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**Diversity of Bacterial and Archaeal Populations in Artificial Rumen
Fermentors Fed Differing Pasture Diets**

by

Smita Singh

**Thesis submitted to the
Davis College of Agriculture, Natural Resources, and Design
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Master of Science
in
Applied and Environmental Microbiology**

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**Division of Plant and Soil Sciences
Morgantown, West Virginia**

2012

**Keywords: Rumen; T-RFLP; Bacterial Diversity; Archaeal Diversity; Pasture; Teff;
Triticale.**

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ABSTRACT

Diversity of Bacterial and Archaeal Populations in Artificial Rumen Fermentors Fed Differing Pasture Diets

SMITA SINGH

Rumens harbor diverse groups of microorganisms. These microorganisms help ruminants in efficient digestion of a wide variety of feed materials. Most of these microorganisms are anaerobic and work symbiotically with the host. The amount of microbial diversity depends on the types of substrates present in the feed. Although some of these microorganisms have been cultured and characterized, the majority of them are unknown because they are unculturable. With the development of molecular techniques such as Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), Automated Ribosomal Intergenic Spacer Analysis (ARISA), Restriction Fragment Length Polymorphism (RFLP), Terminal Restriction Fragment Length Polymorphism (TRFLP) etc., now it is possible to quantify existing rumen microbial diversity, even if the microorganisms are not culturable. Previous studies on rumen ecosystems have shown that microbial populations generally vary with diet. A majority of these studies emphasized changes in bacterial communities in relation to grain diets, and less emphasis was given to traditional pasture diets which provide the main cattle food in several developing countries, and also on effect of changes in diets on archaeal communities in the rumen ecosystem. To understand bacterial and archaeal diversity associated with different pasture diets, I amplified bacterial and archaeal 16S rRNA genes from rumen samples using a universal FAM labeled forward primer and a non-labeled reverse primer, and then these products were digested with different tetrameric restriction enzymes. T-RFLP profiles obtained from *Hha* I and *Rsa* I digested fragments from open canopy (OC) and wooded canopy (WC) pasture diets suggested that bacterial and archaeal community structures were not significantly different in these pasture diets and that harvest time of these diets had no effect on bacterial and archaeal community structure. T-RFLP profiles obtained from *Hha* I, *Rsa* I, and *Hae* III digested fragments from naturalized pasture early cut (NPEC), naturalized pasture late cut (NPLC), teff, and tritcale diets showed highest bacterial and archaeal diversities in tritcale and nplc diets respectively. Diet pairs npec-teff and nplc-tritcale shared more bacterial and archaeal community, and bacterial and archaeal communities associated with diet pairs npec-nplc, npec-tritcale, nplc-teff, and teff-tritcale were significantly different. This study will help in better understanding of the microbial diversity in cattle which in turn will help in optimal utilization of a wide variety of feeds.

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LIST OF TABLES

Table 2.1 List of samples and their collection details. Each sample had one duplicate sample (not shown in table). Harvest 1, 2, 3 and 4 were conducted on 4/25/08, 5/5/08, 5/15/08, and 6/11/08 respectively.	13
Table 2.2 Table showing richness, diversity, and evenness indices of bacterial and archaeal communities associated with open and wooded canopy diets.	24
Table 3.1 List of samples and their collection details. Feed types: 1.) Naturalized pasture early cut (npec), 2.) Naturalized pasture late cut (nplc), 3.) Teff grass (teff), and 4.) Triticale (tri).	49
Table 3.2 Table showing richness, diversity, and evenness indices of bacterial and archaeal communities associated with naturalized pasture early cut (npec), teff, naturalized pasture late cut (nplc), and triticale (tri).	57

LIST OF FIGURES

Figure 2.1 <i>In-vitro</i> single-flow continuous culture system.	15
Figure 2.2 Agarose gel electrophoresis image of PCR products obtained using a.) bacterial universal primers and b.) archaeal universal primers. Lane L = 100 bp DNA ladder from New England Biolabs, lane N= Negative control. In figure 2.2a, lane1=R2F4, lane2=R2F4D, lane3=R1F12, lane4= R1F12D and in figure2.2b lane 1=R2F1, lane2=R2F1D, lane3=R2F2, lane 4=R2F2D, lane5= R2F3, lane6=R2F3D, lane7=R2F4, lane 8=R2F4D.	20
Figure 2.3 Venn diagram showing shared and unique bacterial T-RF's between open canopy (OC) and wooded canopy (WC) pasture diets a.) when digested with <i>Hha</i> I b.) when digested with <i>Rsa</i> I.	21
Figure 2.4 Comparison of <i>Hha</i> I digested bacterial T-RFLP profiles obtained from a single sample of open canopy (OC) and wooded canopy (WC) pasture diets. Red arrow highlight unique bacterial T-RF associated with wooden canopy pasture diets.....	22
Figure 2.5 Venn diagram showing shared and unique archaeal T-RF's between open canopy (OC) and wooded canopy (WC) pasture diets a.) when digested with <i>Hha</i> I b.) when digested with <i>Rsa</i> I.	23
Figure 2.6 Comparison of <i>Hha</i> I digested archaeal T-RFLP profiles obtained from a single sample of open canopy (OC) and wooded canopy (WC) pasture diets. Red arrow highlights unique archaeal T-RF's associated with OC and WC pasture diets.....	23
Figure 2.7 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree of bacterial communities associated with open and wooded canopy pasture. T-RF's generated from <i>Hha</i> I and <i>Rsa</i> I digestion were combined for construction of UPGMA tree.	26
Figure 2.8 UPGMA tree of archaeal communities associated with open and wooded canopy pasture. T-RF's generated from <i>Hha</i> I and <i>Rsa</i> I digestion were combined for construction of UPGMA tree.....	27

Figure 2.9 UPGMA tree of bacterial communities associated with different harvest time. T-RF's generated from <i>Hha</i> I and <i>Rsa</i> I digestion were combined for construction of UPGMA tree.	28
Figure 2.10 Unweighted UniFrac based clustering of bacterial communities associated with different harvest time. UPGMA tree from figure 2.9 was used as an input file for construction of UniFrac tree.	29
Figure 2.11 UPGMA tree of archaeal communities associated with different harvest time. T-RF's generated from <i>Hha</i> I and <i>Rsa</i> I digestion were combined for construction of UPGMA tree.	30
Figure 2.12 Unweighted UniFrac based clustering of archaeal communities associated with different harvest time. UPGMA tree from figure 2.11 was used as an input file for construction of UniFrac tree.	31
Figure 2.13 UPGMA tree of bacterial communities associated with different replications. T-RF's generated from <i>Hha</i> I and <i>Rsa</i> I digestion were combined for construction of the UPGMA tree.	32
Figure 2.14 Unweighted UniFrac based clustering of bacterial communities associated with different replications. UPGMA tree from figure 2.13 was used as an input file for construction of the UniFrac tree.	33
Figure 2.15 UPGMA tree of archaeal communities associated with different replications. T-RF's generated from <i>Hha</i> I and <i>Rsa</i> I digestion were combined for construction of the UPGMA tree.	34
Figure 2.16 Unweighted UniFrac based clustering of Archaeal communities associated with different replications. UPGMA tree from figure 2.15 was used as an input file for construction of the UniFrac tree.	35
Figure 2.17 Nonmetric multidimensional scaling plot of a.) bacterial and b.) archaeal communities associated with open canopy (oc) and wooded canopy (wc) diets. Presence/absence data of T-RF's generated from <i>Hha</i> I and <i>Rsa</i> I digested PCR products were used for construction of the NMDS plot.	37

Figure 2.18 Boxplot of ranked genetic dissimilarity for a.) bacterial and b.) archaeal communities associated with open and wooded canopy diets. Boxes represent median and interquartile ranges.....	38
Figure 2.19 Nonmetric multidimensional scaling plot of a.) bacterial and b.) archaeal communities associated with different harvest time. Presence/absence data of T-RF's generated from <i>Hha</i> I and <i>Rsa</i> I digested PCR products were used for construction of the NMDS plot.....	39
Figure 2.20 Boxplot of ranked genetic dissimilarity for a.) bacterial and b.) archaeal communities associated with different harvest time. Boxes represent median and interquartile ranges. H1= First harvest; H2=Second harvest; H3=Third harvest; H4=Fourth harvest.	40
Figure 2.21 Nonmetric multidimensional scaling plot of a.) bacterial and b.) archaeal communities associated with different replications. Presence/absence data of T-RF's generated from <i>Hha</i> I and <i>Rsa</i> I digested PCR products were used for construction of the NMDS plot.....	41
Figure 2.22 Boxplot of ranked genetic dissimilarity for a.) bacterial and b.) archaeal communities associated with different replications. Boxes represent median and interquartile ranges. R1= First replication; R2=Second replication; R3=Third replication.	42
Figure 3.1 Venn diagram showing shared and unique bacterial T-RF's between naturalized pasture early cut, naturalized pasture late cut, teff, and triticale pasture diets a.) when digested with <i>Hha</i> I, b.) when digested with <i>Rsa</i> I, and c.) when digested with <i>Hae</i> III.....	54
Figure 3.2 Comparison of <i>Hha</i> I digested bacterial T-RFLP profiles obtained from a single sample of npec, nplc, teff and triticale pasture diets. Red arrow highlights unique and shared bacterial T-RF's associated with different diets.	54
Figure 3.3 Venn diagram showing shared and unique archaeal T-RF's between naturalized pasture early cut (npec), naturalized pasture late cut (nplc), teff, and triticale	

pasture diets a.) when digested with *Hha* I, b.) when digested with *Rsa* I, and c.) when digested with *Hae* III..... 55

Figure 3.4 Comparison of *Hha* I digested archaeal T-RFLP profiles obtained from a single sample of naturalized pasture early cut (npec), naturalized pasture late cut (nplc), teff, and triticale pasture diets. Red arrow highlights unique and shared archaeal T-RF's associated with different diets. 55

Figure 3.5 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree of bacterial communities associated with different pasture diets. T-RF's generated from *Hha* I, *Rsa* I, and *Hae* III digestion were combined for construction of UPGMA tree. npec = naturalized pasture early cut, nplc = naturalized pasture late cut, teff = teff grass, and tri = triticale..... 58

Figure 3.6 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree of archaeal communities associated with different pasture diets. T-RF's generated from *Hha* I, *Rsa* I, and *Hae* III digestion were combined for construction of UPGMA tree. npec = naturalized pasture early cut, nplc = naturalized pasture late cut, teff = teff grass, and tri = triticale..... 59

Figure 3.7 Unweighted UniFrac based clustering of bacterial communities associated with different diets. UPGMA tree from figure 3.5 was used as an input file for construction of UniFrac tree. 60

Figure 3.8 Unweighted UniFrac based clustering of archaeal communities associated with different diets. UPGMA tree from figure 3.6 was used as an input file for construction of UniFrac tree. 61

Figure 3.9 Nonmetric multidimensional scaling plot of a.) bacterial and b.) archaeal communities associated with npec (naturalized pasture early cut), nplc (naturalized pasture late cut), teff (teff grass), and tri (triticale) pasture diets. Presence/absence data of T-RF's generated from *Hha* I, *Rsa* I and *Hae* III digested PCR products were used for construction of the NMDS plot..... 62

Figure 3.10 Boxplot of ranked genetic dissimilarity for a.) bacterial and b.) archaeal communities associated with npec (naturalized pasture early cut), nplc (naturalized

pasture late cut), teff (teff grass), and tri (triticale) pasture diets. Boxes represent median and interquartile ranges. Circle above box represent outliers..... 63

TABLE OF CONTENTS

ABSTRACT	II
ACKNOWLEDGEMENTS	III
LIST OF TABLES	IV
LIST OF FIGURES.....	V
TABLE OF CONTENTS.....	X
CHAPTER 1: INTRODUCTION AND OBJECTIVES.....	1
1.1 INTRODUCTION	1
1.1.1 Ruminant digestion	1
1.1.2 Ruminant diets	2
1.1.3 Rumen microbial diversity	2
1.1.4 In-vivo versus in-vitro ruminal fermentors	5
1.1.5 Molecular techniques for the estimation of microbial diversity	5
1.2 RATIONALE	8
1.3 OBJECTIVES	8
1.4 HYPOTHESES	9
CHAPTER 2: DIVERSITY OF BACTERIAL AND ARCHAEAL POPULATIONS IN ARTIFICIAL RUMEN FERMENTORS FED OPEN AND WOODED CANOPY GRASS DIETS.	11
2.1 INTRODUCTION	11
2.2 MATERIALS AND METHODS.....	11
2.2.1 Sample collection / Rumen fermentors	11
2.2.2 DNA extraction	15
2.2.3 DNA amplification	16
2.2.4 Terminal Restriction Fragment Length Polymorphism (T-RFLP).....	17
2.2.5 Data analysis	17
2.3 RESULTS	20
2.3.1 DNA extraction and PCR amplification	20
2.3.2 General characteristics of T-RFLP profiles	21
2.3.3 Biodiversity of T-RFLP profiles	22
2.3.4 Phylogenetic analyses of T-RFLP profiles.....	25
2.3.5 Multivariate analyses of T-RFLP profiles	36
2.4 DISCUSSION	43

CHAPTER 3: DIVERSITY OF BACTERIAL AND ARCHAEAL POPULATIONS IN ARTIFICIAL RUMEN FERMENTORS FED NATURALIZED PASTURE, TEFF, AND TRITICALE DIETS.	47
3.1 INTRODUCTION	47
3.2 MATERIAL AND METHODS	48
3.2.1 <i>Sample collection / rumen fermentors</i>	48
3.2.2 <i>DNA extraction</i>	48
3.2.3 <i>DNA amplification</i>	51
3.2.4 <i>Terminal Restriction Fragment Length Polymorphism (T-RFLP)</i>	51
3.2.5 <i>Data analysis</i>	51
3.3 RESULTS	53
3.3.1 <i>DNA extraction and PCR amplification</i>	53
3.3.2 <i>General characteristics of T-RFLP profiles</i>	53
3.3.3 <i>Biodiversity of T-RFLP profiles</i>	56
3.3.4 <i>Phylogenetic analyses of T-RFLP profiles</i>	56
3.3.5 <i>Multivariate analyses of T-RFLP profiles</i>	64
3.4 DISCUSSION	64
CHAPTER 4: CONCLUSIONS	69
REFERENCES.....	71

CHAPTER 1: INTRODUCTION AND OBJECTIVES

1.1 INTRODUCTION

1.1.1 Ruminant digestion

Ruminants make significant portion of domesticated and wild livestock and play very important role in sustainability of many villages in developing countries. The digestive system of ruminants is unique among mammals because they can efficiently utilize cellulose (Hungate, 1966). Cellulose of plant cell wall constitute more than 50% of total annual global carbon fixation and hence it is important to understand its efficient utilization (Enquist, *et al.*, 2003). Efficient utilization of cellulose by ruminants is mainly due to their symbiotic relationships with microorganisms (Koike, *et al.*, 2003).

Anatomically, the ruminant's digestive system is similar to other mammals except the stomach. A ruminant's stomach has four chambers: rumen, reticulum, omasum, and abomasum. The rumen is the largest compartment (180-240 liters) in cattle and maintains an ideal environment for microbial fermentations (temperature= 38⁰C-42⁰C; pH=6.4-7.2; anaerobic condition) (Bryant, 1959). Microorganisms allow ruminants in digestion and utilization of low quality cellulose rich diets that are generally unsuitable for consumption by other mammals (Hungate, 1966). During the fermentation, cellulose is hydrolyzed to sugar and sugars are converted into volatile fatty acids (VFA): acetate, propionate, and butyrate in decreasing order (Elsden & Phillipson, 1948). These VFAs are absorbed into bloodstream of the rumen wall and serve as main energy source of the ruminants. Ingested proteins are digested by proteases and peptidases of microbial origin. Fermentation end products are methane and carbon dioxide, which are released

into atmosphere by eructation. Rumen microbes synthesize many B vitamins and serve as an additional food source for ruminants (Bryant, 1959).

1.1.2 Ruminant diets

Ruminants gain energy by utilization of several types of diets such as fresh and conserved forages, straws, grains, and many by-products of agriculture. Carbohydrates (fibrous or non-fibrous) and proteins constitute main component of ruminant's diets and also serves as a substrate for microbial fermentation. Carbohydrate is also main source of fats and sugar in cow milk. Both fibrous and non-fibrous carbohydrates have some advantages and disadvantages. Fibrous carbohydrate (cellulose and hemicellulose) is bulkier and hence its microbial fermentation is slower and it is retained in the rumen for longer period of time. Mature plants have more lignin contents in fiber and hence undergo slower fermentation than young plants. Longer fiber particles stimulate rumination process and hence help in faster fermentation. Non-fibrous carbohydrates (starch) undergo rapid fermentation, improve energy supplies, and determine bacterial protein production in the rumen. However, excess of non-fibrous carbohydrates impede fiber fermentation and hence balance of fibrous and non-fibrous carbohydrate diets is important for proper cattle growth and milk production.

1.1.3 Rumen microbial diversity

The numbers and diversity of rumen microorganisms is very high (bacteria: 10^{10} - 10^{11} /ml; > 50 genera; >200 species), (archaea: 10^8 - 10^9 /ml; 5 genera), (protozoan: 10^4 - 10^6 /ml; $\approx$ 25 genera), (fungi: 10^3 - 10^5 zoospores/ml; 5 genera), and (bacteriophage: 10^8 - 10^9 /ml) (Bryant, 1959, Hobson, 1989, Miron, *et al.*, 2001, Kamara, 2005, Kumar, *et al.*,

2009). The majorities of microbial populations are strictly obligate anaerobes and ciliate protozoan's which plays an important role in ruminant's digestion.

In terms of number, bacteria occupy the top position and are the most investigated micro-fauna of rumen (Fernando, *et al.*, 2010). In general, rumen bacterial population are dominated by cellulolytic (*Fibrobacter succinogenes*, *Ruminococcus* spp., *Clostridium* sp., *Eubacterium* sp., *Butyrivibrio fibrosolvens* etc.) and amylolytic (*Streptococcus bovis*, *Ruminobacter amylophilus*, *Prevotella ruminicola* etc.) bacteria (Kamara, 2005). Mechanism of cellulose digestion is very different in *Fibrobacter succinogenes* and *Ruminococcus* spp. *Ruminococcus* produces both exoglucanases and endoglucanases which act together for cellulose digestion. *Ruminococcus* also produces multiprotein complexes cellulosome which acts as a facilitator for attachment of cellulases and bacterial cells to cellulose. Cellulose digestion process is different in *F. succinogenes* because they neither exhibit exoglucanase or cellulosome. Apart from these bacteria, there are group of bacteria in the rumen that helps in nitrogen utilization (*Megasphaera elsdenii*) and lipid degradation (*Anaerovibrio lipolytica*) (Kamara, 2005).

Among archaea seven species of methanogens (*Methanobacterium formicicum*, *Methanobacterium bryanti*, *Methanobrevibacter ruminantium*, *Methanobrevibacter smithii*, *Methanomicrobium mobile*, *Methanosarcina barkeri* and *Methanoculleus olentangyi*) have been reported (Joblin, *et al.*, 1990, Jarvis, *et al.*, 2000). Methanogens helps in making rumen fermentation a continuous process by consuming the fermentation products H₂ and CO₂. To get a continuous supply of H₂, many rumen methanogens live in an epi- or endo-symbiotic relationship with rumen ciliated protozoans (Ushida, 2011).

Although rumen protozoans constitute more than 50% of ruminal biomass and play an important role in ruminant's digestion by fermentation, they are not very critical for survival of the ruminants (Russell, 2002, Ushida, 2011). Morphologically rumen's ciliated protozoans have been divided into two types: 1.) holotrich and 2.) entodiniomorphid. Holotrich protozoans generally help in amylase digestion whereas an entodiniomorphid protozoan generally helps in cellulose and hemicellulose digestion (Mould & Thomas, 1958, Williams & Coleman, 1985). Although more than 15 genera of holotrich protozoa are known from different ruminants, *Isotricha* and *Oligoisotricha* are commonly encountered holotrich protozoans in cattle's rumen (Clarke, 1964, Dehority, *et al.*, 1983, Kamara, 2005). There are several known genera of entodiniomorphid protozoans from ruminants rumen but species belonging to genera *Entodinium*, *Ostracodinium*, *Polyplastron*, *Metadinium*, *Epidinium* etc. are commonly encountered in cattle's rumen (Kamara, 2005).

Rumen fungi play an important role in fiber digestion of the ruminants (Kamara, 2005). The first confirmed existence of fungi in rumen was reported by Orpin in mid-seventies (Orpin, 1975). Since then several genera of fungi have been reported from various ruminants. Species belonging to genera *Neocallimastrix*, *Sphaeromonas*, *Orpinomyces*, *Anaeromyces*, *Ruminomyces* etc. are commonly encountered in cattle's rumen (Kamara, 2005). Bacteriophages are present in large number in rumen. They lyse rumen bacterial cells and hence helps ruminant by providing readily available bacterial proteins (Kamara, 2005).

1.1.4 *In-vivo* versus *in-vitro* ruminal fermentors

In past, most studies on ruminal system were carried-out with fistulated animals (Clarke & Menna, 1961, Emmanuel, 1974, Lund, 1974). These studies were expensive, laborious, and not strictly controlled because of the complexity of rumen system. Use of *in-vitro* rumen fermentors gained popularity during last few decades (Fellner, *et al.*, 1997, Cardozo, *et al.*, 2004, Busquet, *et al.*, 2005, Carro, *et al.*, 2009), mainly because of the above reasons and also because of increased awareness among scientist on ethical use of animals in research. There are several types of *in-vitro* rumen fermentors, but two most common one are continuous-flow fermentors (Hoover, *et al.*, 1976) and semi-continuous Rusitec fermentors (Czerkawski & Breckenridge, 1977). Both systems have some advantages and disadvantages. Continuous-flow fermentors allow higher dilution rates, feeding rates and feeding frequencies than Rusitec fermentors but are less efficient in protozoan population maintenance (Czerkawski & Breckenridge, 1977, Carro, *et al.*, 2009). *In-vitro* rumen fermentors generally give reasonable estimate of rumen fermentation. Hannah *et al.* (1986) observed similar organic matter digestibility, amino acid and crude protein degradability when they compared *in-vitro* rumen fermentors with *in-vivo* rumen fermentation.

1.1.5 Molecular techniques for the estimation of microbial diversity

As discussed above, microbes in the rumen are diverse and are very important for efficient utilization of nutrients from ruminant's diet. Traditional culture based techniques are not suitable for precise estimation of rumen microbial diversity because many rumen microbes are un-culturable (Liu, *et al.*, 1997, Fernando, *et al.*, 2010). Even for those that can be cultured, there is a possibility of an error because many species

(*Prevotella ruminicola*, *Butyrivibrio fibrisolvens* and *Ruminococcus*) although diverse looks morphologically and biochemically very similar (Kamara, 2005) and there is a chance that change in environmental condition from original during cultivation may alter bacterial community structure (Liu, *et al.*, 1997). To overcome the limitations of culture based methods, several culture-independent methods have been proposed for estimation of microbial diversity. The most important among them are 16S rDNA cloning and sequencing (Bond, *et al.*, 1995), denaturing gradient gel electrophoresis (DGGE) (Muyzer, *et al.*, 1993, Kocherginskaya, *et al.*, 2001), restriction fragment length polymorphism (RFLP) (Avgustin, *et al.*, 1994), and terminal restriction fragment length polymorphism (T-RFLP) (Liu, *et al.*, 1997, Ridwan, *et al.*, 2009).

16S rDNA library cloning and sequencing is a culture independent method but construction and screening of bacterial clones are very time consuming and labor intensive (Liu, *et al.*, 1997).

DGGE is a culture and cloning independent electrophoresis based method which uses PCR amplified 16S rRNA gene for microbial profiling. Because DGGE gel contain denaturing reagent in increasing concentration towards the anode it helps in separation of 16S rDNA pcr product of similar size based on sequence heterogeneity and hence helps in microbial diversity estimation. It also helps in identification of dominant microbes because thick bands can be easily excised from the gel and then can be sequenced for microbial identity. DGGE is easy, fast, and comparatively cheap method. Main disadvantages of DGGE are poor resolution of the band on the gel and overlapping of faint bands by thick bands in some cases.

RFLP is an easy culture, cloning and sequencing independent method, where 16S rDNA are amplified using universal primers and then digested with different restriction enzymes for the microbial profiling using gel electrophoresis. RFLP analyses produces large number of bands on polyacrylamide gels, which can be used for differentiation of microbial communities associated with different environments, but RFLP have limited use if one want to know a specific phylogenetic groups associated with a particular environment or species richness or evenness.

T-RFLP is culture, cloning and sequencing independent microbial profiling method like RFLP, but in this method either one or both primers are fluorescently labeled which helps in tracking of terminal fragments and hence in species identification. In T-RFLP method, DNA are PCR amplified for 16S rRNA gene using universal primers, amplified products are cleaned and digested by different restriction enzymes, digested products are separated using capillary electrophoresis. Because primers are fluorescently labeled, only terminal fragments are visible on electropherogram. Size of different terminal fragments are calculated based on fluorescently labeled DNA ladder of known sizes that are run along with the digested products. Software generated T-RFLP profile helps in comparison between samples both in terms species richness (presence or absence of a peaks) and species abundance (relative intensity of peaks from the T-RFLP profiles can be used for estimation of abundance of each terminal fragments in the sample). T-RFLP is highly adaptable, fast and reproducible method (Osborn, *et al.*, 2000). In some cases different species may produce terminal fragments of same size which may cause underestimation of microbial diversity (Engebretson & Moyer, 2003). Many studies have

used T-RFLP method for microbial diversity estimation (Liu, *et al.*, 1997, Nagashima, *et al.*, 2003, Danovaro, *et al.*, 2006, Singh, *et al.*, 2006, Ridwan, *et al.*, 2009).

1.2 RATIONALE

As discussed above, in the last 50 years, our understanding of ruminant's digestion and nutritional requirements have significantly improved and these scientific discoveries have been utilized by farmers for better nutritional management of ruminants (Krause, *et al.*, 2003). Microorganism plays very important role in efficient utilization of nutrients for ruminants. Microbial composition of rumen generally changes with diet and sometimes abrupt change in diet can cause adverse effects on ruminants. This study will be carried out to see if most commonly used local pasture has any effect on bacterial and archaeal diversity. We chose only bacteria and archaea for this study because they are the most abundant micro-fauna in the rumen and play very significant role in ruminant digestion. Protozoan's are very unstable in artificial rumen fermentors and hence were not considered in this study. We chose open (100% sunlight) and wooded (shaded) canopy forage diets for first experiment (chapter 2), because with shading nutritive value of forage improves and we wanted to know if it has any effect on microbial diversity. We chose naturalized pasture (early and late cut) and supplemental grasses (teff, triticale) in the second study (chapter 3) because they have different nutritive quality and we wanted to know if that has any effect on bacterial and archaeal composition in rumen.

1.3 OBJECTIVES

Main objective of this study are to:

- i.) standardize T-RFLP method for estimation of microbial diversity,

- ii.) estimate bacterial diversity of artificial rumen digester fed with differing pasture diet (open canopy pasture, wooded canopy pasture, naturalized pasture early cut, naturalized pasture late cut, teff and triticale)
- iii.) estimate archaeal diversity of artificial rumen digester fed with differing pasture diet (open canopy pasture (OC), wooded canopy pasture(WC), naturalized pasture early cut (NPEC), naturalized pasture late cut (NPLC), teff and triticale).

To accomplish these objectives, I used T-RFLP fingerprinting method, which has several advantages over currently available cloning, culture based and culture independent methods (see above). Main limitation of this method is underestimation of microbial diversity mainly because a.) different microbes may have conserved terminal restriction sites which will lead to similar terminal fragments for different organisms, and b.) T-RFLP is a PCR based method that utilizes conserved primers, it is possible that some microbe may not get amplification because of difference in conserved primer site in these taxa. Next-generation pyrosequencing of 16S rDNA can solve first problem of conserved restriction sites in different taxa, but it is still very expensive and require more technical manpower in data analyses.

1.4 HYPOTHESES

My hypotheses are:

- i.) Open canopy and wooded canopy pasture diet will have different bacterial and archaeal population structure and diversity.
- ii.) NPEC and NPLC pasture will have significantly different bacterial and archaeal community structure

- iii.) Diet pair NPLC -Triticale and NPEC-Teff will have similar bacterial and archaeal composition because of similarity in their nutritive quality.

CHAPTER 2: DIVERSITY OF BACTERIAL AND ARCHAEAL POPULATIONS IN ARTIFICIAL RUMEN FERMENTORS FED OPEN AND WOODED CANOPY GRASS DIETS.

2.1 INTRODUCTION

Approximately 40% of farmland in Appalachian region is occupied by woodlots. Forage production in these woodlots will save ever-shrinking agricultural land for farming purposes and will provide additional income to the farmers. Presence of trees creates shaded area which in turn changes morphology, anatomy, and chemical composition of forage grass and affects both quality and quantity of forage (Kephart & Buxton, 1993, Lin, *et al.*, 2001). Shaded grass have increased leaf area ratio (LAR), decreased specific leaf weight (SLW), decreased cell wall concentration, increased stem length, higher nitrogen concentration, increased crude protein (CP) and increased digestible dry matter compared to non-shaded or open (100% sun-light) grasses (Kephart, *et al.*, 1992, Kephart & Buxton, 1993, Lin, *et al.*, 2001). Kephart and Buxton (1993) observed decrease in neutral detergent fiber (NDF) whereas Lin *et al.*, (2001) observed increase in NDF with shade. These morphological, anatomical and chemical changes increases forage quality of shaded grass compared to non-shaded grass (Kephart & Buxton, 1993, Lin, *et al.*, 2001). The objective of this study was to evaluate rumen bacterial and archaeal populations associated with change in nutritive quality of open and wooded canopy forage diets.

2.2 MATERIALS AND METHODS

2.2.1 Sample collection / Rumen fermentors

Single-flow continuous culture artificial rumen fermentors (Meng et al. (1999) were used for estimation of bacterial and archaeal diversity associated with open canopy (full sunlight) and wooded canopy (shaded) forage diets. Briefly, an individual fermentor consisted of a 2 liter capacity jar with an effluent outlet at the top, which allowed the fermentor volume to be maintained at 1.46 L (Figure 2.1). Fermentor temperature was maintained at 39°C using a water bath equipped with a thermostatically controlled heater (Model 730, Fisher Scientific, Pittsburgh, PA) and a magnetic stirrer. Rumen fluid from two fistulated Holstein cows was collected and pooled, strained through a layer of cheesecloth, and then added to each of 12 fermentors through their overflow ports. Fermentors were fed 50 g of dry material (DM) per day at 6 hours intervals. An anaerobic environment similar to rumen was maintained in each fermentor using a continuous supply of carbon dioxide and anaerobic buffer. Buffer solution contained ingredients as described in Slyter (1990) and were delivered continuously to each fermentors by a peristaltic pump (Masterflex model 7520-10, Cole Palmer Instrument Co., Chicago, IL).

Two forage treatments (open canopy pasture and wooded canopy pasture) were harvested at 4 separate times during late April through mid-June, 2008 from pastures located near Beaver, WV. Forages were harvested during weeks of 4/25/08 (harvest 1), 5/5/08 (harvest 2), 5/15/08 (harvest 3), and 6/11/08 (harvest 4) from same open and wooded canopy pastures. Forages from each harvest time were added into two fermentors (replications 1 & 2) as shown in Table 2.1. Mixture of open canopy and wooded canopy forage diets from each harvest time were added into a single fermentor (replication 3) as shown in Table 2.1.

Table 2.1 List of samples and their collection details. Each sample had one duplicate sample (not shown in table). Harvest 1, 2, 3 and 4 were conducted on 4/25/08, 5/5/08, 5/15/08, and 6/11/08 respectively.

Sr. #	Sample name	Diet name	Run #	Fermentor #	Harvest #	Replication #
1	R1F1	Open canopy	1	1	1	1
2	R1F2	Wooded canopy	1	2	1	1
3	R1F3	Open canopy	1	3	2	1
4	R1F4	Wooded canopy	1	4	2	1
5	R1F5	Open canopy	1	5	3	1
6	R1F6	Wooded canopy	1	6	3	1
7	R1F7	Open canopy	1	7	4	1
8	R1F8	Wooded canopy	1	8	4	1
9	R1F9	Open canopy	1	9	1	2
10	R1F10	Wooded canopy	1	10	1	2
11	R1F11	Open canopy	1	11	2	2
12	R1F12	Wooded canopy	1	12	2	2
13	R2F1	Open canopy	2	1	3	2
14	R2F2	Wooded canopy	2	2	3	2
15	R2F3	Open canopy	2	3	4	2

16	R2F4	Wooded canopy	2	4	4	2
17	R2F5	Open canopy	2	5	1	3
18	R2F6	Wooded canopy	2	6	1	3
19	R2F7	Open canopy	2	7	2	3
20	R2F8	Wooded canopy	2	8	2	3
21	R2F9	Open canopy	2	9	3	3
22	R2F10	Wooded canopy	2	10	3	3
23	R2F11	Open canopy	2	11	4	3
24	R2F12	Wooded canopy	2	12	4	3

Collected samples (1.2 ml from each fermentor after 10 days of the forage addition) were mixed with 3 ml glycerol and stored at -20°C till further processing.

Figure 2.1 *In-vitro* single-flow continuous culture system.

2.2.2 DNA extraction

Glycerol preserved rumen samples were mixed with 10 ml of 5% sodium chloride solution and then centrifuged at 4°C and 4000 rpm for 20 minutes. Centrifuged samples were incubated overnight at 4°C and then supernatant was discarded. Pellet (250 µl) from each sample was used for DNA extraction using Power Soil DNA Isolation Kit (Mo Bio Laboratories Inc. CA), following protocol as suggested by the manufacturer. Extracted DNA was quantified using NanoDrop spectrophotometer (Thermo scientific,

DE, USA) and genomic DNA quality was checked on 1% agarose gel with 2 µl of extracted DNA in each well.

2.2.3 DNA amplification

Approximately 1325 bp of bacterial 16S ribosomal RNA (16S rRNA) gene was amplified using 6-carboxyfluorescein (6-FAM) labeled forward primer 63f (5'-CAGGCCTAACACATGCAAGTC-3') and unlabeled reverse primer 1387r (5'-GGGCGGWTGTACAAGGC-3'), as mentioned in Marchesi et al. (1998). Each PCR reaction consisted of 2 µl of template DNA (10-20 ng/µl), 5 µl of 10X PCR buffer, 4 µl of 25 mM MgCl₂, 1.5 µl of 10 mM dNTP's, 2 µl of each primer (20 pmol/µl), 1.0 µl of Taq polymerase (1U/µl), and rest water to make 50 µl total volume. Bacterial 16S rRNA gene was amplified using touchdown PCR protocol in Thermo Hybaid thermocycler (Thermo Scientific, DE, USA). DNA was initially denatured at 94°C for 5 minutes, followed by 7 cycle of 94°C for 30 seconds; 61°C to 56°C (1°C decrease with each cycle) for 30 seconds; 72°C for 90 seconds, followed by 22 cycle of 94°C for 30 seconds; 55°C for 30 seconds; 72°C for 90 seconds, followed by final extension of 72°C for 7 minutes.

Approximately 800 bp of archaeal 16S rRNA gene was also amplified from all samples using 6-FAM labeled forward primer Ar109f (5'-AC(G/T)GCTCAGTAACACGT-3') and unlabeled reverse primer Ar912r (5'-CTCCCCCGCCAATTCCTTTA-3') as mentioned in Devine (2010). Archaeal specific PCR reaction mixture composition was same as bacterial PCR reaction mixture (see above) but instead of 4 µl, 5 µl of MgCl₂ (25 mM) was used. PCR protocol for archaeal 16S rRNA gene was same as mentioned in Devine (2010).

2.2.4 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Amplified product were cleaned using a QIAquick PCR Purification Kit (Qiagen Inc., CA, USA), and cleaned products were quantified using NanoDrop spectrophotometer (Thermo scientific, DE USA). 500 ng of cleaned PCR product was digested separately in 20 µl total volume by *Hha* I and *Rsa* I restriction enzymes, as per manufacturer's protocol (New England Biolabs, MA, USA). 1.5 µl of digested product was mixed with 0.5 µl of LIZ-labeled GS500 internal size standard (Applied Biosystems, CA, USA) and rest Hi-Di formamide (Applied Biosystems, CA, USA) for a total volume of 10 µl. This mixture was denatured at 95°C for 3 minutes, followed by cooling in ice for 5 minutes. Terminal restriction fragments were separated by capillary electrophoresis in ABI 3730x/ genetic analyzer (Applied Biosystems, CA, USA). Terminal fragments from duplicate samples were also run along with the original samples using capillary electrophoresis in ABI 3730x/ genetic analyzer (Applied Biosystems, CA, USA).

2.2.5 Data analysis

Raw data from capillary electrophoresis were analyzed for T-RFLP profile using GeneMapper® software version 4.0 (Applied Biosystems, CA, USA). Terminal restriction fragments (T-RFs) of size 35bp to 500 bp were selected manually. Terminal fragment length and height of peaks were treated as number and abundance of bacterial/archaeal phylotypes respectively and, were exported to excel sheet for further analysis. Only those peaks (length and height) were considered true that was present in both original and duplicate samples and rest were deleted from further analysis. Peak length and height from original and duplicate samples were averaged and made one for

each sample. Presence/absence (1/0) data were created based on average length and average abundance profiles.

Presence/absence data was used for construction of Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree using default parameters in PAUP* v.4.0b10 (Swofford, 2003). Same UPGMA tree was used as an input tree file for unweighted UniFrac (Lozupone, *et al.*, 2006) based clustering of bacterial and archaeal community associated with different treatments. P-test was performed using 1000 permutations for each pair of samples (diets, harvests, replicates) with Bonferroni correction for multiple comparison and for all samples together in UniFrac (Lozupone, *et al.*, 2006). All trees were edited using FigTree v1.3.1 (<http://tree.bio.ed.ac.uk/>).

Same presence/absence data was used for construction of analysis of similarity (ANOSIM) and nonmetric multidimensional scaling (NMDS) plot using default parameters of Vegan package in R v. 2.13.0 (R, 2011) (see below for sample R-codes). For ANOSIM, Global R test statistics (which measures strength of the results) was used, where R=1 signifies that two groups have different microbial community, and R=0 signifies that two groups have identical bacterial community structure.

R-code used for ANOSIM and NMDS analyses:

```
> library(vegan)

> bac_old = read.table ("C:\\Users\\SMITA
SINGH\\Desktop\\TAMU\\smita\\Smita_2012\\Research\\smita_1_30_12\\old\\NMDS_ba
c_arc_old\\b_old_hha_rsa_nmds.csv",header=T,sep=",")

> bac_old_dist = vegdist (bac_old[,5:180],method = "bray")

> bac_old_anosim = anosim (bac_old_dist, bac_old$diet, perm=999)

> plot(bac_old_anosim)
```

```

> bac_old_nmds=metaMDS(bac_old[,5:180])
Run 0 stress 0.1169503
Run 1 stress 0.1169506
... procrustes: rmse 0.0005220548 max resid 0.001874143
*** Solution reached

> plot(bac_old_nmds$points, type="n")

> text(bac_old_nmds$points, labels=bac_old$Replicate, cex=0.6)

```

Distinctive peak numbers from each sample were counted as species richness (S). Abundance profile (height) from each datasets was used for calculation of Shannon-Wiener diversity (H'), Simpson's diversity (D), Shannon evenness (E) and Simpson evenness ($E_{1/D}$) indices using equations as given below:

$$H' = -\sum(p_i) * (\log_2 p_i)$$

$$D = 1 - \sum(p_i)^2$$

$$E = H' / H_{\max}$$

$$E_{1/D} = (1/D) / S$$

Where,

P_i = Proportion of an individual peak height relative to the sum of all peak heights.

S = Species richness (total number of peaks present in each sample).

$$H_{\max} = \log_2(S).$$

For easy comparison between samples, Simpson's index of diversity (1-D) was calculated instead of the original formulation of the Simpson's index (D), so that the value of the index increases with increasing diversity.

2.3 RESULTS

2.3.1 DNA extraction and PCR amplification

DNA concentration ranged from 3 ng/μl to 17 ng/μl. Bacterial universal primers yielded PCR product of approximately 1325 bp size (Figure 2.2a), whereas Archaeal universal primer pair yielded PCR product of approximately 800 bp size (Figure 2.2b).

Figure 2.2 Agarose gel electrophoresis image of PCR products obtained using a.) bacterial universal primers and b.) archaeal universal primers. Lane L = 100 bp DNA ladder from New England Biolabs, lane N= Negative control. In figure 2.2a, lane1=R2F4, lane2=R2F4D, lane3=R1F12, lane4= R1F12D and in figure2.2b lane 1=R2F1, lane2=R2F1D, lane3=R2F2, lane 4=R2F2D, lane5= R2F3, lane6=R2F3D, lane7=R2F4, lane 8=R2F4D.

PCR product from two samples R1F1 and R1F2 using archaeal primers were very weak and didn't yield T-RFLP profile and hence were not used in any analyses.

2.3.2 General characteristics of T-RFLP profiles

A total of 73 and 102 different T-RF's, each representing different bacterial groups, were present in *Hha* I and *Rsa* I digested samples respectively. Among *Hha* I generated fragments, 10 were unique to open canopy and 9 were unique to wooded canopy (Figures.2.3a & 2.4). Similarly, among *Rsa* I generated fragments, 16 were unique to open canopy and 9 were unique to wooden canopy fed fermentors (Figs.2.3b).

Figure 2.3 Venn diagram showing shared and unique bacterial T-RF's between open canopy (OC) and wooded canopy (WC) pasture diets a.) when digested with *Hha* I b.) when digested with *Rsa* I.

PCR product obtained using archaeal primers yielded 42 and 36 T-RF's after digestion with *Hha* I and *Rsa* I respectively. Among *Hha* I generated fragments, 11 were unique to open canopy and 7 were unique to wooded canopy (Figures 2.5a & 2.6). Similarly, among *Rsa* I generated fragments, 6 terminal fragments were unique to open canopy and 2 terminal fragments were unique to wooded canopy (Figure 2.5b).

2.3.3 Biodiversity of T-RFLP profiles

Shannon-Weiner and Simpson diversity indices were used for determination of bacterial and archaeal diversity associated with open canopy and wooded canopy forage diets. In general open canopy forage diet had higher bacterial and archaeal richness and diversity (both Shannon and Simpson) than wooded canopy forage diets (Table 2.2). Shannon and Simpson evenness indices were almost same for both open and wooded canopy diets.

Figure 2.4 Comparison of *Hha I* digested bacterial T-RFLP profiles obtained from a single sample of open canopy (OC) and wooded canopy (WC) pasture diets. Red arrow highlight unique bacterial T-RF associated with wooden canopy pasture diets.

Figure 2.5 Venn diagram showing shared and unique archaeal T-RF's between open canopy (OC) and wooded canopy (WC) pasture diets a.) when digested with *Hha* I b.) when digested with *Rsa* I.

Figure 2.6 Comparison of *Hha* I digested archaeal T-RFLP profiles obtained from a single sample of open canopy (OC) and wooded canopy (WC) pasture diets. Red arrow highlights unique archaeal T-RF's associated with OC and WC pasture diets.

Table 2.2 Table showing richness, diversity, and evenness indices of bacterial and archaeal communities associated with open and wooded canopy diets.

Sample name	Richness (S)		Shannon diversity (H')		Simpson's index of diversity (1-D)		Shannon Evenness (E)		Simpson's Evenness (E(1/D))	
	Hha I	Rsa I	Hha I	Rsa I	Hha I	Rsa I	Hha I	Rsa I	Hha I	Rsa I
Bacteria										
Open canopy	26.83	32.82	3.23	3.69	0.82	0.82	0.69	0.74	0.24	0.23
Wooded canopy	26.42	30.09	2.97	3.37	0.79	0.76	0.65	0.69	0.19	0.22
Archaea										
Open canopy	11.64	11.55	2.43	2.26	0.71	0.66	0.71	0.65	0.40	0.32
Wooded canopy	10.55	11.18	2.29	2.29	0.69	0.67	0.70	0.68	0.42	0.37

2.3.4 Phylogenetic analyses of T-RFLP profiles

Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree using combined T-RF's profile from *Hha* I and *Rsa* I suggests bacterial and archaeal communities associated with open canopy and wooded canopy pasture diets are not very different (Figures 2.7 & 2.8).

UniFrac based P-test with Bonferroni correction for multiple comparison suggests that bacterial communities associated with different harvest times are also not significantly different (P-value >0.1). Unweighted UniFrac based clustering of harvest time indicates that first (H1) and fourth (H4) harvest times have more similar bacterial community structure than either third (H3) or second (H2) harvest time and H1, H4, and H3 share more bacterial species with each other than H2 (Figures 2.9 & 2.10).

Archaeal communities associated with second harvest time (H2) and fourth harvest time (H4) were very different (P-value = 0.05-0.1), but archaeal communities associated with other harvest times were not significantly different (P-value >0.1). Unweighted UniFrac based clustering suggests that first (H1) and second (H2) harvest times have similar archaeal communities and third (H3) and fourth (H4) have more similar archaeal communities (Figures 2.11 & 2.12).

Figure 2.7 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree of bacterial communities associated with open and wooded canopy pasture. T-RF's generated from *Hha* I and *Rsa* I digestion were combined for construction of UPGMA tree.

Figure 2.8 UPGMA tree of archaeal communities associated with open and wooded canopy pasture. T-RF's generated from *Hha* I and *Rsa* I digestion were combined for construction of UPGMA tree.

Figure 2.9 UPGMA tree of bacterial communities associated with different harvest time. T-RF's generated from *Hha* I and *Rsa* I digestion were combined for construction of UPGMA tree.

Figure 2.10 Unweighted UniFrac based clustering of bacterial communities associated with different harvest time. UPGMA tree from figure 2.9 was used as an input file for construction of UniFrac tree.

Figure 2.11 UPGMA tree of archaeal communities associated with different harvest time. T-RF's generated from *Hha* I and *Rsa* I digestion were combined for construction of UPGMA tree.

Figure 2.12 Unweighted UniFrac based clustering of archaeal communities associated with different harvest time. UPGMA tree from figure 2.11 was used as an input file for construction of UniFrac tree.

Figure 2.13 UPGMA tree of bacterial communities associated with different replications. T-RF's generated from *Hha* I and *Rsa* I digestion were combined for construction of the UPGMA tree.

Figure 2.14 Unweighted UniFrac based clustering of bacterial communities associated with different replications. UPGMA tree from figure 2.13 was used as an input file for construction of the UniFrac tree.

Figure 2.15 UPGMA tree of archaeal communities associated with different replications. T-RF's generated from *Hha* I and *Rsa* I digestion were combined for construction of the UPGMA tree.

Figure 2.16 Unweighted UniFrac based clustering of Archaeal communities associated with different replications. UPGMA tree from figure 2.15 was used as an input file for construction of the UniFrac tree.

UniFrac based P-test with Bonferroni correction for multiple comparison suggests that bacterial communities associated with first replication (R1) and third replication (R3) are significantly different (P-value = 0.01-0.05). Bacterial communities associated with other pairs of replicates are not significantly different (P-value >0.05). Unweighted UniFrac based clustering of replicates suggests that bacterial communities associated with R1 and R2 (second replicate) are more similar to each other than to R3 (Figures 2.13 & 2.14).

Archaeal communities associated with first replication (R1) and third replication (R3) and second replication (R2) and third replication (R3) were significantly different

(P-value = 0.01-0.05) but same was not true between R1 and R2 (P-value >0.10) in UniFrac based P-test with Bonferroni correction for multiple comparison. Unweighted UniFrac based clustering of replicates suggests that archaeal communities associated with R1 and R2 (second replicate) are more similar to each other than to R3 as was observed with bacterial communities (Figures 2.15 & 2.16).

2.3.5 Multivariate analyses of T-RFLP profiles

Nonmetric multidimensional scaling (NMDS) of T-RF's profiles generated from combined presence/absence data of *Hha* I and *Rsa* I digested PCR products demonstrate that bacterial and archaeal communities associated with open and wooded canopy diets are not distinct from one another (Figures 2.17a & 2.17b), an observation that was already confirmed by UPGMA clustering (Figs. 2.7 & 2.8).

The degree of separation of bacterial communities associated with open and wooded canopy diets based on R-value (where R=1 indicate complete separation between groups) were unclear and statistically non-significant (anosim R-value = -0.045 and p-value = 0.748), and same was true for archaeal communities associated with open and wooded canopy diets (anosim R-value=-0.034 and p-value=0.692) (Figures 2.18a & 2.18b).

Figure 2.17 Nonmetric multidimensional scaling plot of a.) bacterial and b.) archaeal communities associated with open canopy (oc) and wooded canopy (wc) diets. Presence/absence data of T-RF's generated from *Hha* I and *Rsa* I digested PCR products were used for construction of the NMDS plot.

Figure 2.18 Boxplot of ranked genetic dissimilarity for a.) bacterial and b.) archaeal communities associated with open and wooded canopy diets. Boxes represent median and interquartile ranges.

Figure 2.19 Nonmetric multidimensional scaling plot of a.) bacterial and b.) archaeal communities associated with different harvest time. Presence/absence data of T-RF's generated from *Hha* I and *Rsa* I digested PCR products were used for construction of the NMDS plot.

Figure 2.20 Boxplot of ranked genetic dissimilarity for a.) bacterial and b.) archaeal communities associated with different harvest time. Boxes represent median and interquartile ranges. H1= First harvest; H2=Second harvest; H3=Third harvest; H4=Fourth harvest.

Figure 2.21 Nonmetric multidimensional scaling plot of a.) bacterial and b.) archaeal communities associated with different replications. Presence/absence data of T-RF's generated from *Hha* I and *Rsa* I digested PCR products were used for construction of the NMDS plot.

Figure 2.22 Boxplot of ranked genetic dissimilarity for a.) bacterial and b.) archaeal communities associated with different replications. Boxes represent median and interquartile ranges. R1= First replication; R2=Second replication; R3=Third replication.

Similarly, bacterial and archaeal communities associated with different harvest time were also not significantly different (Figures 2.19a, 2.19b, 2.20a, and 2.20b). NMDS plots of different replications suggests that first and second replicate samples have more similar bacterial and archaeal community structure than to third replicate samples (Figures 2.21a and 2.21b) , a result similar to one observed in UniFrac based clustering (Figures 2.13 to 2.16). Both bacterial and archaeal communities associated with different replications were significantly separated from one another (Figures 2.22a; R-value =0.43; p-value= 0.001 and 2.22b R-value = 0.305; p-value=0.001).

2.4 DISCUSSION

The bovine rumen is a classical example of host-microbe symbiotic relationship and any disturbance in this strongly balanced ecosystem can cause disease to the animal (Khafipour, *et al.*, 2009). This balance in the rumen ecosystem can get affected by several factors; the most important among them is the type of diets ruminants eat (Khafipour, *et al.*, 2009, Fernando, *et al.*, 2010). Previous studies on rumen ecosystems have shown that microbial populations are generally stable across different animals (Jami & Mizrahi, 2012), location and sampling time points (Li, *et al.*, 2009), but it varies with diets they eat (Tajima, *et al.*, 2001, Khafipour, *et al.*, 2009, Li, *et al.*, 2009, Fernando, *et al.*, 2010). Majority of these studies emphasized on change in bacterial community in relation to grain diets (Khafipour, *et al.*, 2009, Fernando, *et al.*, 2010), and less emphasis was given on traditional pasture diets which is the main cattle food in several developing countries, and also on effect of change in diets on archaeal communities in the rumen ecosystem. Change in microbial population with diets is of

great importance to cattle owners, because it increases energy density, improve feed efficiency, and average daily gain (ADG) (Bevans, *et al.*, 2005).

In this study we examined effect of open canopy and wooded canopy forage diets on rumen bacterial and archaeal community structure using *Hha* I and *Rsa* I digested terminal restriction fragments. We showed that 1.) although bacterial and archaeal community associated with open and wooded canopy diets are not very different from each other, each diets had some unique bacterial and archaeal species 2.) open canopy forage diets generally had slightly higher bacterial and archaeal richness and diversity than wooded canopy forage diets, 3.) bacterial and archaeal community associated with different harvest time were not significantly different, 4.) there was significant difference in bacterial and archaeal communities associated with first and third replications. Archaeal community was significantly different also in between second and third replications, and 5.) bacterial and archaeal community associated with first (R1) and second (R2) replications were more similar to each other than to third replication (R3).

Our results indicate that nutritional difference between open and wooded canopy forage diets don't have a major impact on rumen bacterial and archaeal composition. As expected, bacterial diversity was much higher than archaeal diversity in both diets. These results also suggest to us that different harvest time didn't alter nutritional composition of these diets significantly, and hence we didn't observe any difference in bacterial and archaeal communities associated with different harvest time. This observation is similar to one made by Li *et al.* (2009), where they didn't find significant change in rumen bacterial composition with change in sampling time. Slightly higher

bacterial and archaeal diversity associated with open canopy diets may be linked to its poor forage quality compared to wooded canopy diets. Bacterial and archaeal community differed significantly between first (R1) and third (R3) replicates in anosim (bacteria $R=0.43$, $P=0.01$; archaea $R=0.305$, $P=0.01$) and in UniFrac based P test (p -value <0.05) because third replicate was pseudo replicate and comprised of mixture of open and wooded canopy forage diets. Same was true for archaeal community structure between R2 and R3.

Our study has several strengths. We measured rumen bacterial and archaeal community change with open and wooden canopy diets with emphasis on effect of harvest time over a period of three months. Several studies have shown effect of different diets on rumen microbial community structure (Tajima, *et al.*, 2001, Khafipour, *et al.*, 2009, Li, *et al.*, 2009, Fernando, *et al.*, 2010), but to our knowledge none have measured bacterial and archaeal diversity associated with open and wooden canopy forage diets and also on harvest time. We used *in-vitro* single flow continuous (artificial) fermentors for this experiment, which allowed us in having better control over several parameters that are not possible to control in natural rumen fermentors. Experiments using artificial fermentors are less stressing to animal and are also ethically well accepted among scientific community.

This study has some limitations. Although we had duplicate for each sample, we didn't run PCR in duplicate or triplicate for each sample before restriction digestion. Duplicate or triplicate PCR run helps in avoiding PCR bias (Marsh, *et al.*, 2000, Tajima, *et al.*, 2001) against less abundant microbes, and pooling of duplicate or triplicate PCR products helps in improving sensitivity of PCR for detection of minor communities

(Raymond, *et al.*, 2011). Also for this study we used only two restriction enzymes namely *Hha* I and *Rsa* I, which may not have detected all bacterial diversity associated with open and wooded canopy forage diets in the rumen. It is known from previous studies that no restriction enzyme alone can reveal more than 70% of total species in complex communities such as rumen (Engebretson & Moyer, 2003). Thus, for future studies more than two restriction enzymes should be chosen for better coverage of microbial communities associated with open and wooden canopy forage diets. We used high throughput DNA fingerprinting based approach for the measurement of bacterial and archaeal community structure in response to open and wooden canopy forage intake. Use of 16S rDNA based 454 pyrosequencing for estimation of rumen bacterial diversity have gained popularity among scientific community in recent years (Pitta, *et al.*, 2010, Jami & Mizrahi, 2012, Lee, *et al.*, 2012, Li, *et al.*, 2012), but it is still very expensive for large scale replicated studies, like this one. 454-pyrosequencing cost is decreasing every day, and hopefully in future, repetition of this study with 454-pyrosequencing will give more detail information about bacterial community structure associated with open and wooded canopy forage diets.

CHAPTER 3: DIVERSITY OF BACTERIAL AND ARCHAEAL POPULATIONS IN ARTIFICIAL RUMEN FERMENTORS FED NATURALIZED PASTURE, TEFF, AND TRITICALE DIETS.

3.1 INTRODUCTION

Several carbohydrate based diets such as naturalized pasture, teff grass, triticale etc. are common source of ruminant's nutrition. Naturalized pasture differs from pastureland in absence of cultural management and provides a valuable source of forage for ruminants. Teff is a self-pollinating annual grass that can be harvested multiple times in a year and has high forage quality. Teff is preferred as forage because of its better palatability, fast growth, drought tolerance, wide adaptations etc. On average teff grass contains 9-14% protein, 32-38% acid detergent fiber (ADF), and 53-65% neutral detergent fiber. Teff hay is high in calcium and several other nutrients such as iron, phosphorous, copper, aluminum, barium, and thiamine. As with other forage, teff's fiber content increases with maturity. Triticale is a grain diet and a hybrid product of wheat and rye. It is a high energy (12.6 MJ/kg DM) and medium protein (12.9% as fed) based animal diet and is similar in nutritive value to wheat, but it is much softer than wheat. Triticale contains higher concentration of chloroplast and non-structural carbohydrate whereas naturalized pasture late cut contains least amount of non-structural carbohydrate. Teff and naturalized pasture early cut (npec) have intermediate amount of non-structural carbohydrate. Fiber digestion in the rumen is not optimal because several studies have shown that fiber in feces of ruminants is fermentable (Krause, *et al.*, 2003). As rumen microbes are mainly responsible for rumen fermentation, better management of microbial population in the rumen may help in optimal fermentation of rumen diets.

Microbial diversity in rumen depends on several factors, the most important among them is the type of food ruminants eat (Fernando, *et al.*, 2010, Monteils, *et al.*, 2012). This change in microbial composition with food is mainly because of change in nutrient availability, pH, digesta flow, antimicrobial ingredients etc. (Busquet, *et al.*, 2005, Monteils, *et al.*, 2012). Digestive disorders due to decrease in pH have been observed in ruminants, with abrupt change in feed from forage diet to high-grain diet (van Kessel & Russell, 1996, Klieve, *et al.*, 2003, Khafipour, *et al.*, 2009). Although there are several study on change in microbial community structure with grain diets, literature on effect of pasture diets on rumen bacterial and archaeal community is limited or negligible. The main aim of this study was to see how bacterial and archaeal population changes with change in naturalized (npec, nplc) and supplemental grasses (teff and tritcale).

3.2 MATERIAL AND METHODS

3.2.1 Sample collection / rumen fermentors

In this study artificial rumen fermentors (single-flow continuous culture system) were used for estimation of bacterial and archaeal diversity in response to four pasture diets (naturalized pasture early cut (npec), naturalized pasture late cut (nplc), teff grass (teff) and tritcale (tri)) using fermentors arrangement similar to one described by Meng *et al.* (1999) and as described in section 2.2.1. Six replicates for each diet were used as shown in Table 3.1 and sample collection procedure was same as previously described in section 2.2.1.

3.2.2 DNA extraction

DNA extraction protocol was same as previously described in section 2.2.2.

Table 3.1 List of samples and their collection details. Feed types: 1.) Naturalized pasture early cut (npec), 2.) Naturalized pasture late cut (nplc), 3.) Teff grass (teff), and 4.) Triticale (tri).

Sr. #	Sample name	Diet name	Run #	Fermenter #
1	r1_1_npec	npec	1	1
2	r1_2_npec	npec	1	2
3	r1_3_npec	npec	1	3
4	r1_4_teff	teff	1	4
5	r1_5_teff	teff	1	5
6	r1_6_teff	teff	1	6
7	r1_7_npec	npec	1	7
8	r1_8_npec	npec	1	8
9	r1_9_npec	npec	1	9
10	r1_10_teff	teff	1	10
11	r1_11_teff	teff	1	11
12	r1_12_teff	teff	1	12
13	r2_1_nplc	nplc	2	1
14	r2_2_nplc	nplc	2	2

15	r2_3_nplc	nplc	2	3
16	r2_4_tri	tri	2	4
17	r2_5_tri	tri	2	5
18	r2_6_tri	tri	2	6
19	r2_7_nplc	nplc	2	7
20	r2_8_nplc	nplc	2	8
21	r2_9_nplc	nplc	2	9
22	r2_10_tri	tri	2	10
23	r2_11_tri	tri	2	11
24	r2_12_tri	tri	2	12

3.2.3 DNA amplification

DNA amplification protocol was same as described in section 2.2.3, except for this experiment there were no duplicate samples but each sample was amplified in triplicate and pooled for purification and restriction digestion.

3.2.4 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Triplicate PCR products from each sample were pooled and cleaned using a QIAquick PCR Purification Kit (Qiagen Inc., CA, USA) and quantified using NanoDrop spectrophotometer (Thermo scientific, DE USA).

Approximately 500 ng of cleaned PCR product was digested separately in 20 µl total volume by *Hha* I, *Rsa* I, and *Hae* III restriction enzymes, as per manufacturer's protocol (New England Biolabs, MA, USA). 1.5 µl of digested product was mixed with 0.5 µl of LIZ-labeled GS500 internal size standard (Applied Biosystems, CA, USA) and rest Hi-Di formamide (Applied Biosystems, CA, USA) for a total volume of 10 µl. This mixture was denatured at 95°C for 3 minutes, followed by cooling in ice for 5 minutes. Terminal restriction fragments were separated by capillary electrophoresis in ABI 3730xl genetic analyzer (Applied Biosystems, CA, USA). Terminal fragments from each sample were separated thrice using capillary electrophoresis in ABI 3730xl genetic analyzer (Applied Biosystems, CA, USA).

3.2.5 Data analysis

Raw data from capillary electrophoresis were analyzed for T-RFLP profile using GeneMapper® software version 4.0 (Applied Biosystems, CA, USA). Terminal restriction fragments (T-RFs) of size 35bp to 500 bp and height above 30 RFU were

considered for further analysis., mainly to avoid noisy TRFs due to primer dimer and also to include only those fragments that falls in the linear range of the size standard. Terminal fragment length and height of peaks were treated as number and abundance of bacterial/archaeal phylotypes respectively and, were exported to excel sheet for further analysis. Only those peaks (length and height) were considered true that were present in triplicate samples and rest was deleted from further analysis. Peak length and height from triplicate samples were pooled and averaged.

Presence/absence (1/0) data were created based on average length and average abundance profiles. Presence/absence data was used for construction of Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree using default parameters in PAUP* v.4.0b10 (Swofford, 2003). Same UPGMA tree was used as an input tree file for unweighted UniFrac (Lozupone, *et al.*, 2006) based clustering of bacterial and archaeal community associated with different diets. P-test was performed using 1000 permutations for each pair of diets with Bonferroni correction for multiple comparison (Lozupone, *et al.*, 2006). All trees were edited using FigTree v1.3.1 (<http://tree.bio.ed.ac.uk/>).

Same presence/absence data was used for construction of ANOSIM box plot and NMDS plots using Vegan package in R v. 2.13.0 (R, 2011) following protocol as described in section 2.2.5.

Distinctive peak numbers from each sample were counted as species richness (S). Abundance profile (peak height) from each datasets was used for calculation of Shannon-Wiener diversity (H'), Simpson's diversity (D), Shannon evenness (E) and

Simpson evenness ($E_{1/D}$) indices using equations and protocol as described in section 2.2.5

3.3 RESULTS

3.3.1 DNA extraction and PCR amplification

DNA concentration of samples was in the same range as was observed in section 2.3.1. Sizes of PCR product for bacterial and archaeal 16S rRNA gene were same as observed in section 2.3.1. PCR product from one sample (b_r1_7_npec) using bacterial primers was very weak and didn't yield T-RFLP profile and hence was not used in any analyses.

3.3.2 General characteristics of T-RFLP profiles

A total of 76, 90 and 20 different T-RF's, each representing different bacterial groups, were present in *Hha* I, *Rsa* I, and *Hae* III digested bacterial samples respectively. Among *Hha* I generated bacterial fragments 1, 3, 4, and 16 fragments were unique to npec, teff, nplc, and tri diets respectively (Figures 3.1a & 3.2). Similarly, among *Rsa* I generated fragments, 0, 5, 7, and 12 fragments were unique to npec, teff, nplc, and tri diets respectively (Figure 3.1b). Among *Hae* III generated fragments 1, 5, 1, and 3 fragments were unique to npec, teff, nplc, and tri diets respectively (Figure 3.1c).

Figure 3.1 Venn diagram showing shared and unique bacterial T-RF's between naturalized pasture early cut, naturalized pasture late cut, teff, and triticale pasture diets a.) when digested with *Hha* I, b.) when digested with *Rsa* I, and c.) when digested with *Hae* III.

Figure 3.2 Comparison of *Hha* I digested bacterial T-RFLP profiles obtained from a single sample of npec, nplc, teff and triticale pasture diets. Red arrow highlights unique and shared bacterial T-RF's associated with different diets.

Figure 3.3 Venn diagram showing shared and unique archaeal T-RF's between naturalized pasture early cut (npec), naturalized pasture late cut (npic), teff, and tritcale pasture diets a.) when digested with *Hha* I, b.) when digested with *Rsa* I, and c.) when digested with *Hae* III.

Figure 3.4 Comparison of *Hha* I digested archaeal T-RFLP profiles obtained from a single sample of naturalized pasture early cut (npec), naturalized pasture late cut (npic), teff, and tritcale pasture diets. Red arrow highlights unique and shared archaeal T-RF's associated with different diets.

PCR product obtained using archaeal primers yielded 73, 74, and 63 T-RF's after digestion with *Hha* I, *Rsa* I, and *Hae* III respectively. Among *Hha* I generated fragments 4, 1, 20, and 5 fragments were unique to npec, teff, nplc, and tri respectively (Figures 3.3a & 3.4). Similarly, among *Rsa* I generated fragments 1, 2, 24, and 3 fragments were unique to npec, teff, nplc, and tri diets respectively (Figure 3.3b). Among *Hae* III generated fragments 3, 2, 10, and 4 fragments were unique to npec, teff, nplc, and tri diets respectively (Figure 3.3c).

3.3.3 Biodiversity of T-RFLP profiles

Shannon-Weiner and Simpson diversity indices were used for determination of bacterial and archaeal diversity associated with four diets. In general triticale had highest bacterial richness and diversity (both Shannon and Simpson) than other diets included in this study but same was not true for archaeal communities (Table 3.2). Archaeal richness and diversity was much higher for nplc diet compared to other diets included in this study (Table 3.2). Bacterial community associated with npec diet was more evenly distributed than other diets included in this study (see Shannon and Simpson evenness indices value in table 3.2) but same was not true for archaeal communities. Archaeal communities associated with triticale diet were more evenly distributed than other three diets included in this study.

3.3.4 Phylogenetic analyses of T-RFLP profiles

Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree using combined T-RF's profile from *Hha* I, *Rsa* I and *Hae* III suggests that bacterial and archaeal communities associated with npec and teff are more similar to each other than to other two diets (Figures 3.5 & 3.6).

Table 3.2 Table showing richness, diversity, and evenness indices of bacterial and archaeal communities associated with naturalized pasture early cut (npec), teff, naturalized pasture late cut (nplc), and triticale (tri).

Sample name	Richness (S)			Shannon diversity (H')			Simpson's index of diversity (1-D)			Shannon Evenness (E)			Simpson's Evenness (E(1/D))		
	Hae III	Hha I	Rsa I	Hae III	Hha I	Rsa I	Hae III	Hha I	Rsa I	Hae III	Hha I	Rsa I	Hae III	Hha I	Rsa I
Bacteria															
npec	5.40	18.20	18.40	0.57	3.61	3.51	0.16	0.89	0.88	0.25	0.88	0.85	0.25	0.54	0.47
teff	7.00	23.33	21.00	0.69	3.71	3.72	0.19	0.87	0.90	0.25	0.82	0.85	0.19	0.34	0.49
nplc	6.17	29.00	35.17	0.53	4.01	3.76	0.14	0.90	0.88	0.20	0.83	0.74	0.20	0.35	0.25
tri	6.33	32.83	37.67	0.45	4.19	3.86	0.12	0.91	0.88	0.16	0.84	0.75	0.18	0.35	0.24
Archaea															
npec	19.17	16.17	14.00	3.60	3.51	3.18	0.88	0.88	0.83	0.87	0.89	0.84	0.51	0.57	0.47
teff	16.67	14.50	14.00	3.56	3.37	3.19	0.89	0.88	0.84	0.88	0.88	0.85	0.56	0.58	0.46
nplc	21.50	29.50	28.00	4.05	4.36	4.14	0.92	0.93	0.91	0.94	0.92	0.90	0.69	0.59	0.49
tri	17.33	17.67	16.50	3.78	3.76	3.69	0.91	0.91	0.90	0.95	0.94	0.94	0.76	0.71	0.72

Figure 3.5 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree of bacterial communities associated with different pasture diets. T-RF's generated from *Hha* I, *Rsa* I, and *Hae* III digestion were combined for construction of UPGMA tree. npec = naturalized pasture early cut, nplc = naturalized pasture late cut, teff = teff grass, and tri = triticales.

Figure 3.6 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree of archaeal communities associated with different pasture diets. T-RF's generated from *Hha* I, *Rsa* I, and *Hae* III digestion were combined for construction of UPGMA tree. npec = naturalized pasture early cut, npic = naturalized pasture late cut, teff = teff grass, and tri = triticale.

Figure 3.7 Unweighted UniFrac based clustering of bacterial communities associated with different diets. UPGMA tree from figure 3.5 was used as an input file for construction of UniFrac tree.

Figure 3.8 Unweighted UniFrac based clustering of archaeal communities associated with different diets. UPGMA tree from figure 3.6 was used as an input file for construction of UniFrac tree.

Figure 3.9 Nonmetric multidimensional scaling plot of a.) bacterial and b.) archaeal communities associated with npec (naturalized pasture early cut), npic (naturalized pasture late cut), teff (teff grass), and tri (triticale) pasture diets. Presence/absence data of T-RF's generated from *Hha* I, *Rsa* I and *Hae* III digested PCR products were used for construction of the NMDS plot.

Figure 3.10 Boxplot of ranked genetic dissimilarity for a.) bacterial and b.) archaeal communities associated with npec (naturalized pasture early cut), npic (naturalized pasture late cut), teff (teff grass), and tri (triticale) pasture diets. Boxes represent median and interquartile ranges. Circle above box represent outliers.

Similarly, bacterial and archaeal community associated nplc and tritcale were more similar to each other than to other two diets included in this study (Figures 3.5 & 3.6). Similar relationships were observed for both bacterial and archaeal communities associated different pasture diets included in this study in UniFrac based clustering (Figures 3.7 & 3.8). Bacterial and archaeal communities associated with diet pairs npec-nplc, npec-tritcale, nplc-teff, and teff-tritcale were suggested to be very different (p -value ≤ 0.06) in UniFrac based P-test with Bonferroni correction for multiple comparisons.

3.3.5 Multivariate analyses of T-RFLP profiles

Nonmetric multidimensional scaling (NMDS) of T-RF's profiles generated from combined presence/absence data of *Hha* I, *Rsa* I and *Hae* III digested PCR products demonstrate that bacterial and archaeal communities associated npec and teff are clustered together and bacterial and archaeal communities associated nplc and tritcale diets are also clustered together (Figures 3.9a & 3.9b), an observation that was already confirmed by UPGMA clustering (Figures 3.5 & 3.6). Anosim results suggests that bacterial and archaeal communities were very distinct and significantly different between different pasture diets (Figure 3.10a: R-value = 0.588, p -value = 0.001; Figure 3.10b: R-value=0.499, p -value=0.001).

3.4 DISCUSSION

In this study we examined effect of four pasture diets (naturalized pasture early cut (npec), naturalized pasture late cut (nplc), teff grass (teff), and tritcale (tri)), on rumen bacterial and archaeal community structure using *Hha* I, *Rsa* I, and *Hhe* III digested terminal restriction fragments (T-RF's). We showed that, 1.) each diet and

restriction enzymes, except npec digested with *Rsa* I (see below), produces at least one unique bacterial and archaeal species, 2.) npec digested with *Rsa* I produces only unique archaeal species, 3.) *Hha* I and *Rsa* I generates more bacterial richness but *Hhe* III generates more archaeal richness, 4.) tritcale produces highest bacterial richness and diversity, 5.) nplc produces highest archaeal richness and diversity, 6.) npec diet produces comparatively more even bacterial community structure than other diets included in this study, 7.) tritcale produces comparatively more even archaeal community structure than other diets included in this study, 8.) diet pair npec –teff and nplc-tritcale produces more similar bacterial and archaeal community structure, 9.) bacterial and archaeal communities associated with diet pairs npec-nplc, npec-tritcale, nplc-teff, and teff-tritcale are significantly different.

Because discrimination of bacterial and archaeal populations by T-RFLP depends on 16S rDNA T-RF polymorphism, it is important to choose more than one restriction enzyme, and also to choose those restriction enzymes that have better resolving power for a particular community (Moyer, *et al.*, 1996, Liu, *et al.*, 1997, Engebretson & Moyer, 2003, Schutte, *et al.*, 2008). Previous study using computer simulated restriction fragment length polymorphism of bacterial sequences suggested that for $\geq 99\%$ coverage of operational taxonomic units (OTU's) it is important to include at least three tetrameric restriction enzymes (Moyer, *et al.*, 1996).

Consequently, we used combination of three restriction enzymes (*Hha* I, *Rsa* I, and *Hhe* III) in this study. Bacterial richness and diversity observed with *Hha* I and *Rsa* I restriction enzyme were comparable to other similar studies, but extremely low bacterial richness and diversity was observed for all diet with *Hhe* III restriction enzyme. This may

be mainly because we considered T-RF's sizes only up to 500bp in this study and those *Hhe* III digested bacterial T-RF's that were >500 bp didn't get counted for bacterial richness and diversity and it is known from Moyer *et al.* (1996) computer simulated study on 10 tetrameric restriction enzymes that majority of *Hhe* III digested bacterial restriction fragment fall in size class range 400-1000 bp, whereas it was 250-550 bp for *Hha* I and *Rsa* I restriction enzymes. It is also possible that *Hhe* III restriction sites of rumen bacteria are well conserved and hence we observed fewer numbers of terminal fragments with *Hhe* III restriction enzymes (Schutte, *et al.*, 2008). Interestingly, same was not true in case of rumen archaea (Table 3.2). High bacterial diversity observed with triticale and high archaeal diversity observed with nplc diets, suggests to us that microbial response to nutritional change in rumen is not always same for bacteria and archaea.

Forage maturity at harvest affects supply and utilization of nutrients to ruminants. Generally, mature or late cut forage diets have more dry matter and hence less nutritive quality and digestibility than less mature or early cut forage diets (Kammes, *et al.*, 2012). Lower digestibility associated with mature forage diet also causes greater retention time of these diets in the rumen (Kammes, *et al.*, 2012). We observed greater bacterial richness and diversity associated with late cut (more mature) naturalized pasture diets than in early cut (less mature) naturalized pasture diets (Table 3.2), which may be because of greater retention time and shift in nutritional value of mature (late cut) naturalized pasture diets. Significant difference in microbial community structure between npec and nplc diets suggests to us that nutritional value of naturalized pasture changes significantly with its maturity. Interestingly, mature (late cut) naturalized pasture

diets produces bacterial and archaeal community more similar to triticale diets than to either less mature (early cut) naturalized pasture diet or teff grass. This may be mainly because both triticale and nplc contains more chloroplast compared to other diets hence are protein rich. Similar bacterial and archaeal community structure between less mature (early cut) naturalized pasture and teff grass may be mainly because they have less amount of chloroplast and hence protein, intermediate amount of non-structural carbohydrate, and similar digestibility compared to other two diets.

This study has several strengths. This is the first study to our knowledge, where effect of different pasture diets (npec, nplc, teff, and triticale) on bacterial and archaeal community structure was studied. We showed for the first time that along with nutritional quality and digestibility, maturity of pasture diets also changes bacterial and archaeal community structure significantly. Use of *in-vitro* single flow continuous (artificial) fermentors allowed us in having better control over several parameters that are not possible to control in natural rumen fermentors. In this experiment, for each sample we ran PCR in triplicate and again pooled PCR product after restriction digestion were separated thrice using capillary electrophoresis, which helped us in decreasing PCR bias against less abundant bacteria and archaea (Raymond, *et al.*, 2011). Use of three tetrameric restriction enzymes allowed us in having better bacterial and archaeal coverage than what we would have obtained with one or two restriction enzymes (see chapter 2).

Major limitation of this study was inclusion of T-RF's of only up to 500 bp size. We choose DNA ladder of up to 500 bp size (1200 bp DNA ladder didn't yield accurate standard curve), and hence it was not possible to include fragment size larger than 500

bp in our analyses. This limitation had maximum effect on bacterial diversity and richness associated with *Hhe* III restriction enzymes (Table 3.2).

Use of 16S rDNA based 454 pyrosequencing for estimation of rumen bacterial diversity have gained popularity among scientific community in recent years (Pitta, *et al.*, 2010, Jami & Mizrahi, 2012, Lee, *et al.*, 2012, Li, *et al.*, 2012), but it is still very expensive for large scale replicated studies, like this one. 454-pyrosequencing cost is decreasing every day, and hopefully in future, repetition of this study with 454-pyrosequencing will give more detail information about bacterial community structure associated with different pasture diets.

CHAPTER 4: CONCLUSIONS

This is the first study where effect of six different pastures diets on rumen microbial community structure was tested. We observed that although wooded canopy pasture or silvopasture is better than open canopy pasture in terms of nutritional quality, it has no significant effect on bacterial or archaeal community structure. Similarly harvest time of open or wooded canopy pasture diets had no significant effect on rumen bacterial or archaeal community structure. We did observed significant bacterial and archaeal community structure, when open or wooden canopy pasture was compared with mixture of both wooden and open canopy pasture diets. Bacterial and archaeal diversity was slightly higher in open canopy pasture diets than wooded canopy pasture deits.

Among npec, nplc, teff and tritcale pasture diets, bacterial and archaeal community structure of npec was more similar to teff than to other two diets. Similarly, nplc shared more bacterial and archaeal genera to tritcale than to other two diets. Triticale and nplc produced highest richness and diversity for bacteria and archaea respectively than to other diets. Majority of T-RF's were shared between different pasture diets included in this study but each diet and restriction enzyme except one had at least one unique bacterial and archaeal fauna. Bacterial community was more evenly distributed in npec whereas archaeal community was more evenly distributed in tritcale.

The results from this study is of great importance to cattle owners, because it will help in increasing energy density, improving feed efficiency, and in average daily gain (ADG) of cattle. We can detect microbial community changes among different diets and can fingerprint many pasture types and management system. Although we were not

able to identify dominant rumen bacteria and archaea associated with these diets, its identification will help in improving meat quality. In long term, knowledge of bacterial and archaeal diversity from the proposed research will help in identification of unidentified grass diets for better meat quality. In future, 454-pyrosequencing based approach should be used for identification of dominant bacteria and archaea associated with these diets.

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