydrate metabolism
K03070 Genetic Information Processing
K05708 Amino acid Metabolism
K08503 Genetic Information Processing
K02935 Genetic Information Processing
K03656 Genetic Information Processing
K03784 Nucleotide Metabolism
K08857 Protein Metabolism
K09595 Protein Meatbolism
K03501 Cellular Processes
K00557 Genetic Information Processing
K00705 Carbohydrate Metabolism
K00754 Carbohydrate Metabolism
K00835 Amino acid Metabolism
K00924 Carbohydrate Metabolism
K01095 Lipid Metabolism
K01130 Lipid Metabolism
K01414 Protein Metabolism
K01660 Carbohydrate Metabolism
K01955 Nucleotide Metabolism
K01993 Cellular Processors and Signalling
K02443 Genetic Information Processing
K02559
69 64
KO1524 Nucleotide Metabolism
KO1885 Amino acid Metabolism
KO2035 Environmental Information Processing
KO2078 Lipid Metabolism
KO2841 Glycan Biosynthesis and Metabolism
KO2853 Protein Metabolism
KO3074 Genetic information Processing
KO7024 Unclassified
KO7290 Unclassified
KO0594 Amino acid Metabolism
KO0639 Amino acid Metabolism
KO0591 Nucleotide Metabolism
KO1042 Amino acid Metabolism
KO1043 Unclassified
KO1194 Carbohydrate Metabolism
KO1805 Carbohydrate Metabolism
KO2033 Environmental Information Processing
KO2409 Genetic Information Processing
KO3292 Environmental Information Processing
KO3326 Environmental Information Processing
KO4066 Genetic Information Processing
KO4761 Genetic Information Processing
KO5851 Nucleotide Metabolism
KO7085 Unclassified
KO8225 Cellular Processing and Signalling
KO9687 Environmental Information Processing
KO0440 Environmental Information Processing
69 64
K03496 Cellular Processors
K04763 Genetic Information Processing
K01190 Carbohydrate Metabolism
K01090 Unclassified Metabolism
K04382 Environmental Information Processing
K07497 Genetic Information Processing
K01791 Carbohydrate Metabolism
K07481 Genetic Information Processing
K10124 Environmental Information Processing
K10484 Genetic Information Processing
K00881 Unclassified Metabolism
K07480 Genetic Information Processing
K07481 Genetic Information Processing
K01179 Carbohydrate Metabolism
K02024 Environmental Information Processing
K03279 Glycan Biosynthesis and Metabolism
K03623 Genetic Information Processing
K03654 Genetic Information Processing
K03168 Genetic Information Processing
K03718 Genetic Information Processing
K06867 Unclassified Meatbolism
69 64
K10003
K10017
K07483 Genetic Information Processing
K03046 Genetic Information Processing
K02840 Glycan Biosynthesis and Metabolism
K03279 Glycan Biosynthesis and Metabolism
K01338 Protein Metabolism
K02358 Genetic Information Processing
K03277 Glycan Biosynthesis and Metabolism
K03530 Genetic Information Processing
K03695 Genetic Information Processing
K07489 Genetic Information Processing
K00009 Carbohydrate Metabolism
K02470 Genetic Information Processing
K03445 Cellular Processors and Signalling
K03495 Cellular Processors and Signalling
K03732 Genetic Information Processing
K04077 Genetic Information Processing
K10543 Environmental Information Processing
K00123
K00127
0.0
3.0

Figure 5

Overall COG analysis. The analysis was performed as described in materials and methods. The data was divided to four major categories and is presented.

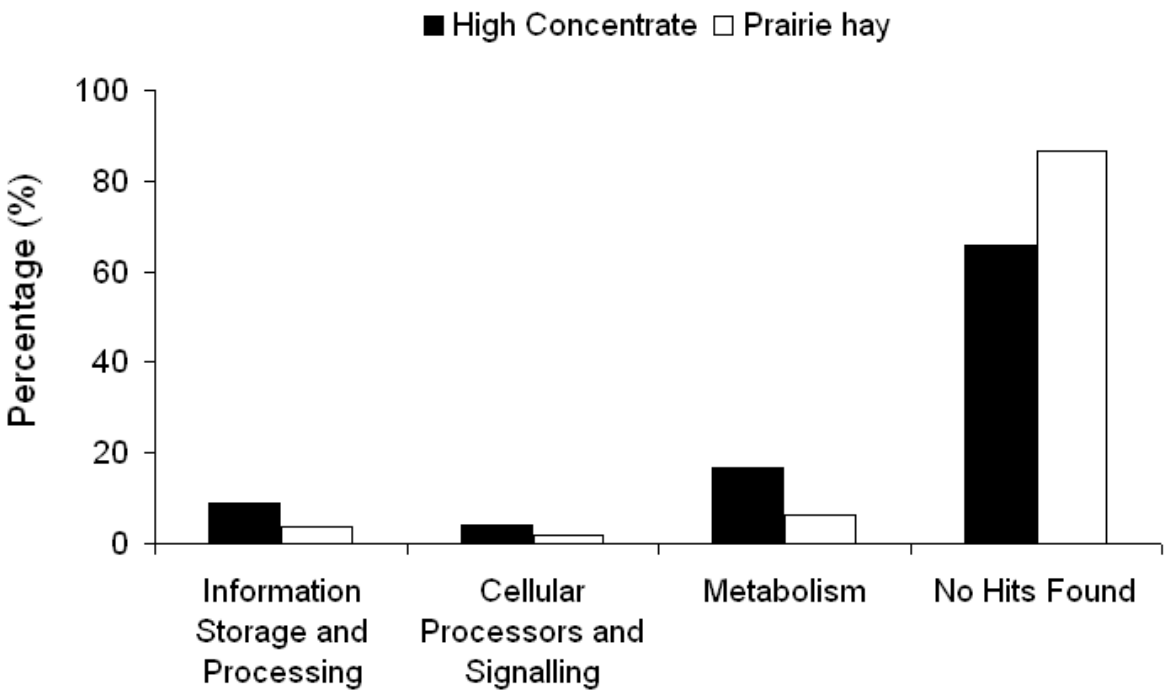

High Concentrate
Prairie hay
Percentage (%)
100
80
60
40
20
0
Information Storage and Processing
Cellular Processors and Signalling
Metabolism
No Hits Found

Figure 6

Distribution of COG analysis. Information storage and processing (a) Cellular processors and signaling (b) Metabolism (c)

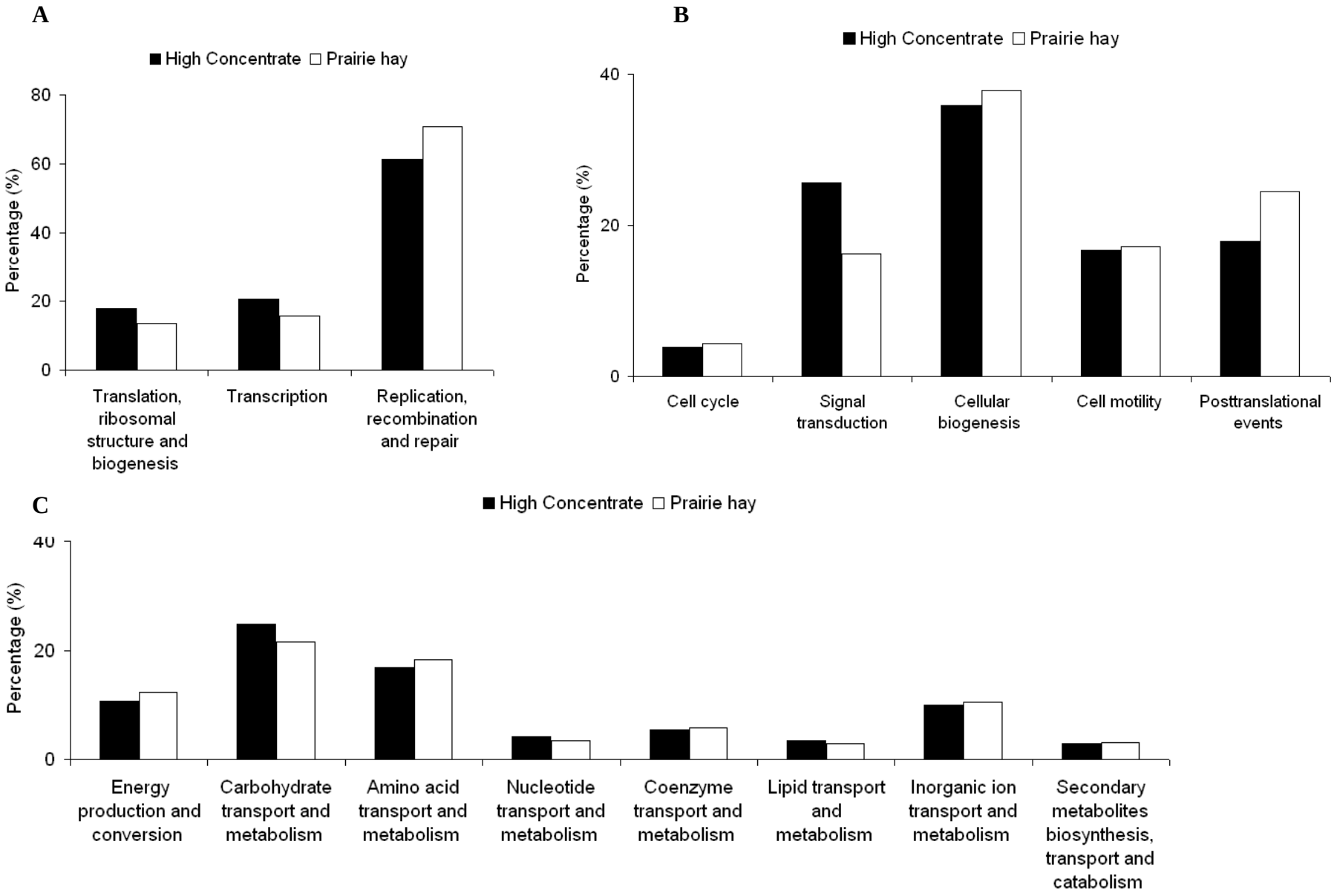

A
High Concentrate
Prairie hay
Percentage (%)
0
20
40
60
80
Translation, ribosomal structure and biogenesis
Transcription
Replication, recombination and repair
B
High Concentrate
Prairie hay
Percentage (%)
0
20
40
Cell cycle
Signal transduction
Cellular biogenesis
Cell motility
Posttranslational events
C
High Concentrate
Prairie hay
Percentage (%)
0
20
40
Energy production and conversion
Carbohydrate transport and metabolism
Amino acid transport and metabolism
Nucleotide transport and metabolism
Coenzyme transport and metabolism
Lipid transport and metabolism
Inorganic ion transport and metabolism
Secondary metabolites biosynthesis, transport and catabolism

Figure 7

Distribution of bacterial species at phylum level using “MEGAN” (Huson et al., 2007). The numbers next to each phylum represents the hits observed for the corresponding phylum.

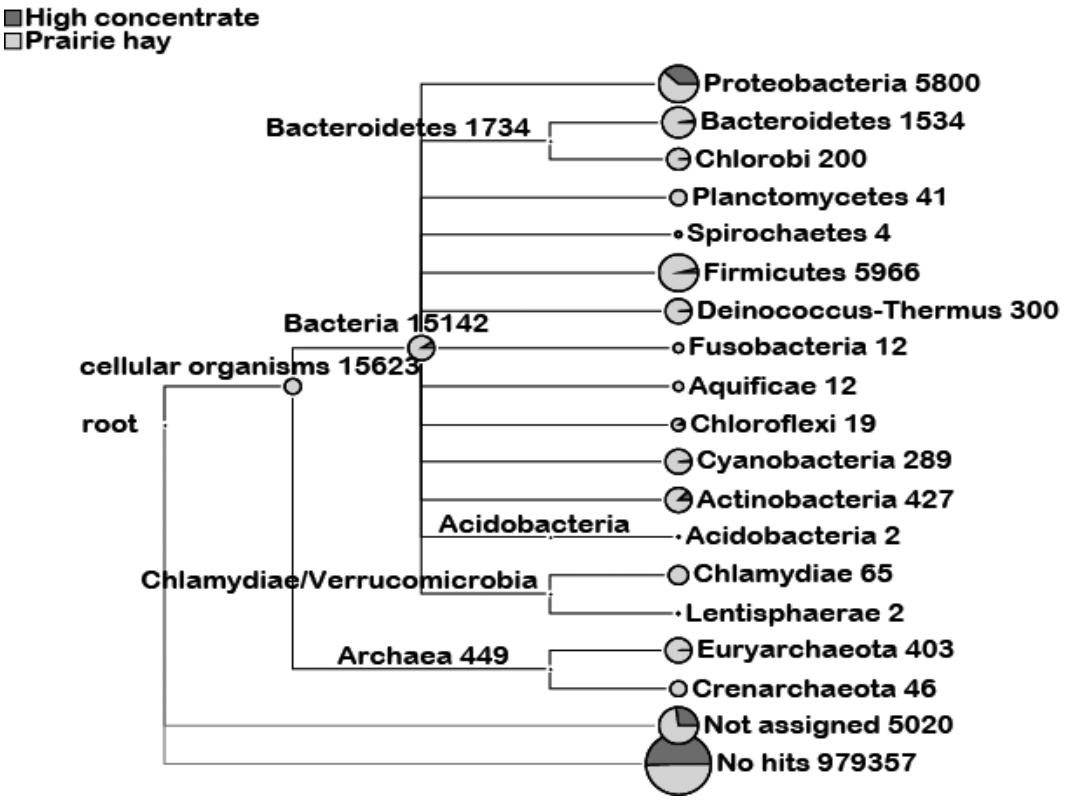
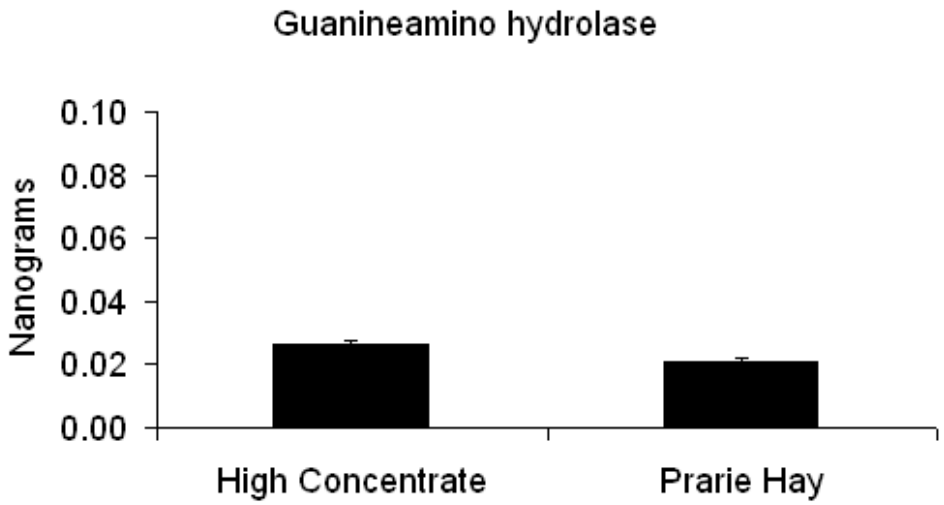
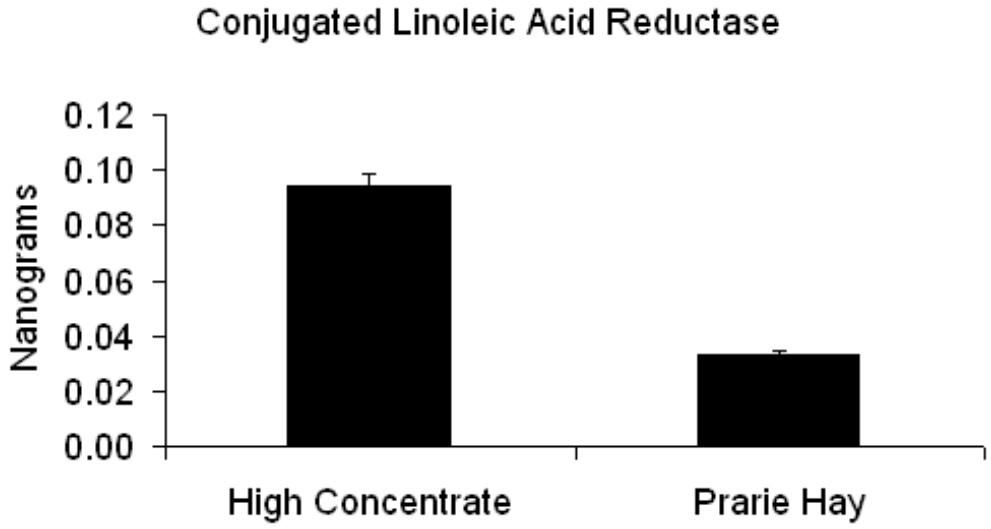
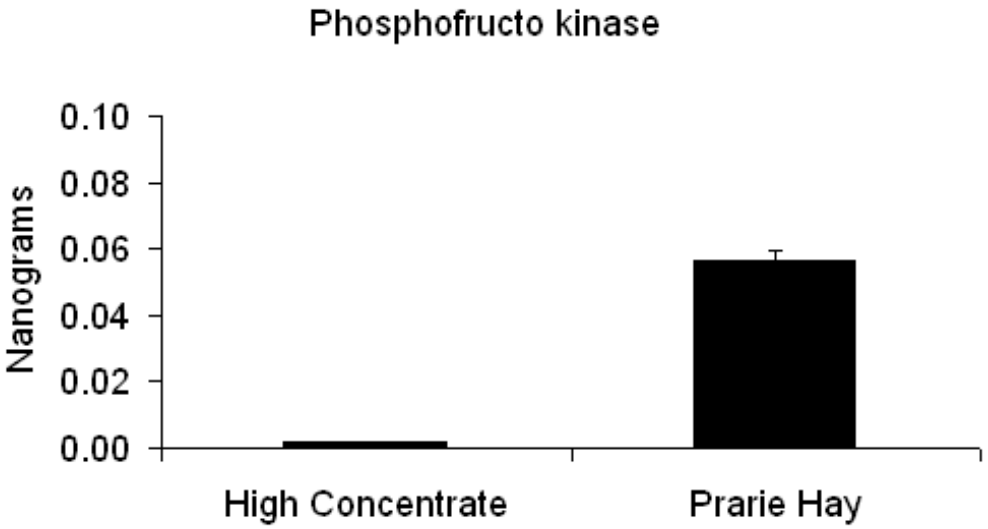

High concentrate
Prairie hay
Proteobacteria 5800
Bacteroidetes 1734
Bacteroidetes 1534
Chlorobi 200
Planctomycetes 41
Spirochaetes 4
Firmicutes 5966
Deinococcus-Thermus 300
Bacteria 15142
Fusobacteria 12
cellular organisms 15623
Aquificae 12
root
Chloroflexi 19
Cyanobacteria 289
Actinobacteria 427
Acidobacteria
Acidobacteria 2
Chlamydiae/Verrucomicrobia
Chlamydiae 65
Lentisphaerae 2
Archaea 449
Euryarchaeota 403
Crenarchaeota 46
Not assigned 5020
No hits 979357

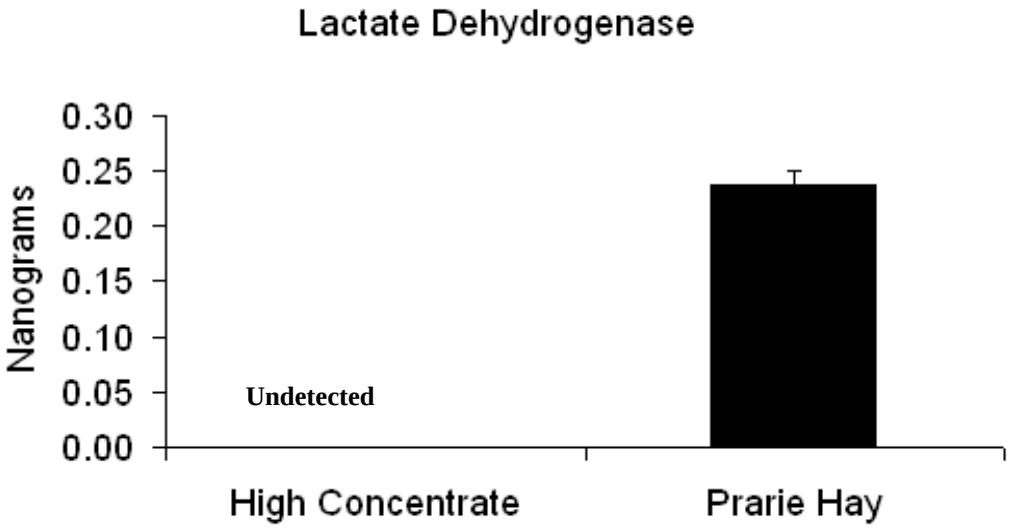
Figure 8

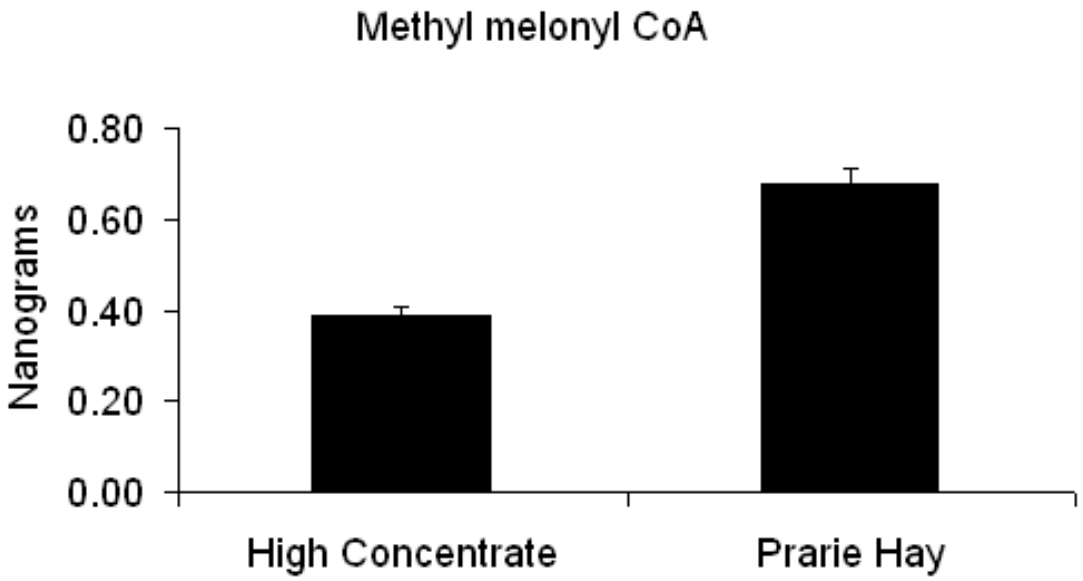
Real-time PCR results generated for a selected set of enzymes that play a key role in nutrient digestion. Real-time PCR analysis was performed using absolute quantification.


Guanineamino hydrolase
Nanograms
0.10
0.08
0.06
0.04
0.02
0.00
High Concentrate
Prarie Hay


Conjugated Linoleic Acid Reductase
Nanograms
0.12
0.10
0.08
0.06
0.04
0.02
0.00
High Concentrate
Prarie Hay


Phosphofructo kinase
Nanograms
0.10
0.08
0.06
0.04
0.02
0.00
High Concentrate
Prarie Hay


Lactate Dehydrogenase
Nanograms
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Undetected
High Concentrate
Prarie Hay


Methyl melonyl CoA
Nanograms
0.80
0.60
0.40
0.20
0.00
High Concentrate
Prarie Hay

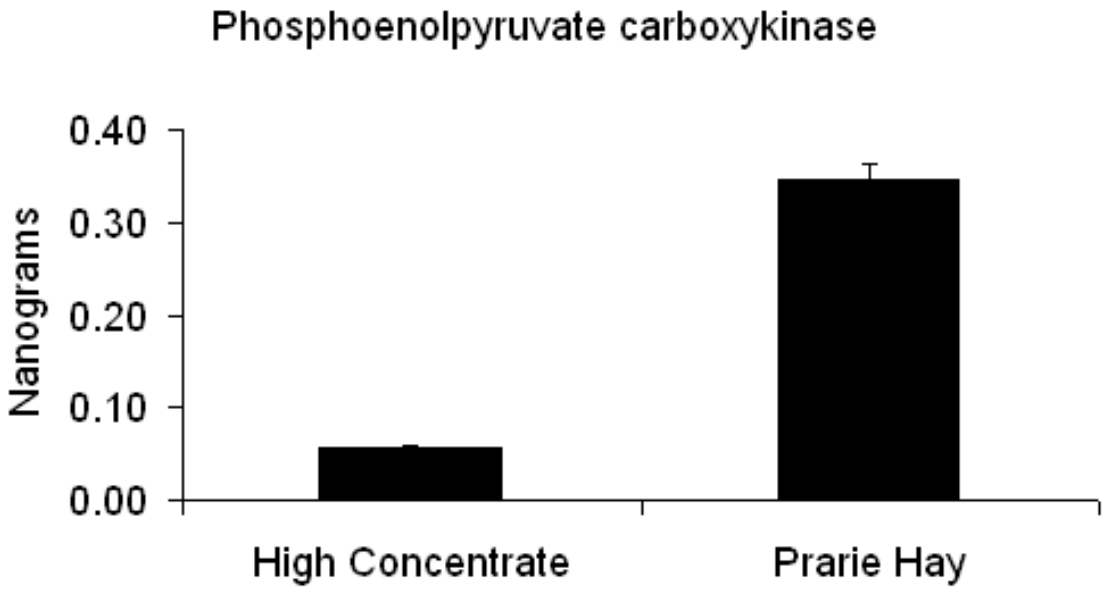

Phosphoenolpyruvate carboxykinase
Nanograms
0.40
0.30
0.20
0.10
0.00
High Concentrate
Prarie Hay

Table 2. EST library statistics from 454 pyro-sequencing.

	High Concentrate		Prairie Hay	
Total number of flows	17357		40772	
Total number of contigs	579		2135	
Total flows within contigs	5387	31.0%	17023	41.8%
Singlet flows	11970	69.0%	23749	58.2%

Table 3. The distribution of the annotated genes among metabolic pathways with respect to metabolic function.

Carbohydrate Metabolism	
Glycolysis / Gluconeogenesis	4.17%
Pentose and glucuronate interconversions	16.67%
Fructose and mannose metabolism	16.67%
Galactose metabolism	8.33%
Ascorbate and aldarate metabolism	4.17%
Starch and sucrose metabolism	8.33%
Aminosugars metabolism	16.67%
Pyruvate metabolism	4.17%
Glyoxylate and dicarboxylate metabolism	8.33%
Butanoate metabolism	12.50%
Energy Metabolism	
Oxidative phosphorylation	25.00%
ATP synthesis	25.00%
Photosynthesis	8.33%
Methane metabolism	16.67%
Nitrogen metabolism	16.67%
Sulfur metabolism	8.33%
Lipid Metabolism	
Bile acid biosynthesis	9.09%
Androgen and estrogen metabolism	9.09%
Glycerolipid metabolism	45.45%
Glycerophospholipid metabolism	18.18%
Arachidonic acid metabolism	18.18%

Nucleotide Metabolism	
Purine metabolism	73.33%
Pyrimidine metabolism	26.67%

Amino Acid Metabolism	
Glutamate metabolism	9.76%
Alanine and aspartate metabolism	9.76%
Glycine, serine and threonine metabolism	7.32%
Methionine metabolism	7.32%
Valine, leucine and isoleucine degradation	2.44%
Valine, leucine and isoleucine biosynthesis	9.76%
Lysine biosynthesis	9.76%
Lysine degradation	2.44%
Arginine and proline metabolism	2.44%
Phenylalanine metabolism	4.88%
Tryptophan metabolism	2.44%
Phenylalanine, tyrosine and tryptophan biosynthesis	4.88%
Tyrosine metabolism	2.44%
Other Amino acids	17.07%
Urea cycle and metabolism of amino groups	7.32%

Glycan Biosynthesis and Metabolism	
N-Glycan degradation	3.85%
Glycosaminoglycan degradation	3.85%
Lipopolysaccharide biosynthesis	42.31%
Peptidoglycan biosynthesis	3.85%
Glycosphingolipid metabolism	7.69%
Glycan structures - biosynthesis 2	34.62%
Glycan structures - degradation	3.85%

Metabolism of Cofactors and Vitamins	
Pantothenate and CoA biosynthesis	12.50%
Biotin metabolism	12.50%
Folate biosynthesis	6.25%
One carbon pool by folate	6.25%
Diterpenoid biosynthesis	18.75%
Limonene and pinene degradation	6.25%
Stilbene, coumarine and lignin biosynthesis	6.25%
Alkaloid biosynthesis I	6.25%
Tetracycline biosynthesis	6.25%
Novobiocin biosynthesis	6.25%

Gamma-Hexachlorocyclohexane degradation	6.25%
Fluorene degradation	6.25%
1- and 2-Methylnaphthalene degradation	6.25%
Genetic Information Processing	
Transcription	4.40%
Translation	92.31%
Environmental Information Processing	
Membrane Transport	92.86%
Signal Transduction	7.14%

Discussion

Microbial communities play an integral role in nutrient digestion and material recycling in the ecosystem (Fernando et al., 2008d; Fernando et al., 2008c; Girguis et al., 2005; Hallam et al., 2003). Yet, our understanding of the different microbial communities and their functional role within different environments is in its infancy. Furthermore, the molecular mechanisms used by these microbes in material recycling and nutrient digestion are yet to be discovered. Recent 16S rRNA sequencing projects (Fernando et al., 2008d; Fernando et al., 2008a; Fernando et al., 2008c; Tringe et al., 2005), and large insert Bacterial Artificial Chromosome (BAC) clones and small insert libraries constructed from environmental samples (Schloss and Handelsman, 2005; Tringe et al., 2005; Tyson et al., 2004; Venter et al., 2004) have offered a glance into the phylogenetic diversity of uncultured microbial populations in different environments, and have provided valuable information into the lifestyles and capabilities of these microbial populations. However, this metagenomics approach of sequencing large or small insert clones from the microbial population, only provides the opportunity to identify the capabilities of the population, and does not help identify the metabolic activity or the functional role of the microbial population at a given time under a given environmental condition. Therefore we propose a community transcriptomics approach to study the functional role of microbes to identify the metabolic pathways in action in a given environment. In this study, we evaluate the complex rumen microbial ecosystem as a proof of concept and to identify the metabolic pathways involved in nutrient digestion within the ruminant animal.

The greatest challenge in microbial community transcriptomics is the ability to extract intact good quality RNA from different, somewhat harsh, environments. The rumen is no different as it is a very dynamic environment where microbial cells are consistently being lysed and degraded. The rumen is chock full of nucleases and has shown to degrade naked DNA in as low as 15 min (Fernando et al. unpublished data). Our attempt to isolate good quality RNA from the rumen was successful (figure 2), where a majority of the intact total RNA was prokaryotic in origin. Further purification of this total RNA yielded intact good quality mRNA which was used for community transcriptomics using 454 pyro-sequencing (Figure 2).

454 pyro-sequencing generated ~60,000 reads from the two EST libraries (prairie hay and high concentrate diet) with redundancies of <18%, suggesting large diversity in terms of function within each microbial environment. The larger number of unique reads detected in the prairie hay suggest more diverse metabolic activity with this environment than in high concentrate. The EST reads generated were analysed to identify metabolic pathways active within the two metabolic environments. Overall EST analysis only annotated ~25% of the genes identified within the two populations. This suggests that the rumen microbial population is highly diverse, and is composed of many currently unknown microbial species which utilize unknown metabolic pathways in nutrient digestion and cellular processors. This observation was consistent with the COG analysis results were 65% and 85% of the genes were un-annotated in high concentrate and prairie hay respectively. ~15.08% of the annotated genes belonged to genes with no metabolic hits, suggesting rapid turnover of bacteria within the rumen. This notion was further supported by analyzing the ESTs involved in genetic information and

processing (Figure 3 & Table 3) and the COG results observed under information storage and processing (Figure 6a) which shows increased number of genes involved in DNA replication, recombination, repair, ribosomal structure and biogenesis, transcription and translation. KEGG annotation of the combined ESTs displayed diverse metabolic pathways in action within the ruminal environments. Among these, energy metabolism pathways were interesting as we detected genes involved in oxidative phosphorylation. The detection of genes involved in oxidative phosphorylation within an anaerobic environment is surprising but is not uncommon as, most bacteria within the rumen are facultative aerobes and species such as *Megasphera elsdenii* found within the rumen is known to use an electron transport chain that contains ferredoxin and an “electron transferring” flavoprotein in propionate production (Russell, 2002). Genes involved in energy metabolism also belonged to photosynthesis, Methane metabolism, Nitrogen metabolism and sulphur metabolism, suggesting the presence of diverse bacterial populations which utilized different energy substrates. A majority of the genes involved in lipid metabolism were implicated in glycerolipid metabolism, glycerophospholipid metabolism and arachidonic acid metabolism, but we also detected a few genes that were involved in bile acid biosynthesis and androgen and estrogen metabolisms. These genes may have resulted from sloughed off cells of the animal that was not removed during removal of poly A mRNA. We detected more genes involved in purine metabolism compared to genes involved in pyrimidine metabolism. ESTs involved in amino acid metabolisms and carbohydrate metabolism was among the highest detected and accounted for more than 50% of the genes detected in metabolism. These genes were involved in a variety of metabolic processes, and were likely due to the fact that

the ruminant environments contained large amounts of carbohydrates and protein substrates. COG analysis displayed a greater number of genes involved in carbohydrate metabolism in high concentrate than in prairie hay. This was expected as the high concentrate diet has more carbohydrate substrates that can be utilized by the bacteria than in prairie hay. Genes involved in transcription was significantly lower than the genes involved in translation, suggesting that translation is more active than transcription. This can be explained if the mRNA transcripts stay longer within the cell which allows the translation of these mRNA transcripts to occur repeatedly leading to a more active translation process than transcription. COG analysis of the EST sequences revealed that genes involved in translation, ribosomal structure and biogenesis, transcription were higher in the bacterial population on high concentrate while, genes involved in replication, recombination and repair were higher in the population on prairie hay (Figure 6a). This suggests that the microbial population on high concentrate is transcriptionally more active, while the microbial population on prairie hay is a rapidly replicating population. Among the ESTs involved in environmental information processing, genes implicated in membrane transport was significantly higher in high concentrate, but we also detected genes involved in the cell cycle, signal transduction, cellular biogenesis, cell motility, and posttranslational events. This increase in membrane transport may help move partially digested and fermentable substrates into the cell for further digestion and energy production. COG analysis detected more genes (4.22%) involved in cellular processors and cell signalling in high concentrate than in prairie hay (1.75%). This may be due to one of two reasons; 1) as many more bacterial genomes belonging to Proteobacteria which increase in high concentrate (Fernando et

al., 2008c) has been sequenced (Liolios et al., 2008) which allows more genes to be annotated in high concentrate or 2) due to the fermentable carbohydrates in high concentrate, there may be more membrane transport occurring in this environment or both. We also detected a large number of genes involved in glycan biosynthesis and metabolism and in vitamins and cofactor metabolism (Table 3).

KEGG cluster analysis performed on the ESTs (Figure 4) displayed a common set of genes that are expressed in both populations. Interestingly, a majority of the genes detected in the two bacterial populations were unique to each of the two populations. A majority of these unique genes found in each population displayed functions related to information storage and processing, cellular transport, cellular processing and signaling, and Metabolism, suggesting that the unique genes detected in the two bacterial populations may have come from the bacteria that are specific to each environment. This unique EST fingerprints from the two populations display the role of the bacterial community within that ruminal environment. The unique EST sequences found in the two different ruminal environments show similar metabolic function, and suggest that these two bacterial populations may be using different metabolic pathways to accomplish the same overall function. Similar to the KEGG cluster analysis results, the genes detected through COG analysis displayed expression of a unique set of genes within each ruminal environment, further suggesting the presence of unknown bacterial populations and unknown metabolic pathways within each environment. COG analysis of the two EST populations displayed more annotated genes in high concentrate than in prairie hay. Previously, we reported an increase of Proteobacteria when cattle are put on a high concentrate diet (Fernando et al., 2008c). Therefore, this higher number of

annotated genes in high concentrate is due to the fact that currently the majority of the genomes sequenced belong to phylum Proteobacteria (Liolios et al., 2008).

To identify bacterial diversity of the population and to identify bacterial species based on the EST sequences generated, “MEGAN” (MEta Genome ANalysis) (Huson et al., 2007) was performed on the BLASTX results. MEGAN analysis at Phylum level displayed a larger diversity of bacterial species in prairie hay compared to high concentrate diet. A majority of the ESTs had no significant hits suggesting that the EST signatures detected have never been seen before. Further suggesting that these ESTs would have come from currently unknown bacterial species. Among the ESTs that had BLASTX results, the phylogenetic analysis using “MEGAN” identified bacteria belonging to phyla *Proteobacteria, Bacteroidetes, Chlorobi, Planctomycetes, Spirochaetes, Firmicutes, Deinococcus-Thermus, Fusobacteria, Aquificae, Chloroflexi, Cyanobacteria, Actinobacteria, Acidobacteria, Chlamydiae, Lentisphaerae,* and *Euryarchaeota* on prairie hay (Figure 7). This shows that the bacterial population on prairie hay is highly diverse with a significant number of unknown bacterial species. The ESTs identified from high concentrate belonged to *Proteobacteria, Bacteroidetes, Firmicutes,* and *Actinobacteria,* where a majority was from phylum *Proteobacteria*. This observation of high amounts of *Proteobacteria* within the high concentrate sample is consistent with the COG analysis results and our previous observation of increasing Proteobacterial populations on high concentrate diet (Fernando et al., 2008c). Further analysis of the Proteobacterial population shows that a majority belongs to *gamma Proteobacteria*. A closer analysis of the BLASTX results at genus and species level allows the

identification of the bacterial species involved in each metabolic process identified (see appendix 1).

Therefore, community transcriptomics provides new opportunities and hope in uncovering the functional role of microbial communities and the organisms that govern these processors. This RNA based approach will also provide the opportunity to identify new metabolic pathways and will be of great value in understanding the functional role of microbial communities. More importantly, transcriptomics based approach will allow estimation of rates of material recycling by quantification of gene expression using copy numbers of genes.

We also performed realtime PCR analysis on a selected set of genes that are known to be active within the rumen (Russell, 2002) (figure 8). As no gene is known that is universally expressed in constant levels in microbial communities to be used as a normalizer gene, we evaluated gene expression using absolute quantification. Guanine aminohydrolase, which is involved in purine metabolism (Vogels and Vanderdrift, 1976) displayed similar levels of expression within the two ruminal environments suggesting that similar levels of nucleotide metabolism occurs within the two environments. Phosphofructokinase gene expression in the two bacterial populations were different where, the amount of phosphofructokinase detected on prairie hay was higher than in high concentrate diet. Phosphofructokinase is a key enzyme in glycolysis, that converts fructose-6-phosphate to fructose-1,6-bisphosphate (Voet and Voet, 2005). The increase of phosphofructokinase in the bacterial population on prairie hay suggests that glycolysis occurs at a higher rate in prairie hay than in high concentrate diet. This may be due to the time of sampling, as we sampled 6-8 hours after feeding, by this time a

majority of the digestion in high concentrate ruminal environment may have completed due to faster digestion rates. Whereas, in the ruminal environment with prairie hay it would take longer to digest the fibrous material, therefore the glycolytic pathway may have been more active at the time of collection. Similar to Phosphofructokinase, higher levels of phosphoenolpyruvate carboxykinase and methyl melonyl CoA was higher in bacterial populations on prairie hay when compared to high concentrate. Phosphoenolpyruvate carboxykinase, “catalyzes the reversible carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate (OAA)” (Schocke and Weimer, 1997) (favorably in the direction of OAA to PEP), and is known to have a gluconeogenic role (Chau and Ng, 1986). Furthermore, methyl melonyl CoA is implicated in propionate production (Russell, 2002), suggesting that these two enzymes may be acting in opposite directions to balance propionate production and gluconeogenesis. The bacterial population on high concentrate diet displayed larger amounts of conjugated linoleic acid reductase expression when compared to the bacterial population on prairie hay.

This increase in high concentrate may be due to higher amounts of lipid in the high concentrate diet. To our surprise, we were unable to detect the expression of lactate dehydrogenase in animals on high concentrate diet. Lactate dehydrogenase catalyzes the reversible reaction of converting lactate to pyruvate (Evans and Martin, 2002). Thus, the increase in lactate dehydrogenase in pararie hay animals may be to generate more pyruvate for ATP production, whereas the reason for no lactate dehydrogenase expression in high concentrate may be due to higher amounts of ATP generated through fermentable carbohydrates and propionate production (Russell, 2002).

Although, we used degenerate primers to evaluate key enzymes involved in rumen metabolic functions, these enzymes were not conserved enough to detect gene expression from the total bacterial population. Therefore we may be missing the expression of certain bacterial species. But, with the current advances in microarray technology, we could design oligonucleotide probes specific for each bacterial species from the EST sequences to detect gene expression changes of selected genes in the population. This approach provides new opportunities to study the gene expression changes of a single bacterial species within complex bacterial environments. Thus, use of community transcriptomics will provide the opportunity to identify novel molecular mechanisms in action and to determine the rate of these novel pathways in a given environment. The transcripts can also be used to identify the bacterial diversity present within the environment and therefore provides a better view of the role of each bacterial species within the environment to build a structure function relationship. Therefore, using community transcriptomics we propose a new method to study microbial populations that allows identification of function as well as identity of bacterial species within an environment. With more and more bacterial genomes being sequenced, this new approach will provide great potential to identify functional roles of bacteria in a given environment.

Reference List

Abulencia,C.B., Wyborski,D.L., Garcia,J.A., Podar,M., Chen,W.Q., Chang,S.H. et al. (2006) Environmental whole-genome amplification to access microbial populations in contaminated sediments. *Applied and Environmental Microbiology* **72:** 3291-3301.

Agarwal,K. and Agarwal,S. (2008) Helicobacter pylori vaccine: From past to future. *Mayo Clinic Proceedings* **83:** 169-175.

Amann,R.I., Binder,B.J., Olson,R.J., Chisholm,S.W., Devereux,R., and Stahl,D.A. (1990) Combination of 16S Ribosomal-Rna-Targeted Oligonucleotide Probes with Flow-Cytometry for Analyzing Mixed Microbial-Populations. *Applied and Environmental Microbiology* **56:** 1919-1925.

Chau,H.S. and Ng,S.K. (1986) Activation of Phosphoenolpyruvate Carboxykinase Isolated from Veillonella-Parvula. *Biochemistry and Cell Biology-Biochimie et Biologie Cellulaire* **64:** 898-905.

Church,D.C. (1993) *Ruminant Animal: Digestive Physiology and Nutrition*. New Jersey: Prentice Hall.

Edwards,J.E., Mcewan,N.R., Travis,A.J., and Wallace,R.J. (2004) 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **86:** 263-281.

Evans,J.D. and Martin,S.A. (2002) Cloning of the L-lactate dehydrogenase gene from the ruminal bacterium Selenonionas ruminantium HD4. *Current Microbiology* **44:** 155-160.

Ewers,C., Lubke-Becker,A., and Wieler,L.H. (2004) Mannheimia haemolytica and the pathogenesis of pneumonic pasteurellosis. *Berliner und Munchener Tierarztliche Wochenschrift* **117:** 97-115.

Ewing,B. and Green,P. (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Research* **8:** 186-194.

Ewing,B., Hillier,L., Wendl,M.C., and Green,P. (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Research* **8:** 175-185.

Fernando,S.C., Horn,G.W., Purvis II,H.T., Najar,F.Z., Sukharnikov,L., Nagaraja,T.G. et al. Bacterial population dynamics during ionophore treatment. 2008a.
Ref Type: Unpublished Work

Fernando,S.C., Purvis II,H.T., Najar,F.Z., Sukharnikov,L., Nagaraja,T.G., Krehbiel,C.R. et al. Rumen microbial changes during adaptation to high concentrate diet. 2008d.
Ref Type: Unpublished Work

Fernando,S.C., Purvis II,H.T., Najar,F.Z., Sukharnikov,L., Nagaraja,T.G., Krehbiel,C.R. et al. Rumen microbial population dynamics during sub acute acidosis. 2008c.
Ref Type: Unpublished Work

Girguis,P.R., Cozen,A.E., and Delong,E.F. (2005) Growth and population dynamics of anaerobic methane-oxidizing archaea and sulfate-reducing bacteria in a continuous-flow bioreactor. *Applied and Environmental Microbiology* **71:** 3725-3733.

Goo,Y.A., Roach,J., Glusman,G., Baliga,N.S., Deutsch,K., Pan,M. et al. (2004) Low-pass sequencing for microbial comparative genomics. *Bmc Genomics* **5**.

Hallam,S.J., Girguis,P.R., Preston,C.M., Richardson,P.M., and Delong,E.F. (2003) Identification of methyl coenzyme M reductase A (mcrA) genes associated with methane-oxidizing archaea. *Applied and Environmental Microbiology* **69:** 5483-5491.

Huson,D.H., Auch,A.F., Qi,J., and Schuster,S.C. (2007) MEGAN analysis of metagenomic data. *Genome Research* **17:** 377-386.

Lebeau,A., Reverchon,S., Gaubert,S., Kraepiel,Y., Simond-Cote,E., Nasser,W., and Van Gijsegem,F. (2008) The GacA global regulator is required for the appropriate expression of Erwinia chrysanthemi 3937 pathogenicity genes during plant infection. *Environmental Microbiology* **10:** 545-559.

Liolios,K., Mavromatis,K., Tavernarakis,N., and Kyrpides,N.C. (2008) The Genomes On Line Database (GOLD) in 2007: status of genomic and metagenomic projects and their associated metadata. *Nucleic Acids Research* **36:** D475-D479.

Margulies,M., Egholm,M., Altman,W.E., Attiya,S., Bader,J.S., Bemben,L.A. et al. (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437:** 376-380.

Moriya,Y., Itoh,M., Okuda,S., Yoshizawa,A.C., and Kanehisa,M. (2007) KAAS: an automatic genome annotation and pathway reconstruction server. *Nucleic Acids Res* **35:** W182-W185.

oki-Kinoshita,K.F. and Kanehisa,M. (2007) Gene Annotation and Pathway Mapping in KEGG. *Methods Mol Biol* **396:** 71-92.

Russell,J.B. (2002) *Rumen microbiology and its role in ruminant nutrition*. Ithaca, NY: James B. Russell.

Sambrook,J. and Russel,D.W. (2001) *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schloss,P.D. and Handelsman,J. (2005) Metagenomics for studying unculturable microorganisms: cutting the Gordian knot. *Genome Biology* **6**.

Schocke,L. and Weimer,P.J. (1997) Purification and characterization of phosphoenolpyruvate carboxykinase from the anaerobic ruminal bacterium Ruminococcus flavefaciens. *Archives of Microbiology* **167:** 289-294.

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

Stangegaard,M., Dufva,I.H., and Dufva,M. (2006) Reverse transcription using random pentadecamer primers increases yield and quality of resulting cDNA. *Biotechniques* **40:** 649-657.

Sturn,A., Quackenbush,J., and Trajanoski,Z. (2002) Genesis: cluster analysis of microarray data. *Bioinformatics* **18:** 207-208.

Tatusov,R.L., Fedorova,N.D., Jackson,J.D., Jacobs,A.R., Kiryutin,B., Koonin,E.V. et al. (2003) The COG database: an updated version includes eukaryotes. *Bmc Bioinformatics* **4**.

Tatusov,R.L., Koonin,E.V., and Lipman,D.J. (1997) A genomic perspective on protein families. *Science* **278:** 631-637.

Tringe,S.G., von Mering,C., Kobayashi,A., Salamov,A.A., Chen,K., Chang,H.W. et al. (2005) Comparative metagenomics of microbial communities. *Science* **308:** 554-557.

Tyson,G.W., Chapman,J., Hugenholtz,P., Allen,E.E., Ram,R.J., Richardson,P.M. et al. (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428:** 37-43.

Venter,J.C., Remington,K., Heidelberg,J.F., Halpern,A.L., Rusch,D., Eisen,J.A. et al. (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304:** 66-74.

Voet,D. and Voet,J.G. (2005) *Biochemistry*. Jhon Wiley & Sons Inc.

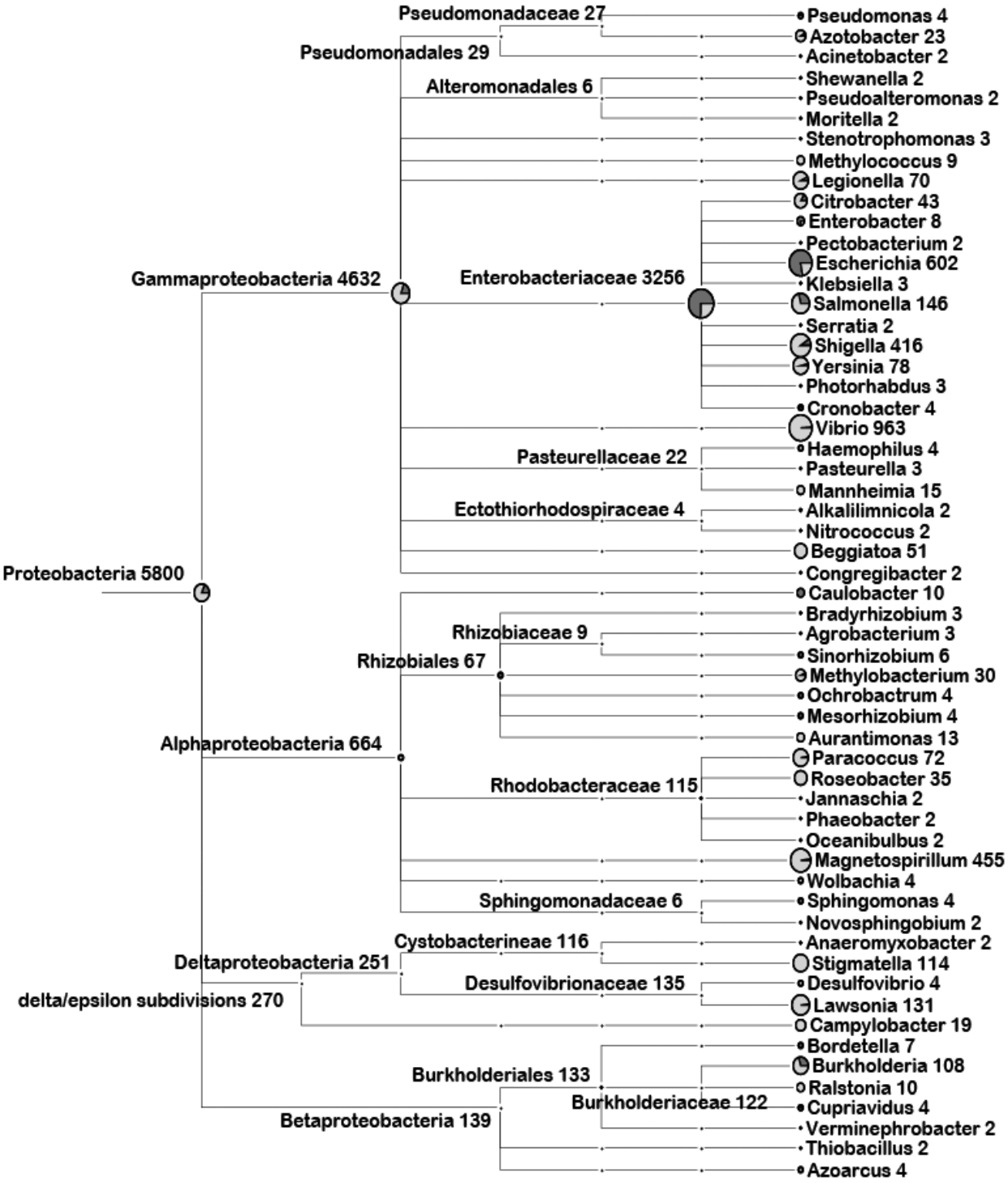
Vogels,G.D. and Vanderdrift,C. (1976) Degradation of Purines and Pyrimidines by Microorganisms. *Bacteriological Reviews* **40:** 403-468.

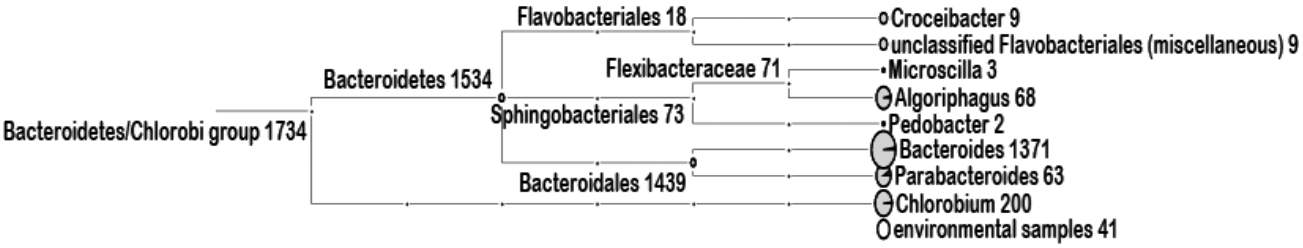
Woyke,T., Teeling,H., Ivanova,N.N., Huntemann,M., Richter,M., Gloeckner,F.O. et al. (2006) Symbiosis insights through metagenomic analysis of a microbial consortium. *Nature* **443:** 950-955.

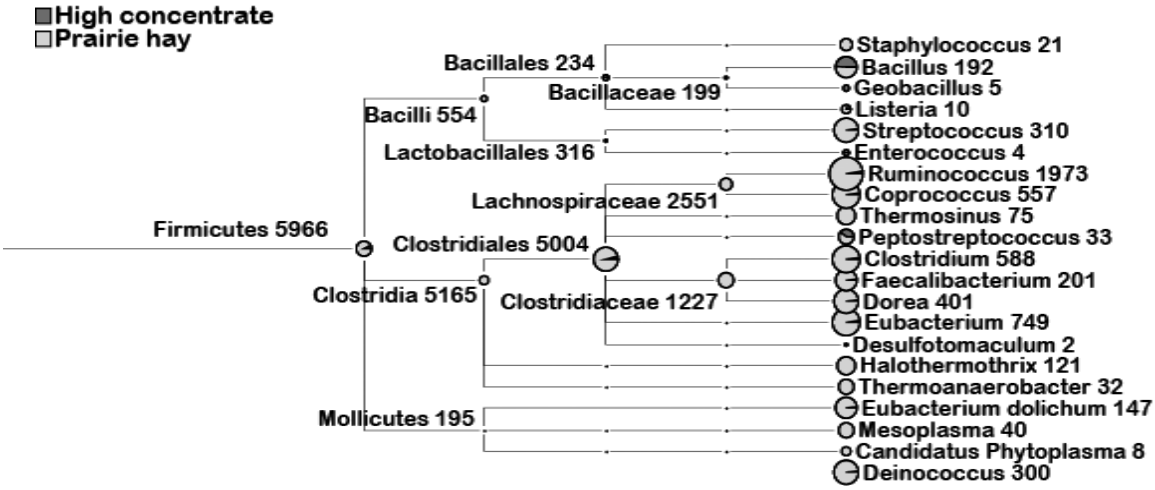
Zengler,K., Toledo,G., Rappe,M., Elkins,J., Mathur,E.J., Short,J.M., and Keller,M. (2002) Cultivating the uncultured. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **99:** 15681-15686.

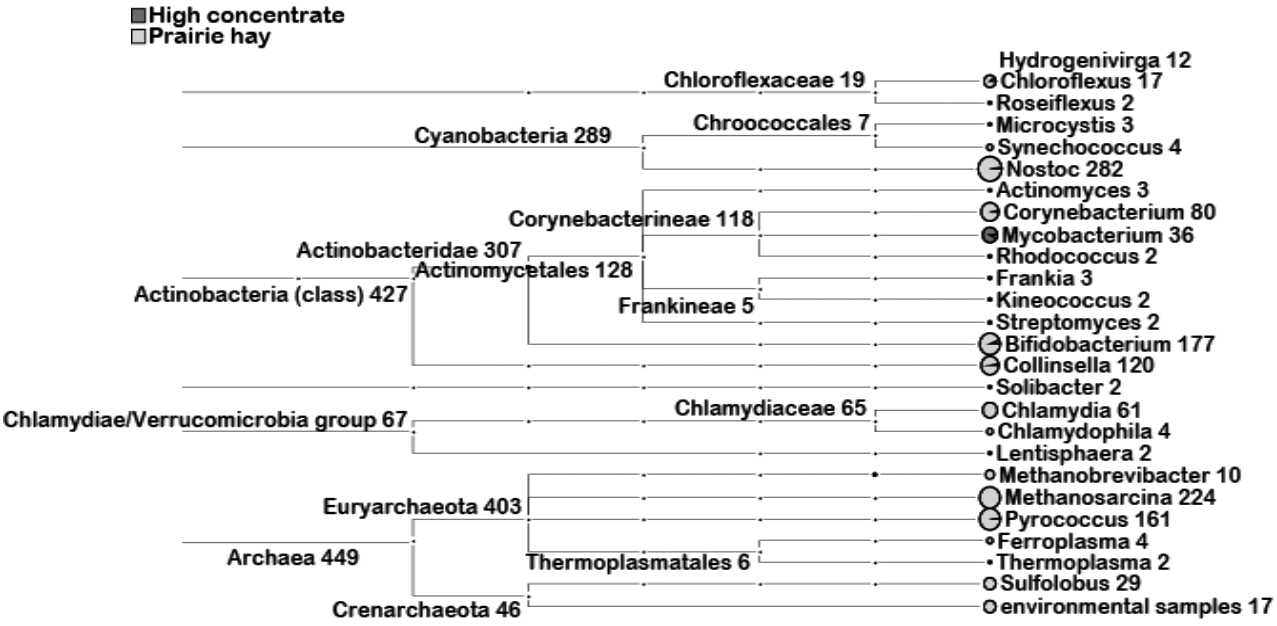
Appendix 1

Distribution of bacteria within each phyla at genus level.




Flavobacteriales 18
Croceibacter 9
unclassified Flavobacteriales (miscellaneous) 9
Flexibacteraceae 71
Microscilla 3
Bacteroidetes 1534
Algoriphagus 68
Sphingobacteriales 73
Pedobacter 2
Bacteroidetes/Chlorobi group 1734
Bacteroides 1371
Bacteroidales 1439
Parabacteroides 63
Chlorobium 200
environmental samples 41


High concentrate
Prairie hay
Staphylococcus 21
Bacillales 234
Bacillus 192
Bacillaceae 199
Geobacillus 5
Bacilli 554
Listeria 10
Streptococcus 310
Lactobacillales 316
Enterococcus 4
Ruminococcus 1973
Lachnospiraceae 2551
Coprococcus 557
Thermosinus 75
Firmicutes 5966
Clostridiales 5004
Peptostreptococcus 33
Clostridium 588
Clostridia 5165
Faecalibacterium 201
Clostridiaceae 1227
Dorea 401
Eubacterium 749
Desulfotomaculum 2
Halothermothrix 121
Thermoanaerobacter 32
Mollicutes 195
Eubacterium dolichum 147
Mesoplasma 40
Candidatus Phytoplasma 8
Deinococcus 300


High concentrate
Prairie hay
Hydrogenivirga 12
Chloroflexaceae 19
Chloroflexus 17
Roseiflexus 2
Chroococcales 7
Cyanobacteria 289
Microcystis 3
Synechococcus 4
Nostoc 282
Actinomyces 3
Corynebacterineae 118
Corynebacterium 80
Mycobacterium 36
Actinobacteridae 307
Rhodococcus 2
Actinomycetales 128
Frankia 3
Actinobacteria (class) 427
Kineococcus 2
Frankineae 5
Streptomyces 2
Bifidobacterium 177
Collinsella 120
Solibacter 2
Chlamydiaceae 65
Chlamydiae/Verrucomicrobia group 67
Chlamydia 61
Chlamydophila 4
Lentisphaera 2
Methanobrevibacter 10
Euryarchaeota 403
Methanosarcina 224
Pyrococcus 161
Archaea 449
Ferroplasma 4
Thermoplasmatales 6
Thermoplasma 2
Sulfolobus 29
Crenarchaeota 46
environmental samples 17

CHAPTER VII

CULTURING THE UNCULTURED FROM THE BOVINE RUMEN

Introduction

Based on microscopic observations from different environments it is estimated that 99% of the bacteria found in any given environment is resistant to invitro culture (Pace, 1997). This observation is also common to the ruminal environment where, based on direct microscopic counts it is estimated that 10^9-10^{11} (Grubb and Dehority, 1975; Maki and Foster, 1957) bacterial cells are present per gram of ruminal contents, but we have only isolated a handful of the species we observe under a microscope. Thus, traditional culture dependent techniques have only helped us identify and evaluate the functional role of a few microbes that reside within the rumen (Goad et al., 1998; Hungate et al., 1952; Hungate, 1968). However, recent studies carried out in the rumen using 16S rRNA based techniques (Fernando et al., 2008d; Fernando et al., 2008a) have provided a glimpse into the phylogenetic diversity present within the rumen, suggesting that there is an imperative need to identify and culture more organisms from the rumen to identify their physiological role within the rumen in nutrient digestion and to compliment the molecular investigations. Traditional bacterial isolation techniques have been laborious and time consuming (Bryant and Robinson, 1961; Ferguson et al., 1984). Furthermore, these traditional techniques have been bias towards specific groups of organisms (Bryant and Robinson, 1961; Ferguson et al., 1984). Traditional culture based techniques have failed to culture most rumen bacteria due to the complex nutrient environment that is present within the rumen and the inability to replicate such an environment in petri-dishes and culture tubes. Recent studies in other environments have proposed that using a chemical composition similar to the natural environment of the bacterial species will help grow uncultured bacterial species from these

environments (Connon and Giovannoni, 2002; Kaeberlein et al., 2002; Rappe et al., 2002). Several studies have utilized this approach to culture new rumen bacterial species where, rumen fluid has been used as the culture media for growth (Bryant and Robinson, 1961; Hungate et al., 1964). But, these studies have used laborious and time consuming techniques for isolation and screening for the new bacterial species.

In this study we use a high throughput culturing method that allows isolating and identifying new bacterial species from the rumen. We propose a 16S rRNA based screening approach to scan the cultured organisms to identify the microbial species and also provide an approach to identify the phylogeny of the new bacteria based on sequence analysis methods using bioinformatic programs.

Experimental Approach

Preparation of media

Rumen fluid was collected from steers on prairie hay and high concentrate diet. The samples collected were strained through 4 layers of cheese cloth and was autoclaved to kill all microbes present in the rumen fluid. Minimal media containing 6g/L Na_2HPO_4, 3g/L KH_2PO_4, 1g/L NH_4Cl, 0.5g/L NaCl, 0.12g/L $MgSO_4$ and 0.01g/L $CaCl_2$ was supplemented with 20% autoclaved rumen fluid to prepare the growth medium used for culturing rumen bacteria. The plates used in the culturing process contained 15% of agar in addition to the recipe described above.

Preparation of inoculum

Rumen samples were collected in a 4L closed flasks and was transported to the lab. In the laboratory, total rumen content (fluid+solid) collected was homogenized in a blender and ~1g of the homogenized sample was used to make a 10-fold dilution series ranging from 10^{1}-10^{-10} to be used for inoculation. The overall strategy that was used for isolation of novel rumen bacteria is shown in figure 1.

Culturing rumen bacteria

100 µl of the dilution series generated was used to inoculate plates (minimal media + rumen fluid). The plates were incubated at 37°C under anaerobic conditions in anaerobic chambers (Fisher Scientific, Pittsburgh, PA). Gaspak™ EZ (BD Biosciences, San joes, CA) gas packs were used to create the anaerobic conditions, and indicator strips (BD Biosciences, San joes, CA) were used to ensure that anaerobic conditions were maintained throughout the incubation period. Upon incubation the plates were examined daily for any growth of bacterial colonies on the plates. The colonies that were present on each plate were picked to 96 well culture plates (BD Biosciences, San joes, CA) and were incubated in liquid media under anaerobic conditions. After picking any grown colonies from the plates, the plates were further incubated to identify slow growing organisms. The plates were incubated for a total of 4 weeks and colonies that grew were picked routinely. The bacterial species grown in 96 well plates were replicated and glycerol stocks were made and stored at -80°C.

DNA extraction from the bacterial cultures

One of the replicated 96-well plates was used for DNA extraction. DNA extractions from the bacterial cultures were performed using the CTAB (cetyltrimethylammoniumbromide) method in the 96-well format. Briefly, the plates containing the bacterial cultures were centrifuged at 4000 rpm to pellet the cells. The media was removed by aspiration and the bacteria was resuspended in 100 µl of CAB buffer (100 mM Tris-HCl (pH8.0), 100 mM EDTA (pH8.0), 1.5 M NaCl and 1% CTAB). 10 µl of proteinase K (10 mg/ml) was added to the cells and was vortexed to break any pellets formed. 10 µl of 20% SDS was added to this solution and was mixed by inverting. The cell lysate formed was incubated at 65°C for 1 hour with gentle agitation. A phenol-chloroform extraction was performed in the 96-well format (Sambrook and Russel, 2001) and the DNA was precipitated. The precipitated DNA was resuspended in 50 µl of water and was used for PCR analysis to amplify the 16S rRNA gene.

Screening for previously uncultured bacteria

100 ng of the bacterial DNA isolated was used for amplification of the 16S rRNA gene. The PCR amplification was performed in a MJ dyad thermocycler (MJ research, Watertown, MA) using universal BacT0008F 5'-AGAGTTTGATCCTGGCTCAG-3' and BacT0805R 5'-GGACTACCAGGGTATCTAATCCC-3' primers to amplify a 809 bp fragment (in *E. coli*) of the 16S rRNA gene present in all Eubacteria (Fernando et al., 2008d). A 50 µl PCR reaction contained, 100 ng of DNA, 1X PCR buffer (Promega, Madison, WI, USA) 1.5 mM $MgCl_2$, 400 nM of each primer, 200 µM dNTPs, 100 ng/µl BSA and 3 U Taq DNA Polymerase (Promega, Madison, WI, USA). The cycling

conditions were: one cycle of 4 min at 95ºC, 30 sec at 52ºC, 1 min at 72ºC followed by 34 additional cycles of 30 sec at 95ºC, 30 sec at 52ºC, 1 min at 72ºC and a final extension of 3 min at 72ºC. The PCR products generated were electrophoresed on a 1.2% gel to ensure the amplification of the desired amplicon.

The correct size PCR products generated were cleaned by treating the PCR products in the 96-well format with shrimp alkaline phosphatase (1 unit/µl) and Exonuclease III (10 units/µl) (Amersham Biosciences, Piscataway, NJ, USA) using a volume equaling 10% of the PCR product as described by Desilva et *al.* (2003). The reaction was incubated at 37°C for 30 min followed by 80°C for 10 min. 1–3 µl of the resulting cleaned PCR product was used to sequence the 16S rRNA gene. The forward primer BacT0008F 5'-AGAGTTTGATCCTGGCTCAG-3' was used as the sequence primer in all sequencing reactions. The sequence reactions were performed in a 10 µl reaction volume. The reaction volume contained, 1–5 µl PCR product, 1 µmol sequencing primer, 2 µl BigDye™ (Applied Biosystems, Foster City, CA) and 1X buffer (Applied Biosystems, Foster City, CA). The sequencing reactions were performed in a MJ dyad thermocycler (MJ research, Watertown, MA) using the following conditions: one cycle of 4 min at 95ºC, 95°C for 30 sec, 50ºC of 30 sec, 60ºC for 1 min, followed by 60 additional cycles of 95ºC for 30 sec, 50ºC for 30 sec, and 60ºC for 1 min, followed by a 4°C hold. The sequence reaction was ethanol precipitated (Sambrook and Russel, 2001) and was analyzed on an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA). The data collected was analyzed using the sequence analysis software and initial base calling was done using the KB base caller. The sequences generated were analyzed using BLASTN (Altschul et al., 1997) and "Classifier" (Wang et al., 2007) software

analysis programs. The sequences were compared against the NCBI and RDP databases (Cole et al., 2003; Cole et al., 2005) to indentify if the 16S sequences generated were from cultured bacterial species. If the bacterial 16S sequences generated did not have a significant hit to any known bacterial species, these bacteria were used for biochemical analysis. The bacterial species identified as previously uncultured, were used to extract pure DNA using the High Pure PCR Template Preparation kit (Roche Diagnostics, Indianapolis, IN) and this DNA was used to amplify the full-length 16S rRNA gene from the bacterial species to further characterize these organisms. The PCR reaction was performed as decribed above using the following primer set; BacT0008F 5'-AGAGTTTGATCCTGGCTCAG-3' and BacT1510R 5'-GGTTACCTTGTTACGACTT-3'

Phylogenetic and Comparative Analyses

The assembled data was aligned using the ClustalW program. The alignment generated was used for Phylogenetic and Molecular evolutionary analyses using MEGA 4.0 (Tamura et al., 2007). Phylogenetic trees were constructed using the neighbor-joining method using the p-distance matrix. The phylogenetic tree constructed was tested by bootstaping using 1000 replications.

Biochemical analysis

The cultures selected based on the 16S rDNA sequences were used for biochemical testing. The selected bacterial cultures were sub-cultured 3 times before using for biochemical testing. The media used for each bacterial species differed based on the

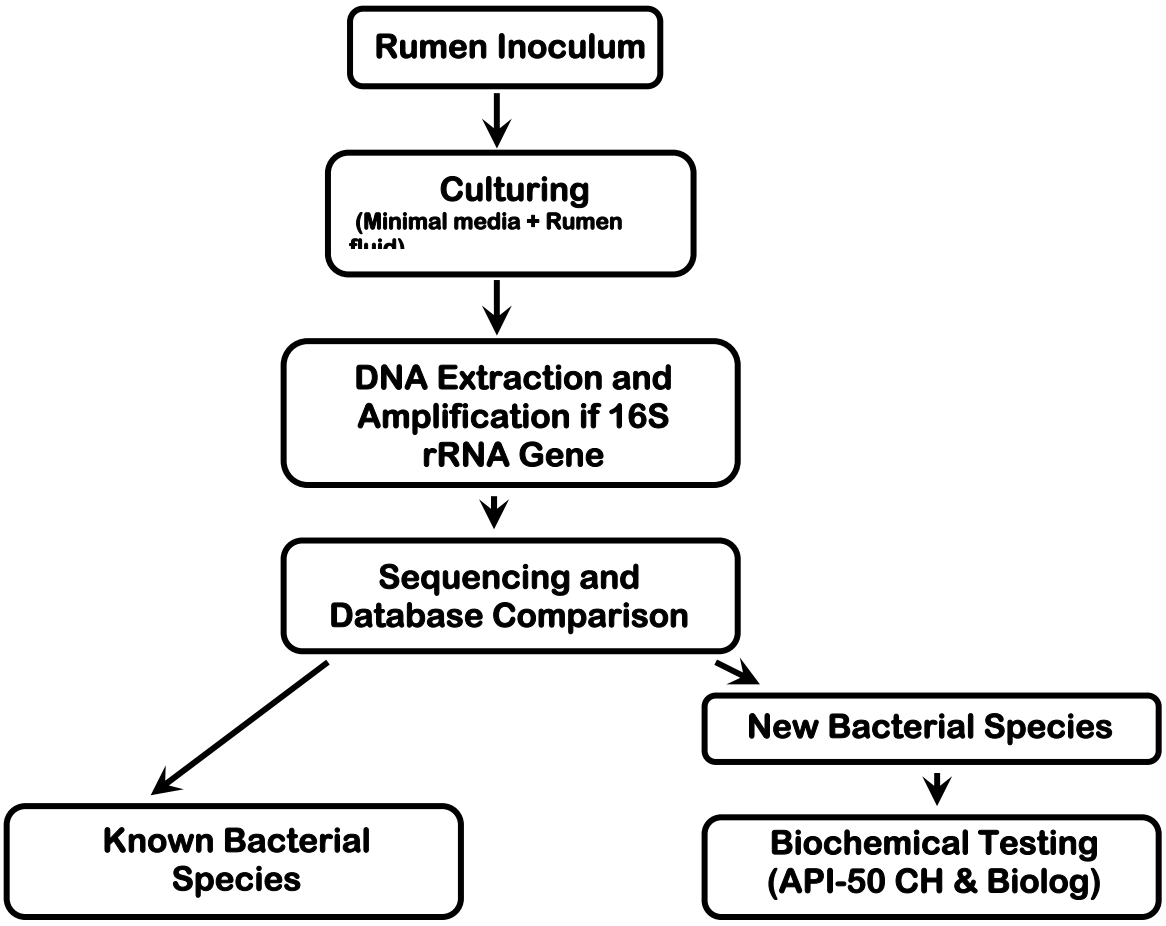
bacterial species analyzed. *Lactobacillus, Streptococcus* and *Bifidobacterium* species were cultured in MRS medium (BD Biosciences, San joes, CA), while some of the *Aceintobacter* and *Spingomonas sp.* were cultured on MRS supplemented with 20% rumen fluid. A series of 6 characterization tests were carried out to as described by McCoy and Gilliland (2007) (Mccoy and Gilliland, 2007). These tests include; 1) Gram stain, 2) Growth at 45°C, 3) Growth at 15°C, 4) Catalase test, 5) Arginine hydrolysis test, and 6) Carbohydrate fermentation patterns using API 50 CH kit (Biomeriux, Hazelwood, MO). The Gram stains on the selected cultures were carried out as described by Gerhardt et al. (1994). The growth of the isolated bacterial cultures at 15°C and 45°C was carried out by inoculating 10 ml of culture media with 10% inoculum and incubating the cultures under anaerobic conditions at the respective temperatures for 2-5 days and 48 hrs respectively. The catalase test (Gerhardt et al., 1994) was performed by growing the bacterial cultures for 24 hrs anaerobically on agar plates containing the correct inoculation media and flooding the plate with 3% hydrogen peroxide. If the cultures were positive to the catalase enzyme, effervescence was observed around the colonies. Arginine hydrolysis test was performed by inoculating 10 ml of Arginine broth (17.0 g/L casein, 3.0 g/L soymeal, 0.5 g/L D (+) glucose, 5.0 g/L NaCl, and 10 g/L L-Arginine monohydro-chloride) with 10% inoculum and incubating at 37°C for 48 hrs. Upon growth on arginine media, a drop of the culture was mixed with a drop of Nesseler's reagent (Sigma-Aldrich, St. Louis, MO) to observe the development of an orange/gold precipitate.

The carbohydrate fermentation patterns of the isolated bacterial cultures were evaluated using API 50 CH kit (Biomeriux, Hazelwood, MO). The cultures were subcultured 3

times before being used for API testing. The cultures were transferred to centrifuge tubes and the bacterial cells were precipitated by centrifugation at 11600X*g* for 10 min at 4°C. The supernatant was discarded and the cells were resuspended in Analytab Products Inc. (API) identification broth. The API broth was prepared according to manufacturer's protocols (Biomeriux, Hazelwood, MO). This centrifugation and resuspention steps were repeated a second time. After the second wash and centrifugation the cells were resuspended in 10 ml of API broth and were used for API testing. The 50 different carbohydrate substrates present in the API test strips were inoculated with the bacterial cultures and were incubated at 37°C under anaerobic conditions. The API strips were read at 24 and 48 hour intervals and the identities of the bacterial cultures were determined by comparing the carbohydrate fermentation fingerprint with bacterial species listed in the Bergey's Manual of Systematic Bacteriology (2001).

Figure 1

The overall strategy that was used for isolation of novel rumen bacterial species.


Rumen Inoculum
Culturing
(Minimal media + Rumen
DNA Extraction and
Amplification if 16S
rRNA Gene
Sequencing and
Database Comparison
New Bacterial Species
Known Bacterial
Species
Biochemical Testing
(API-50 CH & Biolog)

Results

The bacterial colonies that grew faster belonged to previously isolated bacterial species and mainly belonged to *Streptococcus bovis*. Therefore, we concentrated on the slow growing bacterial colonies on the plates. The 16S rRNA gene sequences of these slow growing bacterial species were analyzed using BLAST. The BLASTN (Altschul et al., 1997) analysis of the bacterial sequences identified 38 bacterial species with no hits to any known 16S rRNA gene sequence. These sequences with no know hit was analyzed using the "Classifier" (Wang et al., 2007) software program to classify the sequences and to identify the bacterial genus that these unknown bacterial species belong. The bacterial species classified using "classifier" (Wang et al., 2007) is shown in table 1. The previously uncultured bacterial species identified through BLASTN and "classifier", were compared against known bacterial species from the same genera to identify the differences of the new species against the isolates identified previously. Bootstrapped phylogenetic trees constructed for each genera using the 16S rRNA gene sequences from the known isolates and the new bacterial species identified during this study is shown in figures 2-7. Out of the 38 new bacterial species identified using the 16S rRNA sequence, 13 were selected for biochemical analysis to identify the fermentation patterns of these organisms. The fermentation patterns on 50 different carbohydrate substrates and results of the arginine and catalase tests are shown in table 2. The fermentation patterns of the bacterial species were different and within each genera (Table 2). The results of growth at 15°C and at 45°C and the gram staining are also included in table 2. A representative set of gram stain pictures are shown in figure 8.

Figure 2

Bootstrapped phylogenetic tree constructed for *Lactobacillus spp.* using the 16S rRNA gene sequences from the known isolates and the new bacterial species identified.

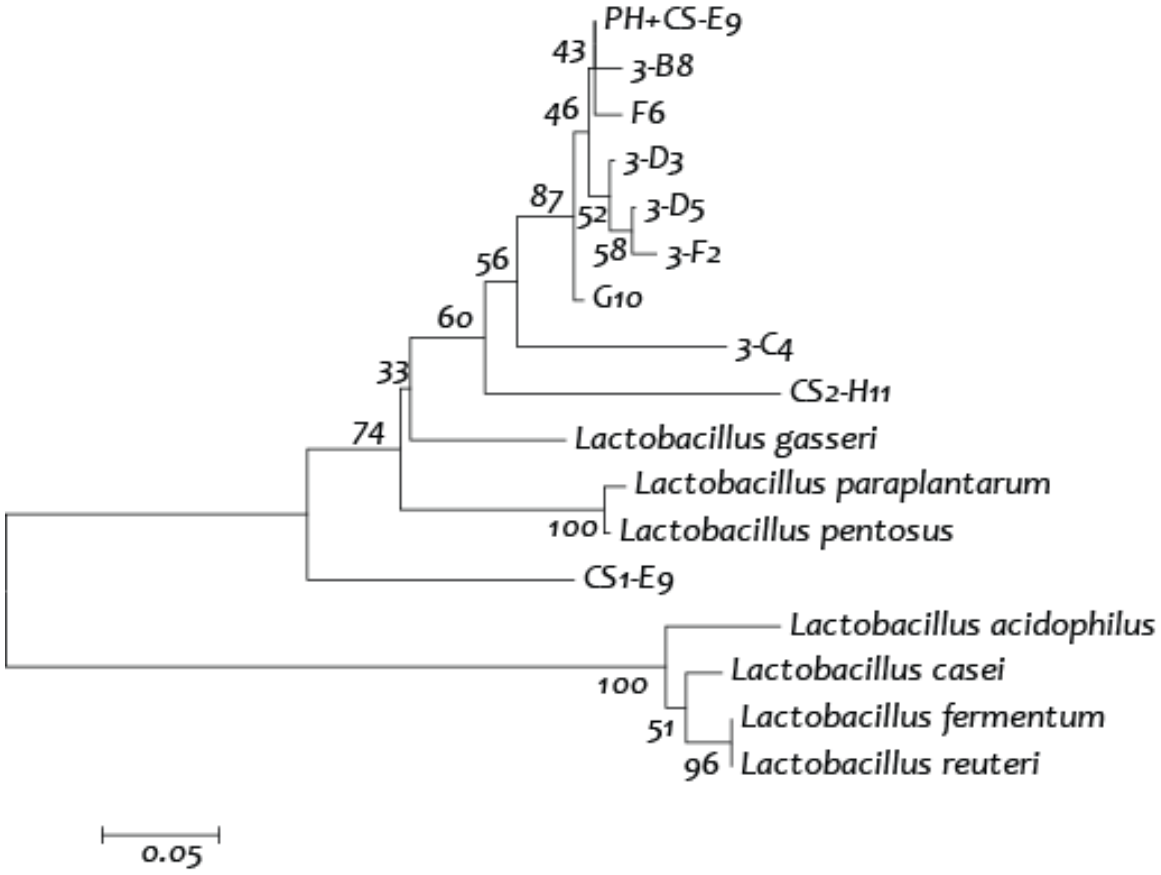

PH+CS-E9
43
3-B8
46
F6
3-D3
87
52
3-D5
58
3-F2
56
G10
60
3-C4
33
CS2-H11
74
Lactobacillus gasseri
Lactobacillus paraplantarum
100
Lactobacillus pentosus
CS1-E9
Lactobacillus acidophilus
100
Lactobacillus casei
51
Lactobacillus fermentum
96
Lactobacillus reuteri
0.05

Figure 3

Bootstrapped phylogenetic tree constructed for *Acinetobacter spp.* using the 16S rRNA gene sequences from the known isolates and the new bacterial species identified.

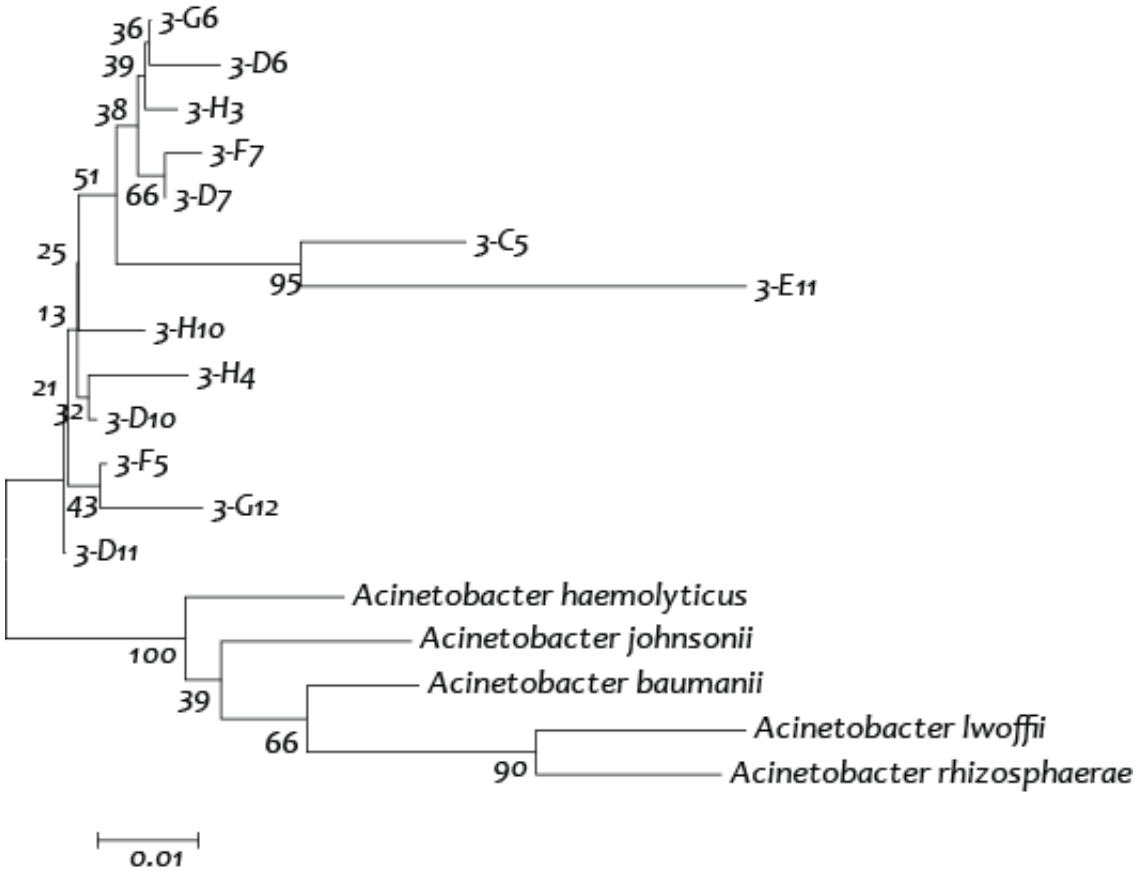

36
3-G6
39
3-D6
38
3-H3
3-F7
51
66
3-D7
25
3-C5
95
3-E11
13
3-H10
3-H4
21
32
3-D10
3-F5
43
3-G12
3-D11
Acinetobacter haemolyticus
Acinetobacter johnsonii
100
Acinetobacter baumanii
39
Acinetobacter lwoffii
66
90
Acinetobacter rhizosphaerae
0.01

Figure 4

Bootstrapped phylogenetic tree constructed for *Streptococcus spp.* using the 16S rRNA gene sequences from the known isolates and the new bacterial species identified.

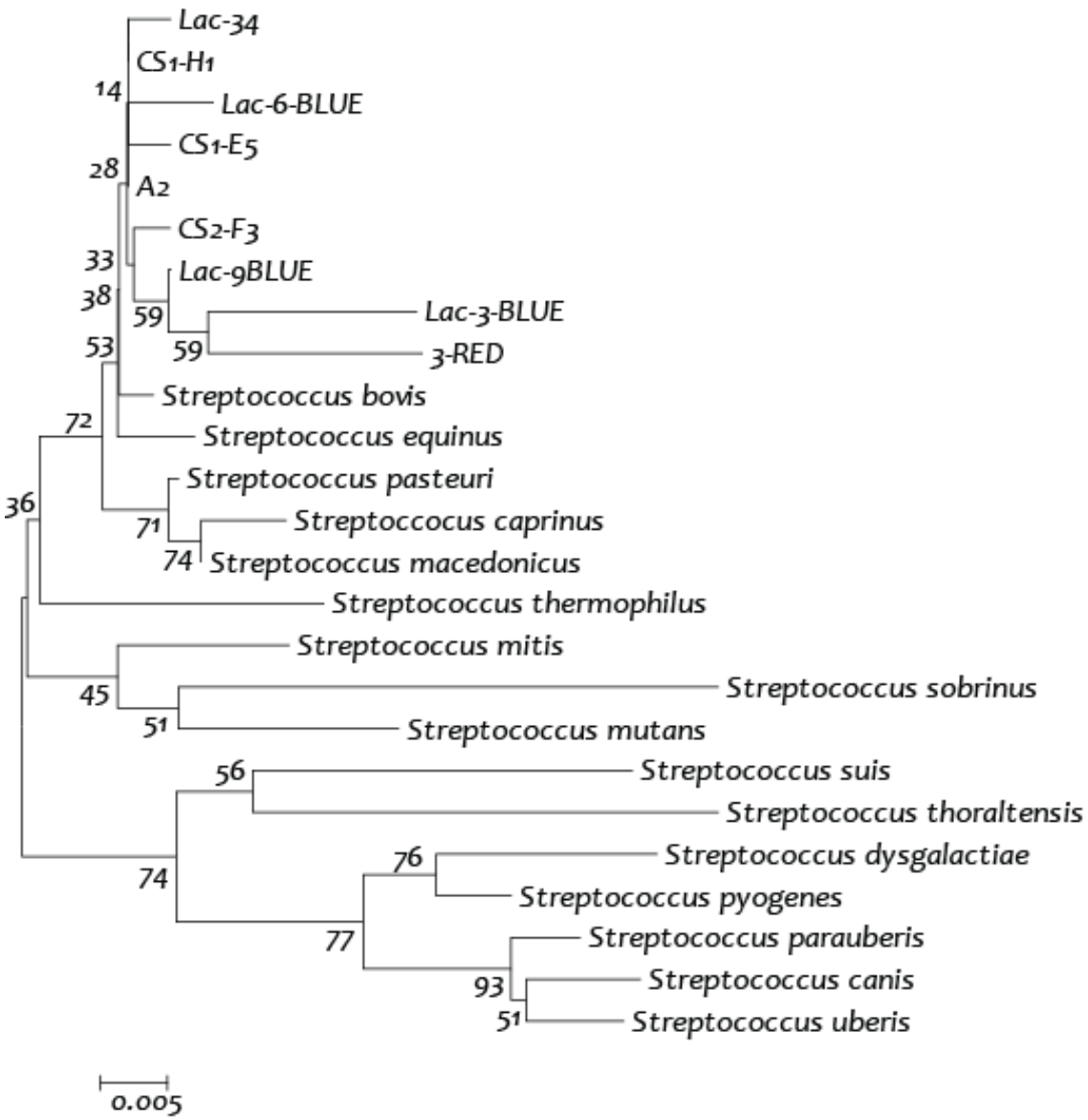

Lac-34
CS1-H1
14
Lac-6-BLUE
CS1-E5
28
A2
CS2-F3
33
Lac-9BLUE
38
59
Lac-3-BLUE
53
59
3-RED
Streptococcus bovis
72
Streptococcus equinus
Streptococcus pasteuri
36
71
Streptoccocus caprinus
74
Streptococcus macedonicus
Streptococcus thermophilus
Streptococcus mitis
45
Streptococcus sobrinus
51
Streptococcus mutans
56
Streptococcus suis
Streptococcus thoraltensis
76
Streptococcus dysgalactiae
74
Streptococcus pyogenes
77
Streptococcus parauberis
93
Streptococcus canis
51
Streptococcus uberis
0.005

Figure 5

Bootstrapped phylogenetic tree constructed for *Bifidobacterium spp.* using the 16S rRNA gene sequences from the known isolates and the new bacterial species identified.

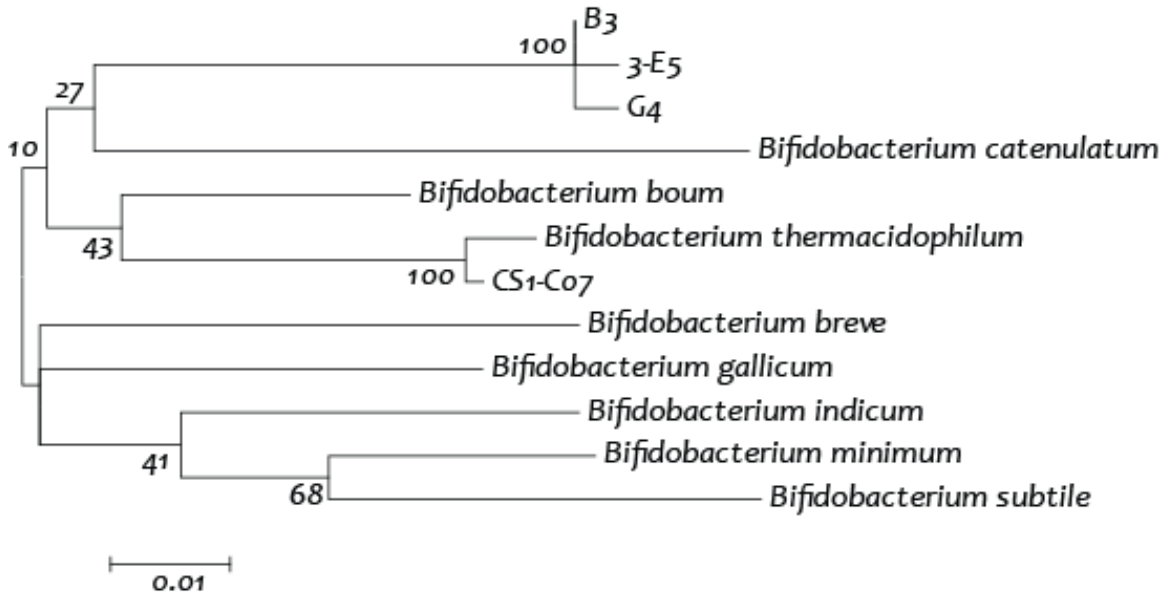

B3
100
3-E5
27
G4
10
Bifidobacterium catenulatum
Bifidobacterium boum
Bifidobacterium thermacidophilum
43
100
CS1-C07
Bifidobacterium breve
Bifidobacterium gallicum
Bifidobacterium indicum
41
Bifidobacterium minimum
68
Bifidobacterium subtile
0.01

Figure 6

Bootstrapped phylogenetic tree constructed for *Comamonas spp.* using the 16S rRNA gene sequences from the known isolates and the new bacterial species identified.

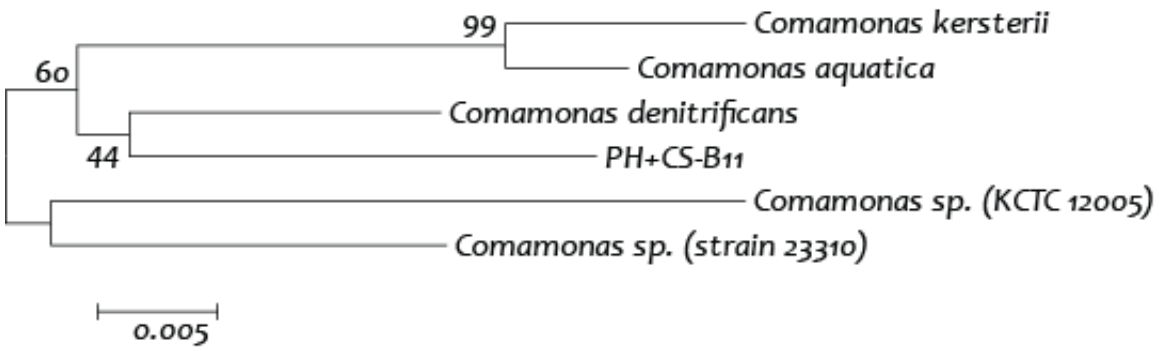

99
Comamonas kersterii
Comamonas aquatica
60
Comamonas denitrificans
44
PH+CS-B11
Comamonas sp. (KCTC 12005)
Comamonas sp. (strain 23310)
0.005

Figure 7

Bootstrapped phylogenetic tree constructed for *Sphingomonas spp.* using the 16S rRNA gene sequences from the known isolates and the new bacterial species identified.

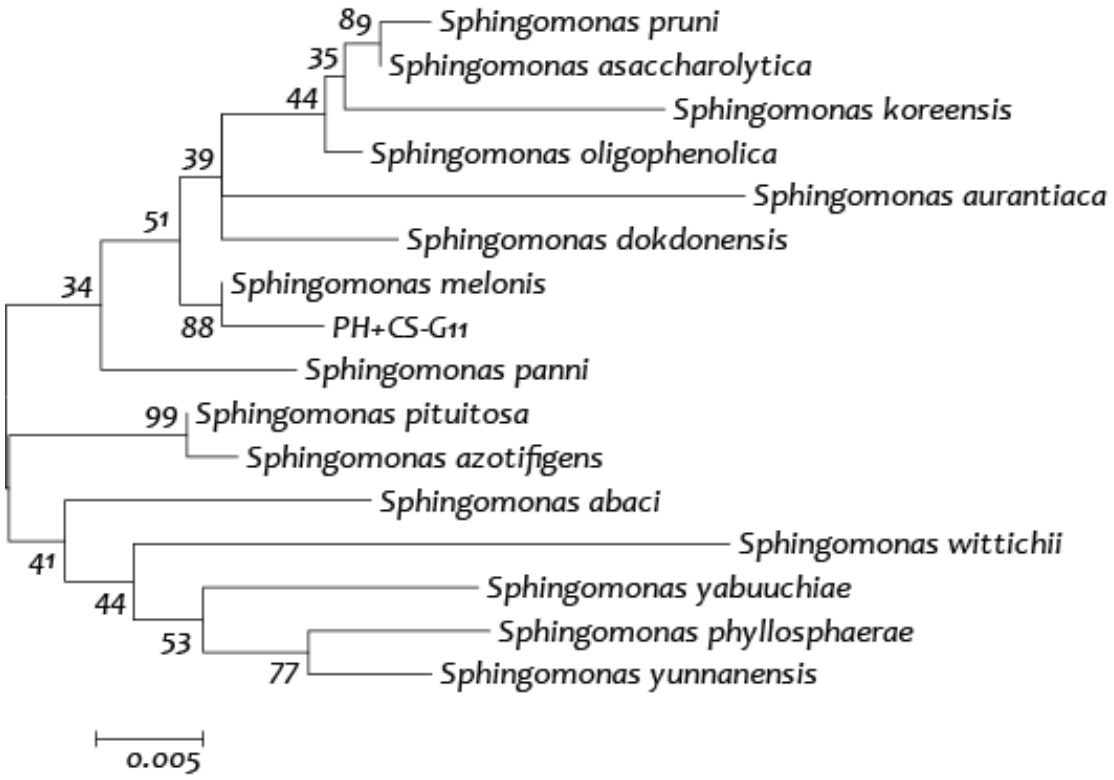
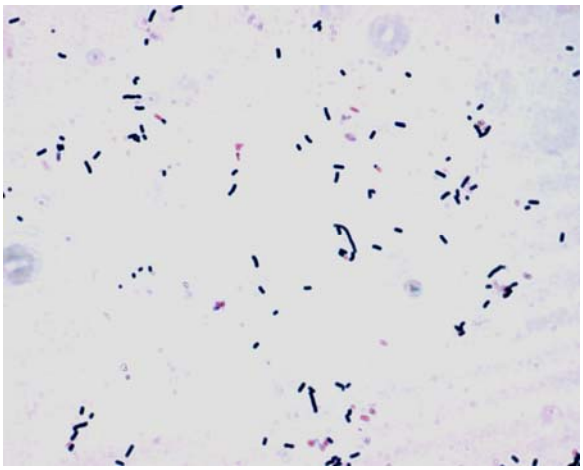
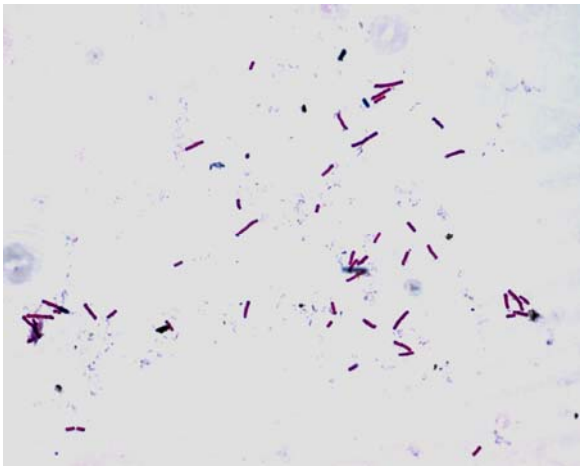

89
Sphingomonas pruni
35
Sphingomonas asaccharolytica
44
Sphingomonas koreensis
Sphingomonas oligophenolica
39
Sphingomonas aurantiaca
51
Sphingomonas dokdonensis
34
Sphingomonas melonis
88
PH+CS-G11
Sphingomonas panni
99
Sphingomonas pituitosa
Sphingomonas azotifigens
Sphingomonas abaci
Sphingomonas wittichii
41
44
Sphingomonas yabuuchiae
53
Sphingomonas phyllosphaerae
77
Sphingomonas yunnanensis
0.005

Figure 8

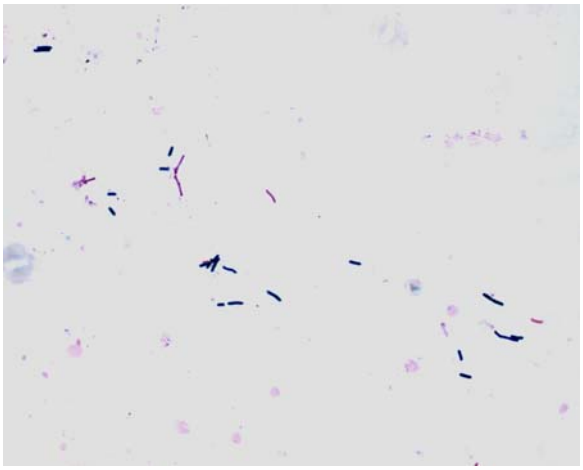
Representative set of gram stains from a selected set of new isolates.



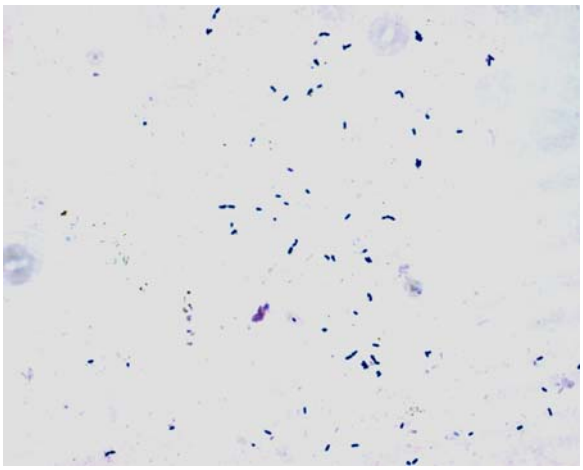
PH+CS-E9 (Lactobacillus sp.)



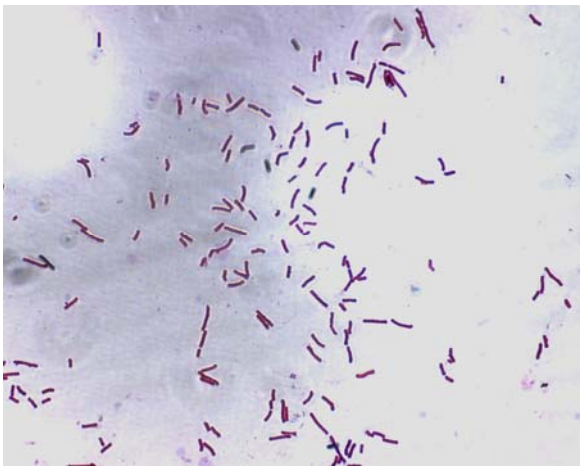
PH+CS-B11 (Comamonas sp.)



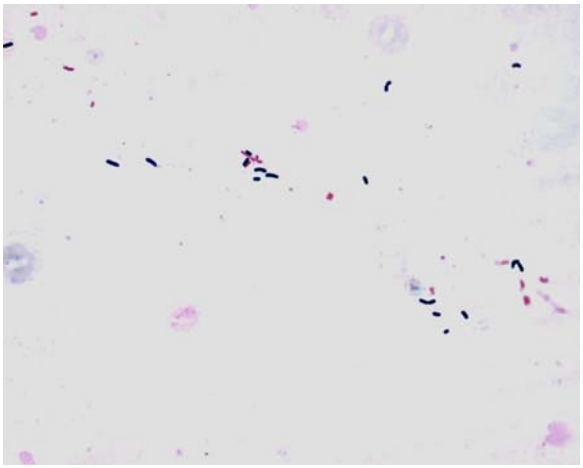
CS1-f9 (Lactobacillus sp.)



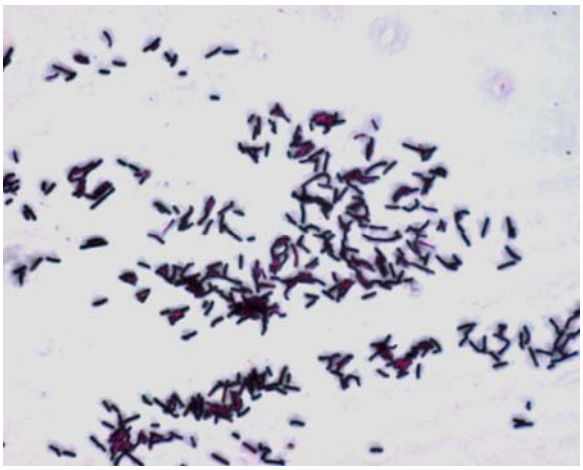
CS1-C7 (Bifidobacterium sp.)



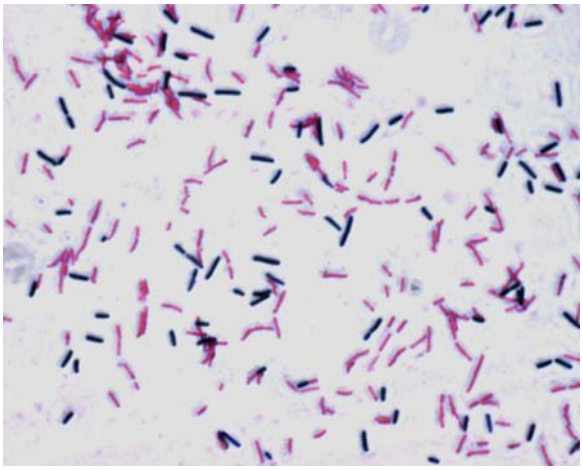
CS1-E9 (Lactobacillus sp.)



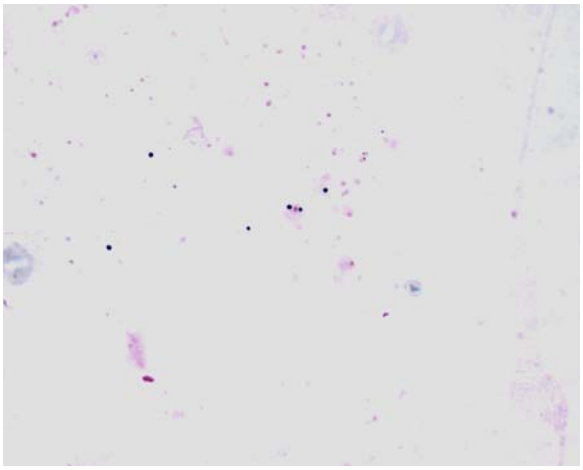
3-B8 (Lactobacillus sp.)



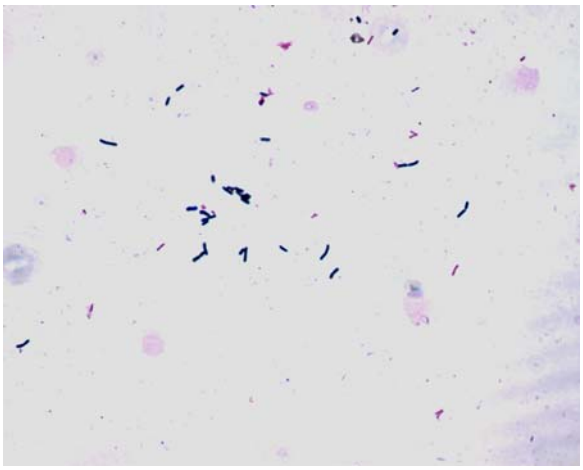
CS2-H11 (Lactobacillus sp.)



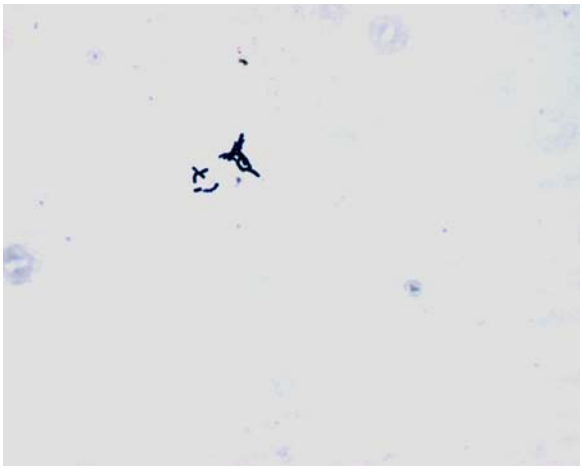
CS1-E5 (Streptococcus sp.)



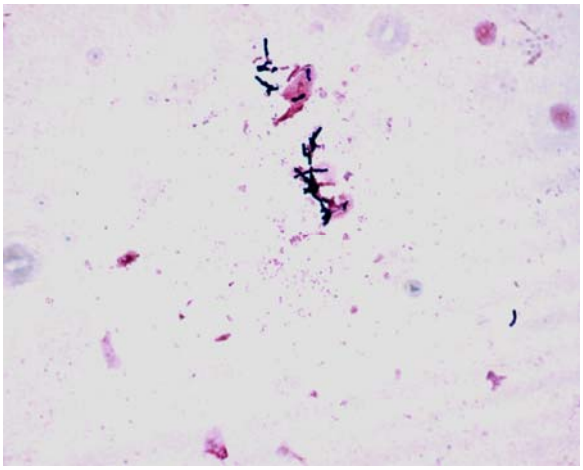
3-H4 (Acinetobacter sp.)



PHCS-G11 (Spingomonas sp.)



3-F5 (Acinetobacter sp.)



3-F4 (Bifidobacterium sp.)

Table 1. New Isolates identified based on 16S sequence analysis. The sequences were classified using the “Classifier” software analysis program as described in materials and methods.

Lactobacillus sp.	***Streptococcus sp.***
PH+CS-E9	*Lac-34*
CS2-H11	*CS1-H01*
CS1-E9	*Lac-6-Blue*
3-B08	*CS1-E05*
3-D03	*A02*
3-D05	*CS2-F03*
3-F02	*Lac-9-Blue*
3-C04	*Lac-3-11*
G10	*Red-03*
F06	

Acinetobacter sp.	***Bifidobacterium sp.***
3-G06	*3-B03*
3-D06	*3-E05*
3-H03	*G04*
3-H07	*CS1-C07*
3-D07	
3-C05	***Comamonas sp.***
3-E11	
3-H10	*PH+CS-B11*
3-H04	
3-D10	***Sphingomonas sp.***
3-F05	
3-G12	*PH+CS-G11*
3-D11	

Table 2. API-CH 50 analysis results.

Tube / Substrate	CS1-E9	CS2-H11	PH+CS-E9	3-B8	F-06
	Lactobacillus	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*
0 CONTROL					
1 GLYcerol					
2 ERYthritol					
3 D ARAbinose				+	
4 L ARAbinose	+	+	+	+	+
5 RIBose	+	+	+	+	+
6 D XYLose	+	+	+	+	+
7 L XYLose					
8 ADOnitol					
9 β-Methyl-D-Xyloside	+	+	+/-	+	+
10 GALactose	+	+	+/-	+	+
11 GLUcose	+	+	+	+	+
12 FRUctose	+	+	+/-	+/-	+/-
13 MaNnosE		+			
14 SorBosE					
15 RHAmnose					
16 DULcitol					
17 INOsitol					
18 MANnitol					
19 SORbitol					
20 α-Methyl-D-Mannoside					
21 α-Methyl-D-Glucoside					
22 N-Acetyl-Glucosamine	+/-	+/-			
23 AMYgdalin					+/-
24 ARButin					
25 ESCulin			+	+	+
26 SALicin					
27 CELlobiose	+	+			
28 MALtose	+	+		+	+/-
29 LACtose	+	+	+/-	+	+
30 MELibiose	+	+	+/-	+	+
31 Sucrose	+	+		+/-	+/-
32 TREhalose					
33 INUlin					
34 MeLeZitose					
35 RAFfinose	+	+		+/-	+/-
36 Starch	+	+			
37 GLYcoGen	+	+			
38 XyLiTol					
39 GENtiobiose					
40 D TURanose					
41 D LYXose					

42 D TAGatose					
43 D FUCose					
44 L FUCose					
45 D ARabitoL					
46 L ARabitoL					
47 GlucoNaTe					
48 2-Keto-Gluconate					
49 5-Keto-Gluconate					
Arginine Test	-	-	+		
Catalase Test	-	-	-	-	-
Growth at 15°C	-	-	-		
Growth at 45°C	+	+	+	+	+
Gram Stain	+	+	+		

Tube / Substrate	CS1-C7	3-G4	PH+CS-G11	PH+CS-B11
	Bifidobacterium	*Bifidobacterium*	*sphingomonas*	*comamonas*
0 CONTROL				
1 GLYcerol				
2 ERYthritol				
3 D ARAbinose				
4 L ARAbinose		+	+	+
5 RIBose		+	+	+
6 D XYLose		+	+	+
7 L XYLose				
8 ADOnitol				
9 β-Methyl-D-Xyloside		+	+	
10 GALactose	+	+	+/-	+/-
11 GLUcose	+	+	+	+
12 FRUctose	+	+	+/-	+/-
13 MaNnosE				
14 SorBosE				
15 RHAmnose				
16 DULcitol				
17 INOsitol				
18 MANnitol				
19 SORbitol				
20 α-Methyl-D-Mannoside				
21 α-Methyl-D-Glucoside	+			
22 N-Acetyl-Glucosamine				
23 AMYgdalin				
24 ARButin	+/-	+/-		
25 ESCulin	+	+	+	+
26 SALicin	+/-			
27 CELlobiose				
28 MALtose	+	+	+	+/-
29 LACtose		+	+	+
30 MELibiose	+	+	+	+
31 Sucrose	+	+/-	+/-	
32 TREhalose				
33 INUlin				
34 MeLeZitose				
35 RAFfinose	+	+/-	+/-	
36 Starch	+	+		
37 GLYcoGen	+	+/-		
38 XyLiTol				
39 GENtiobiose				
40 D TURanose	+			
41 D LYXose				
42 D TAGatose				

43 D FUCose				
44 L FUCose				
45 D ARabitoL				
46 L ARabitoL				
47 GlucoNaTe				
48 2-Keto-Gluconate				
49 5-Keto-Gluconate				
Arginine Test	-		+	-
Catalase Test	-	-	-	-
Growth at 15°C	-		-	-
Growth at 45°C	+	+	+	+
Gram Stain	+		+	+

Tube / Substrate	3-H3	3-H4	CS1-E5	CS1-H1
	Acinetobacter	*Acinetobacter*	*Streptococcus*	*Streptococcus*
0 CONTROL				
1 GLYcerol				
2 ERYthritol				
3 D ARAbinose				
4 L ARAbinose	+			
5 RIBose	+			
6 D XYLose	+			
7 L XYLose				
8 ADOnitol				
9 β-Methyl-D-Xyloside	+			
10 GALactose	+/-			+
11 GLUcose	+	+/-	+	+
12 FRUctose	+/-	+/-		+
13 MaNnosE				+
14 SorBosE				
15 RHAmnose				
16 DULcitol				
17 INOsitol				
18 MANnitol				
19 SORbitol				
20 α-Methyl-D-Mannoside				
21 α-Methyl-D-Glucoside				
22 N-Acetyl-Glucosamine				+/-
23 AMYgdalin				
24 ARButin				
25 ESCulin	+			
26 SALicin				
27 CELlobiose				+
28 MALtose	+		+	+
29 LACtose	+			+
30 MELibiose	+			
31 Sucrose		+/-	+/-	+
32 TREhalose				
33 INUlin				
34 MeLeZitose				
35 RAFfinose				+
36 Starch			+/-	+
37 GLYcoGen			+/-	+
38 XyLiTol				
39 GENtiobiose				
40 D TURanose				
41 D LYXose				
42 D TAGatose				

43 D FUCose				
44 L FUCose				
45 D ARabitoL				
46 L ARabitoL				
47 GlucoNaTe				
48 2-Keto-Gluconate				
49 5-Keto-Gluconate				
Arginine Test			-	
Catalase Test	-	+	-	
Growth at 15°C			-	-
Growth at 45°C	+	+	+	+
Gram Stain			+	+

Discussion

Early studies of microbial populations in environments were carried out using culture dependent techniques (Pace, 1997). However, several studies that have estimated microbial diversity based on microscopic observations suggests that 99% of the microbes found in most environments are resistant to in-vitro culture (Pace, 1997). Recent meta-genome and 16S sequencing projects (Fernando et al., 2008c; Fernando et al., 2008a; Fernando et al., 2008d) of environmental samples have provided valuable information into the phylogenetic diversity of microbial species and their capabilities in different environments (Schloss and Handelsman, 2005; Tringe et al., 2005; Tyson et al., 2004; Venter et al., 2004). However, it has been difficult to identify the microbial species that contain such capabilities due to the inability to grow them in pure culture. Nevertheless, having the organism in pure culture is of critical importance to identify the functional role of the organism in a given environment and to identify how the organism adapts to changes in the environments.

Within the rumen, recent studies using molecular techniques (Fernando et al., 2008d; Fernando et al., 2008a; Fernando et al., 2008c; Tajima et al., 1999) have displayed large diversity of microbial species and have suggested that the microbial diversity is far greater than anticipated. A recent EST study carried out by Fernando et. *al.* 2008 (Fernando et al., 2008b) on rumen microbial populations, identified a large number of un-annotated EST sequences also suggesting there is a large number of unknown bacterial species within the rumen microbial community. Therefore, in this study we use a new screening strategy to successfully culture new bacterial species from the rumen, and display the fermentation capabilities of a selected set of bacterial species isolated.

The high throughput screening process using the 16S rRNA gene enabled us to ramp up the screening process. This 16S rRNA based screening approach for uncultured bacteria identified 38 different bacterial species. Further analysis of the 16S sequences using “Classifier” (Wang et al., 2007) revealed that the sequences belonged to genus *Lactobacillus (10), Streptococcus (9), Bifidobacterium (4), Acinetobacter (14), Sphingomonas (1),* and *Comamonas (1) (*Table 1). Phylogenetic analyses were performed on the new isolates to identify the relatedness to previously cultured bacterial species that belonged to the same genus. The phylogenetic analysis of the *Lactobaciullus sp.* revealed that a majority of the new bacterial species were distant from known *Lactobacillus sp.* The bacterial species labelled as CS1-E9 was closely related to *Lactobacillus pentosus* and *Lactobacillus paraplantarum* than any other species. *Lactobacillus pentosus* and *Lactobacillus paraplantarum* has been reported to play a role in fermentation of pentoses such as xylose and arabinose (Rabinowitz, 1959), suggesting that the new isolate CS1-E9 may also play a role in pentose metabolism. This new bacterial species was isolated from the rumen of animals on high concentrate diet which has high amount os pentoses and hexoses. Therefore, isolate CS1-E9 may have better carbohydrate fermentation capabilities. Isolates CS2-H11 and 3-C4 were distantly related to other new isolates, but were closer to the new isolates than known isolates. The remaining new *Lactobacillus spp.* were closely related to each other than any known *Lactobacillus sp*. The gram stains generated for the *Lactobacillus sp*. were all gram positive but differed in size. The bacterial cells of isolate CS2-H11 and CS1-E9 were larger than the cells observed for CS1-F9, 3-B8 and PH+CS-E9. The *Acinetobacter* species identified were distant from known isolates of *Acinetobacter sp.*

According to the Bergey's manual of systematic bacteriology (Garrity et al., 2005a), *Acinetobacter spp.* are considered as strict aerobes and are defined as gram variable. We did our incubations of these *Acinetobacter* isolates under anaerobic conditions. Therefore this may be the first report of anaerobic *Acinetobacter spp.* Phylogenetic analysis of the *Acinetobacter spp.* displayed that these isolates were distant from known *Acinetobacter* isolates, where isolates 3-C5, 3-E11, 3-G12, 3-H4, and 3-H10 were more distant than others. The gram stains for the *Acinetobacter spp.* displayed that these isolates were gram positive, but were morphologically variable as we detected *Acinetobacter sp.* belonging to both cocci and rods. The *Acinetobacter sp*. did not grow well in the absence of rumen fluid and the growth rate was relatively slow. Similar to the *lactobacillus spp.* and *Acinetobacter spp*., the *Streptococcus* isolates were clustered together and were away from the known bacterial isolates. Among the isolates identified, Lac-3-Blue and 3-RED were more distant from other isolates but were more closely related to each other. Interestingly, although *Streptococcus spp.* are expected to have a cocci morphology, isolate CS1-E5 displayed a rod shaped morphology. This observation was puzzling to us, but re-sequencing and analysis of the full length 16S rRNA gene confirmed that this rod shaped bacterium belonged to genus *Streptococcus.* Out of the four *Bifidobacterium* isolates identified, isolate CS1-C7 was closely related to *Bifidobacterium thermacidophilum*, while the closest to the other three isolates is *Bifidobacterium catenulatum*. *Bifidobacterium* species are known to be hexose fermenters and use the fructose-6-phosphate pathway in carbohydrate metabolism (Ventura et al., 2004). The Bifidobacteria we isolated were from animals on high concentrate which is high in hexoses, suggesting that the new isolates may also be

good carbohydrate fermenters. Among the gram stains for *Bifidobacterium spp.* the cell sizes were different for the different isolates, where 3-F4 was larger than CS1-C7. Only one *Comamonas spp*. was identified during this study. The phylogenetic analysis of this isolate against the 16S rRNA sequences available at NCBI revealed that this isolate was closer to *comamonas denitrificans*. The gram stain revealed that this *comamonas spp*. Is a gram positive rod shaped organism. The *sphingomonas spp.* Isolated was close to *S. melonis*. The gram stain of this isolate revealed that it is a gram positive rod. Out of the 38 new isolates, 13 Isolates were selected and were used to evaluate the fermentation patterns and the substrate specificity. The substrate specificity and fermentation patterns were evaluated using the API-CH 50 substrate kits. The fermentation substrates of *Lactobacillus* isolates were different among the isolates. Isolate CS1-E9 and CS2-H11 displayed similar substrate utilization patterns, but only CS2-H11 fermented D-manose, while only isolate 3-B8 fermented arabinose. Although, isolates CS1-E9 and CS2-H11 displayed similar fermentation patterns, these two organisms were phylogenetically distant based on the 16S rRNA sequences, suggesting that biochemical testing alone may not help identify bacterial species (Boyd et al., 2005). However, Lactobacillus isolates PH+CS-E9, 3-B8, and F-06 had fermentation patterns, where D-arabinose, L-Arabinose, ribose, xylose, β-Methyl-D-Xyloside, galactose, glucose, fructose, ferric citrate, maltose, lactose, melibiose, sucrose, and raffinose were utilized at different levels. Isolate (PH+CS-E9) utilized less substrates than the rest of the *Lactobacills* isolates. This difference in fermentation substrates is consistent with the 16S rRNA based phylogenetic tree which shows that the isolates tested were different bacterial species. The fermentation pattern of the two

Streptococcus species analyzed using the API kit displayed significant differences. Isolate CS1-H1 fermented an array of substrates which included, galactose, glucose, fructose, manose, celibiose, maltose, lactose, sucrose, rafinose, starch, and glycogen. However, isolate CS1-E5 had a narrower range of substrate utilization, and utilized glucose, maltose, sucrose, starch and glycogen. Biochemical testing performed on the *Acinetobacter spp.* displayed fermentation capabilities of only a few different substrates. This may be due to the slow growth rates of *Acinetobacter spp.* and/or because the API kit we utilized is designed for *Lactobacillus sp.,* and *Bifidobacterium sp*. We hope to use the BioLog (Biolog, Inc. Hayward, CA) to evaluate the fermentation patterns of *Acinetobacter* species in the future. The *Bifidobacterium* isolates evaluated (CS1-C7) using the API kit utilized galactose, glucose, fructose, α-Methyl-D-Glucoside, ferric citrate, maltose, melibiose, sucrose, rafinose, starch glycogen, and turansoe. However *Bifidobacterium* isolate 3-G4 fermented many more substrates which included arabinose, ribose xylose, β-Methyl-D-Xyloside, glalactose, glucose, fructose, ferric citrate, maltose, lactose, and melibiose. These two isolates (CS1-C7 and 3-G4) were phylogenetically distant from each other. However, CS1-C7 was closely related to *Bifidobacterium thermacidophilum* . *Bifidobacterium thermacidophilum,* is known to utilize arabinose, galactose, Methyl-AD-glucopyranoside, amygladin. Arbutin, ferric citrate, and salicin (von Ah et al., 2007), which displays a different fermentation pattern than the new isolate. The *Sphingomonas* isolate utilized arabinose, ribose, xylose, β-Methyl-D-Xyloside, glucose, ferric citrate, maltose, lactatose, and melibiose while the *Comamonas* isolate displayed a similar fermentation pattern but did not utilize β-Methyl-D-Xyloside.

In this investigation we have isolated several new bacterial isolates from the rumen. Recently, *Lactobacillus* and *Bifidobacterium* species have been utilized as probiotics in animals and humans (Leahy et al., 2005). Thus, our new isolates may have the potential of being used as probiotics in cattle and needs further investigation. The *Acinetobacter* species identified are the first to be reported that can grow anaerobically, our investigation detected large amounts of *Acinetobacter* in our screening process suggesting that, they may have a significant role within the rumen. We believe that this new high throughput screening strategy for culturing new bacterial isolates together with the media formulation from the original source provides the opportunity to increase the efficiency of culturing new bacterial species from any given environment.

Reference List

(2001) *Bergey's manual of Systematic Bacteriology*. New York: Springer.

(2005) *Bergey's Manual of Systematic Bacteriology: The Proteobacteria; Introductory essays*. Springer.

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

Boyd,M.A., Antonio,M.A.D., and Hillier,S.L. (2005) Comparison of API 50 CH strips to whole-chromosomal DNA probes for identification of Lactobacillus species. *Journal of Clinical Microbiology* **43:** 5309-5311.

Bryant,M.P. and Robinson,I.M. (1961) An Improved Nonselective Culture Medium for Ruminal Bacteria and Its Use in Determining Diurnal Variation in Numbers of Bacteria in Rumen. *Journal of Dairy Science* **44:** 1446-&.

Cole,J.R., Chai,B., Farris,R.J., Wang,Q., Kulam,S.A., McGarrell,D.M. et al. (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Research* **33:** D294-D296.

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

Connon,S.A. and Giovannoni,S.J. (2002) High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Applied and Environmental Microbiology* **68:** 3878-3885.

DeSilva,U., Guo,X., Kupfer,D.M., Fernando,S.C., Pillai,A.T.V., Najar,F.Z. et al. (2003) Allelic variants of ovine prion protein gene (PRNP) in Oklahoma sheep. *Cytogenetic and Genome Research* **102:** 89-94.

Ferguson,R.L., Buckley,E.N., and Palumbo,A.V. (1984) Response of Marine Bacterioplankton to Differential Filtration and Confinement. *Applied and Environmental Microbiology* **47:** 49-55.

Fernando,S.C., Horn,G.W., Purvis II,H.T., Najar,F.Z., Sukharnikov,L., Nagaraja,T.G. et al. Bacterial population dynamics during ionophore treatment. 2008a.
Ref Type: Unpublished Work

Fernando,S.C., Najar,F.Z., Sukharnikov,L., Wiley,G., Macmil,S., Krehbiel,C.R. et al. Meta-functional Genomics of the bovine rumen. 2008b.
Ref Type: Unpublished Work

Fernando,S.C., Purvis II,H.T., Najar,F.Z., Sukharnikov,L., Nagaraja,T.G., Krehbiel,C.R. et al. Rumen microbial changes during adaptation to high concentrate diet. 2008c. Ref Type: Unpublished Work

Fernando,S.C., Purvis II,H.T., Najar,F.Z., Sukharnikov,L., Nagaraja,T.G., Krehbiel,C.R. et al. Rumen microbial population dynamics during sub acute acidosis. 2008d. Ref Type: Unpublished Work

Gerhardt,P., Murray,R.G.E., Wood,W.A., and Krieg,N.R. (1994) *Methods of General and Molecular Microbiology*. Washington, DC: American Society of Microbiology.

Goad,D.W., Goad,C.L., and Nagaraja,T.G. (1998) Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. *Journal of Animal Science* **76:** 234-241.

Grubb,J.A. and Dehority,B.A. (1975) Effects of An Abrupt Change in Ration from All Roughage to High Concentrate Upon Rumen Microbial Numbers in Sheep. *Applied Microbiology* **30:** 404-412.

Hungate,R.E. (1968) Ruminal fermentation. In *Handbook of Physiology*. Code,C.F. (ed). Washington, D.C.: American Physiology Society, pp. 2725-2745.

Hungate,R.E., Bryant,M.P., and Mah,R.A. (1964) The rumen bacteria and protozoa. *Annual Review of Microbiology* **18:** 131-&.

Hungate,R.E., Dougherty,R.W., Bryant,M.P., and Cello,R.M. (1952) Microbiological and Physiological Changes Associated with Acute Indigestion in Sheep. *Cornell Veterinarian* **42:** 423-449.

Kaeberlein,T., Lewis,K., and Epstein,S.S. (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* **296:** 1127-1129.

Leahy,S.C., Higgins,D.G., Fitzgerald,G.F., and van Sinderen,D. (2005) Getting better with bifidobacteria. *Journal of Applied Microbiology* **98:** 1303-1315.

Maki,L.R. and Foster,E.M. (1957) Effect of Roughage in the Bovine Ration on Types of Bacteria in the Rumen. *Journal of Dairy Science* **40:** 905-913.

Mccoy,S. and Gilliland,S.E. (2007) Isolation and characterization of Lactobacillus species having potential for use as probiotic cultures for dogs. *Journal of Food Science* **72:** M94-M97.

Pace,N.R. (1997) A molecular view of microbial diversity and the biosphere. *Science* **276:** 734-740.

Rabinowitz,J.C. (1959) Fermentative Metabolism. *Annual Review of Microbiology* **13:** 441-464.

Rappe,M.S., Connon,S.A., Vergin,K.L., and Giovannoni,S.J. (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418:** 630-633.

Sambrook,J. and Russel,D.W. (2001) *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schloss,P.D. and Handelsman,J. (2005) Metagenomics for studying unculturable microorganisms: cutting the Gordian knot. *Genome Biology* **6**.

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

Tamura,K., Dudley,J., Nei,M., and Kumar,S. (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24:** 1596-1599.

Tringe,S.G., von Mering,C., Kobayashi,A., Salamov,A.A., Chen,K., Chang,H.W. et al. (2005) Comparative metagenomics of microbial communities. *Science* **308:** 554-557.

Tyson,G.W., Chapman,J., Hugenholtz,P., Allen,E.E., Ram,R.J., Richardson,P.M. et al. (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428:** 37-43.

Venter,J.C., Remington,K., Heidelberg,J.F., Halpern,A.L., Rusch,D., Eisen,J.A. et al. (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304:** 66-74.

Ventura,M., van Sinderen,D., Fitzgerald,G.F., and Zink,R. (2004) Insights into the taxonomy, genetics and physiology of bifidobacteria. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **86:** 205-223.

von Ah,U., Mozzetti,V., Lacroix,C., Kheadr,E.E., Fliss,I., and Meile,L. (2007) Classification of a moderately oxygen-tolerant isolate from baby faeces as Bifidobacterium thermophilum. *Bmc Microbiology* **7**.

Wang,Q., Garrity,G.M., Tiedje,J.M., and Cole,J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73:** 5261-5267.

VITA

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Thesis: META-FUNCTIONAL GENOMICS OF THE RUMEN MICROBIOME

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2004–2008 Completed the requirements for the Doctor of Philosophy in Animal Genetics at Oklahoma State University, Stillwater, Oklahoma in May, 2008.

2002 – 2004 M.S. in Animal Genomics, Oklahoma State University, Stillwater, OK.
Dissertation : Isolation, Characterization, Mapping and Expression Analysis of Porcine Tissue Kallikreins

1998 – 2002 B.Sc. in Microbiology with first class honors, University of Kelaniya (Sri Lanka)
Special Project : Designing a Diagnostic Kit for the Detection of Citrus Greening Organism (*Liberobacter asiaticum*) using the ELISA Technique

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Pages in Study: 331 Candidate for the Degree of Doctor of Philosophy

Major Field: Animal Genomics

Scope and Method of Study:

Findings and Conclusions:

Rumen microbes play an important role in nutrient digestion. The synergistic relationship between the bacteria within the rumen and the animal provides ruminants with nutrients that are not available to monogastric animals. This relationship between the animal and rumen microbes is a complex relationship. Understanding this relationship and the functional role of these microbes within the rumen is of critical importance. However, the current understanding of the diversity and functional role of rumen bacterial species is very limited as most existing studies were based on culture dependent techniques while around 99.9% of the rumen bacteria are resistant to in-vitro culture. In an effort to better understand rumen microbial population diversity and function, we undertook a meta-genomics approach to analyze rumen biome. We have analyzed real-time population fluctuations within the rumen during the adaptation of an animal to a high concentrate diet from a forage-based diet, during ionophore treatment and also as an animal develops acidosis, an important metabolic disorder in cattle, using T-RFLP analyses, sequence analysis of 16S rDNA libraries and by real-time PCR analysis. We also performed a large-scale, community level transcriptome, analysis of the rumen microbes using a pyro-sequencing strategy combined with several bioinfomatic approaches. This helped us better understand the functional role of bacteria within communities as well as determine the rate of metabolic pathways within the rumen. Finally, we have in-vitro cultured and biochemically characterized several previously unknown rumen microbes.

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