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IN

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APPLICATION OF DNA TECHNOLOGY TO ASSESS THE GENETIC DIVERSITY AND DYNAMICS OF UNCULTURED MICROORGANISMS OF RUMINAL FLUID OF BUFFALO (*Bubalus bubalis*)

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DECLARATION

I, **Mr. Krishna Mohan Singh**, the undersigned hereby solemnly declare that the work presented in this thesis entitled "**Application of DNA Technology to Assess the Genetic Diversity and Dynamics of Uncultured Microorganisms of Ruminal Fluid of Buffalo (*Bubalus bubalis*)** " is original and independent. I declare further that this work has not been submitted for any degree or diploma to any other Universities or institutions.

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LIST OF ACRONYMS

%	Percent
@	At the rate of
µg	Microgram
µg/g	Microgram/gram
µl	Microliter
EDTA	Ethylene diamine tetra acetic acid
e.g.	exampli gratia
et al.	*et alibi*
etc	*et cet-er-a*
g	Grams
i.e.	id est (that is)
mg	Milligram
ml	Milliliter
spp.	Species
bp	Base pair
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate
M	Molar
Min	Minutes
mM	Millimolar
N	Normal
ng	Nanograms
nm	Nanometer
nt	Nucleotide
OD	Optical Density
PCR	Polymerase Chain Reaction
pM	Picomole
SDS	Sodium dodecyl sulphate
sec	Seconds
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris-EDTA

UV	Ultra Violet
V	Volts
viz.	Videlicet (namely)
MEGA	Molecular Evolutionary Genetics Analysis
PHYLIP	PHYlogeny Inference Package
CPCSEA	Committee for the purpose of control and supervision of experiments on animals

Chapter 1. Introduction and Literature Review

INTRODUCTION

Environmental biotechnology is undergoing a dramatic revolution because of increasing accumulation of biological information and contextual environmental parameters. This will not only enable a better identification of diversity patterns, but will also shed more light on the associated environmental conditions, spatial locations, and seasonal fluctuations, which could explain such patterns. Microbial transformations in the rumen ecosystem have a major impact on our ability to meet the challenge of reducing the environmental footprint of ruminant livestock agriculture, as well as enhancing product quality. Population size and microbial diversity in the rumen ecosystem enhances the resistance of the network of metabolic pathways present, as well as increasing the potential number of new pathways available. The resulting stability of rumen function is further promoted by the existence of rumen microbiota within biofilms. These protected, structured communities offer potential advantages, but very little is currently known about how ruminal microorganisms interact on feed-surfaces and how these communities develop.The temporal and spatial development of biofilms is strongly linked to the availability of dietary nutrients; the dynamics of which must also be given consideration, particularly in fresh-forage-based production systems. Nutrient dynamics, however, impact not only on pathway inputs but also the turnover and output of the whole ecosystem. Knowledge of the optimal balance of metabolic processes and the corresponding microbial taxa required to provide a stable, balanced ecosystem will enable a more holistic understanding of the rumen.

The microbial eco-system of the rumen is stable and at the same time dynamic. The eco-system is stable as it is well established and has been performing the function of bioconversion of feed into volatile fatty acids. In a healthy ruminant the contamination of the eco-system does not occur in spite of the fact that millions of microbes invade the rumen everyday through feed, drinking water and air. The eco-system is dynamic as the microbial population changes considerably on change of diet so as to adapt it to the new feed ingredients. This happens because the rumen microbes are adapted to survive in a set of constraints prevalent in the rumen and any

contaminant which cannot survive these constraints is eliminated. The major environmental constraints are anaerobiosis, high buffering capacity and osmotic pressure and saprophytic competition among the microbes for their survival. The anaerobiosis relation inside the rumen is one of the major constraints in the rumen eco-system, which help in conserving the energy ultimately to be used by the host animal. The amount of energy released by electron transfer reaction depends upon the terminal electron acceptor. In aerobic microbes, the terminal electron acceptor is oxygen and the release of energy in the form of ATP is much higher compared to energy released when the terminal electron acceptor is an organic compound as in the case of anaerobic bacteria. The anaerobic conditions in the rumen are maintained by gases generated during fermentation, e.g. carbon dioxide, methane and traces of hydrogen. Some of the oxygen entrapped in the feed consumed by the animal is utilized by the facultative anaerobes present in the rumen and thus a perfect anaerobic condition is generated and maintained.

Livestock production is subsidiary to plant agriculture in India, in the sense that animals are fed on agricultural byproducts like cereal straws, sugarcane bagasse, tree foliages and cakes of oil seeds like groundnut, cotton, mohua, neem and mustard. The efficiency of ruminants to utilize such a wide variety of feeds. This is due to highly diversified rumen microbial ecosystem consisting of bacteria, ciliate protozoa, methaogens, anaerobic fungi and bacteriophages (Hobson, 1989; Hespell *et al.,* 1997; Klieve and Bauchop, 1988; Klieve and Swain, 1993). They change feedstuff into volatile fatty acids (VFA), microbial cells and vitamins. The net result of these reactions going on in the rumen is responsible for the bioconversion of feed into such form that is utilizable by the animal as a source of energy, protein and other nutrients (short chain volatile fatty acids, microbial cells). These microbes survive in the rumen under different constraints which may be either natural or feed associated as some of the feeds contain a significant amount of anti-nutritional factors, which sometimes limit the growth of some of these natural microbial inhabitants. The synergism and antagonism among the different groups of microbes and even among different genera of the same group is so diverse and complicated that it is difficult to quantify the role played by any particular group of microbes present in the rumen.

The rumen microbial ecology

The rumen, a specialized foregut in herbivorous animals (Ruminantia), hosts microbiota of archaea, eubacteria, bacteriophages, anaerobic fungi, and ciliated protozoa. These microorganisms constitute the rumen microbial ecosystem. In the analysis of this system, interactions among the microbes are of paramount importance.

In nature the adhesion of bacteria to solid substances is a common phenomenon and this is also true in the rumen microbial ecosystem. There is a difference in the distribution of various types of ruminal microorganisms among rumen fluids, feed particles, the rumen epithelium, and surface of ciliate protozoa. Minato *et al.,* (1966) demonstrated that microbial populations associated with feed particles are predominant and account for 50-75% of the total microbial population in the rumen. They described the adhesion of microbes to solid substances as an important factor in successful competition and survival in the rumen, as well as in the digestion of solid feed (Minato *et al.,* 1966; Mitsumori and Minato, 1997). In many anaerobic cellulolytic bacteria, the adhesion of bacterial cells to insoluble cellulose is an essential step in the degradation of cellulosic materials (Minato and Suto, 1978).

Methane-producing archaea are a distinct group of organisms which are a normal component of the animal gastrointestinal microbial ecosystem. Methanogens residing in the animal gastrointestinal tract belong to the genera *Methanobacterium*, *Methanobrevibacter*, *Methanosphaera*, *Methanomicrobium*, *Methanogenium* and *Methanosarcina* (Miller and Wolin, 1986; Jarvis *et al*., 2000; Miller and Lin, 2002).

Most methanogens obtain energy by reduction of carbon dioxide to methane by using hydrogen as the electron donor. This process maintains the low partial pressure of hydrogen in the rumen and promotes the production of hydrogen and other products by the non-methanogenic fermentative microbial community (Wolin *et al*., 1997). Methanogenesis accounts for 2 - 12% of the dietary energy loss in ruminants (Johnson and Johnson, 1995); therefore, there has been considerable interest in limiting methane production and avoiding energy loss.

Several species of methanogens have been isolated from ruminants, but few have been consistently found in high numbers (Stewart *et al*., 1997) and it is likely that major species of rumen methanogens are yet to be identified (Raskin *et al.,* 1994

and Wolin *et al.,* 1997). The most common species of methanogens isolated from the rumen are strains of *Methanobrevibacter*, *Methanomicrobium*, *Methanobacterium* and *Methanosarcina* (Wolin *et al*., 1997; Jarvis *et al.,* 2000).

The rumen protozoa were first described in 1843 (Gruby and Delafond), and with their striking appearance it was assumed that they must be important for the welfare of their host. However, despite the fact that protozoa can contribute up to 50% of the bio-mass in the rumen, the role of protozoa and in particular the role of individual genera in ruminal fermentation remains unclear. This is largely because of the difficulties in maintaining cultures of rumen protozoa in the laboratory.

There are hundreds of species of ciliate protozoa classified within many different genera; however, commonly identified ruminal species are primarily classified in the family's Isotrichidae and Ophryoscolecidae (Dehority, 2003). The Isotrichids are completely covered by cilia whereas the Ophryoscolecids contain ciliary zones only at the anterior ends. Morphological classification was used based on cilia positioning, number of ciliary zones, as well as the presence and number of skeletal plates and caudle spines.

Ciliates are the most abundant protozoa found in the rumen of both domesticated and wild ruminants. Rumen ciliates are involved in host metabolism and digestion of plant material (Williams and Coleman, 1992) and play an important role in the rumen microbial ecosystem by producing hydrogen as a by-product of plant digestion. The hydrogen is then used by methanogenic archaea (i.e., methanogens) to reduce carbon dioxide to methane, a potent greenhouse gas. Removal of protozoa from the rumen (i.e., defaunation) has been shown to reduce methane emission by an average of 13% (Hegarty, 1999).

Studies on defaunated animals have shown that exclusion of protozoa from the rumen has a beneficial effect on the growth rate, wool growth and feed conversion efficiency of animals under certain feeding conditions (Bird and Leng, 1984; Demeyer, 1992; Ivan *et al*., 1992; Bird *et al*., 1994; Santra and Karim, 2000). Contrary to these reports, some experiments have concluded that protozoa are an essential component of the microbial ecosystem in the rumen, and their exclusion has a harmful effect on the productivity of ruminants (Eadie and Gill, 1971; Ramprasad and Raghavan, 1981).

Molecular techniques for rumen microbiology

The enumeration of a specific species of microbes in the ecosystem (to quantify its role in rumen fermentation) is difficult with the conventional techniques due to a large number of biochemical tests to be performed and imprecision of the technique even for the most predominant microbe present in the ecosystem. This is perhaps due to selection pressure of the medium used for enumeration as the relative numbers of these microbes will change when cultivated in a Petri-dish in comparison to that metabolically active in the ecosystem. There is no single culture medium available which can support growth of all the culturable bacteria of rumen. In addition, very large proportion of rumen microbes (like in any other eco-system) is non-culturable, but is active in the rumen. Therefore, it is essential to search for some better technique of quantifying specific microbes in this ecosystem, which can take care of the drawbacks of the conventional techniques of studying microbial ecology of the rumen. After the discovery that rRNA is present in every cell and that its nucleotide sequence can be used for phylogenetic classification (Woese, 1987; Woese *et al.,* 1990), the introduction of the so-called 16S rRNA/18S r RNA approach has demonstrated that the majority of rumen microbes are phylogenetically different from those described in culture.

Newer molecular approaches are available to identify and characterize the eukaryotes and prokaryotes, based on detecting highly conserved gene regions-16S rRNA and 18S rRNA, respectively for bacteria and protozoa. These techniques are fast, accurate and robust, and provide ample scope to study the diversity of microorganism. As a result of the application of molecular biology, it has been revealed that in many ecosystems, the culturable bacterial species represent only a small proportion of the total number of bacterial species that are actually present. It is also possible to design tools for detecting strains of the microbes/protozoa by use of these techniques.

A dramatic increase in application of approaches based on the sequence diversity of 16S rRNA (for bacteria and methanogen;) and 18S rRNA (for protozoa) gene has been made during last decade to explore the diversity of microbes in rumen The techniques used have included the polymerase chain reaction (PCR) (Wright *et al*., 1997; Karnati *et al*., 2003), restriction fragment length polymorphism (RFLP)

analysis (Peek *et al*., 2004; Regensbogenova *et al*., 2004a), real-time PCR (Bergen, 2004; Ozutsumi *et al*., 2005; Skillman *et al*., 2006b; Sylvester *et al.,* 2005), denaturing gradient gel electrophoresis (DGGE) (Regensbogenova *et al*., 2004b) and phylogenetic analysis of protozoa based on 18S rDNA libraries generated by PCR amplification (Shin *et al*., 2004a; Karnati *et al*., 2003). Despite this information the enumeration and identification of community members have tremendous limitations, as there is no complete phylogenetic classification of the rumen microbes. The application of these molecular techniques has been changing our perspectives about ruminal and GI microbiota.

Sequence comparison of nucleic acids isolated from complex microbial ecosystem can be used to provide molecular characterization and thus to provide classification system. It can also be used to study microbial ecology from nutritional viewpoints like diet dependent shift in microbial population, contribution of bacterial and protozoal nitrogen to host, properties and regulation of microbial enzymes, characterization and transcription of genes encoding enzymes involved in various fermentative process in rumen, identification of microbial tolerant to various harmful substances (e.g. Tannins) present in feeds and fodder etc. These new techniques may also help in understanding the mechanism of feed utilization in the rumen.

Different kind of ruminants, due to the diet and inhabiting environment variation, could harbor a distinct population of rumen microbes. The microbial ecosystem is well studied for the rumen of domesticated animals like cattle, sheep and goat, but it is poorly studied in buffalo rumen.

LITERARTURE REVIEW

Ruminants have least competition with humans for the supply of their nutritional requirements because they are capable of utilizing nutrients from the crop residues and agro-industrial byproducts which are otherwise unsuitable for the consumption of human beings and other simple stomached animals. This ability in the ruminants is due to specialized evolution of their digestive systems which provide suitable environment for the establishment and development of micro-organisms capable of digesting large amount of fibrous herbages. These micro-organisms are also able to utilize non protein nitrogenous compounds for the synthesis of amino

acids and proteins suitable for the supply of protein requirements of the host animals. These microbes also synthesize almost all vitamins of the B-complex group due to which these are normally not required to be supplemented in the diet of the ruminants. In addition to these qualities, the rumen micro-organisms play a significant role in the detoxification and neutralization of different kinds of anti-nutritional factors in the natural as well as processed feeds. The number and ratio of different kinds of rumen micro-organisms change with the diet composition, and such changes often provide considerable protection to the host against dietary imbalances like an increase in the number of ciliate protozoa, on the feeding of high grain diets, reduces lactic fermentation by engulfing considerable amount of starchy feeds.

The digestive system of ruminants

The ruminants are basically herbivorous animals and derive their major nutritional requirements from the ingestion of a variety of herbages. Since herbages are mostly fibrous and concentration of energy and protein is usually much lower than the grains, the ruminants are required to consume large amount of such fibrous feeds. Accordingly their digestive system has been evolved to accommodate large quantity of bulky fibrous feeds. A major modification has occurred in the stomach and the large intestine of the ruminants.

The alimentary canal of the ruminants extends from oral cavity and ends with the anal opening. This musculo-tubular organ undergoes several modifications in different regions for specific digestive functions. The various compartments of the digestive tract are the mouth cavity, the oesophagus, the stomach complex (rumen, reticulum, omasum, abomasums and pylorus), and the intestines (duodenum, small intestine, caecum, colon, rectum and anus). The accessory glands actively associated with the digestion are the liver, the pancreas and the spleen.

The reticulo-rumen, a part of the four compartments of ruminants; stomach harbours a mixed microbial population, which performs some specific functions for the host animals. These microbes exist in the gastro-intestinal tract of the animals as ecto-symbiotic partners. The weight of reticulo-rumen (10 -15% total live weight of animals) explains its importance in the metabolism of ruminants.

Feedstuffs consumed by ruminants are all initially exposed to the fermentative activity in the rumen prior to gastric and intestinal digestion. None of the

enzymes of animal origin have ever been found to degrade lignocellulosic feeds. This appears to be the sole proprietary function of the microbes which release enzymes responsible for the degradation of lignocelluloses. Dietary polysaccharides and protein are degraded by the ruminal micro-organisms into characteristic end products, which in turn provide nutrients for metabolism by the host animal.

Bacterial diversity and ecology

Rumen, the so-called fourth stomach of cow, buffaloes, and the likes, harbors a plethora of microorganisms represented by bacteria, ciliate protozoa, anaerobic fungi, archaea and bacteriophages. What is indeed of great interest is the balance of equilibrium by way of species richness represented by such a large consortia of aerobic to anaerobic forms that does not get disturbed even when the animal diet undergoes a change. Kamra (2001) describes that this balancing act is a consequence of the prevailing anaerobiosis, high buffering capacity of the system, resulting in osmotic pressure and the ensuing competition among the microbial members for survival. Rumen is a storehouse of anaerobic fungi found nowhere else that play a very dominant role in degradation of lignocellulosic component of the feed.

Ruminants in India are fed mainly on lignocellulosic agricultural by-products which are rich in cellulose, hemicellulose, and lignin. The rumen harbours various types of bacteria which are active in degradation of these components of the feed (Table1.1). The interaction among themselves and with other microbial groups in the rumen is also responsible for synergistic effect on the production of volatile fatty acids and microbial proteins in the rumen. Some of the common features of bacteria found in the rumen of animals fed on high roughage diet are as follows:

- Majority of the bacteria are Gram-negative. The number of Gram-positive bacteria tends to increase on increasing high energy diets in the ration.
- Most of the bacteria are obligate anaerobes. Some of them are so sensitive to oxygen that these are killed on exposure to oxygen. A few rumen bacteria require a very low redox potential (indicating a high degree of anaerobiosis) and grow at a redox potential lower than –350 mV.
- The optimum pH for the growth of rumen bacteria lies between 6.0 and 6.9.
- The optimum temperature is 39ºC.

- The bacteria can tolerate a considerably higher level of organic acids without affecting adversely their metabolism.

Table 1.1: Bacterial diversity of the rumen microbial ecosystem of domestic and wild animals

Substrate	Bacteria	Reference
Bacteria active in carbohydrate utilization		
	Fibrobacter succinogenes	Hungate, 1950 ; Flint *et al*.,
	(Bacteroides succinogenes)	1990
Cellulose	*Ruminococcus flavefaciens*	Dehority, 1986; Dehority and
	Ruminococcus albus	Scott,1967; Bryant, 1986
	Clostridium cellobioparum	Hungate, 1944
	Clostridium longisporum	Hungate, 1957
	Clostridium lochheadii	
	Eubacterium cellulosolvens	Bryant *et al*., 1958; Van
	(Cillobacterium cellulosolvens)	Gylswyk and Hoffman, 1970
	Butyrivibrio fibrisolvens	Bryant and Burkey, 1953;
		Bryant and Small, 1956
Hemicellulose	*Prevotella ruminicola*	Cotta, 1992
	(Bacteroides ruminicola)	
	Eubacterium xylanophilum,	Van Gylswyk and Van der
	E. uniformis	Toorn, 1985
	Streptococcus bovis	Latham *et al.*, 1979
	Ruminobacter amylophilus	Hamlin and Hungate, 1956;
Starch	*(Bacteroides amylophilus)*	Stackebrandt and Hippe ,1986
	Prevetella ruminicola	Cotta,1992
	(Bacteroides ruminicola)	
	Succinivibrio dextrinosolvens	Bryant and Small, 1956
	Succinivibrio amylolytica	Bryant *et al*., 1958
	Selenomonas ruminantium	Caldwell and Bryant,1966
	Lactobacillus acidophilus,	Hungate ,1966; Stewart,1992

	L.casei, L.fermentum,	
Sugars/dextrins	*L. plantarum, L. brevis,*	
	L. helveticus	
	Bifidobacterium globosum, B.	Scardovi,1981
	longum, B. thermophilum	
	B. ruminale	Scardovi *et al*., 1969
	B. ruminantium	Biavati and Mattarelli,1991
Pectin	*Treponema saccharophilum*	Paster and Canale-Parola, 1985 ; Bryant *et al.,* 1960
	Lachnospira multiparus	Bryant and Small, 1956
Bacteria active in nitrogen utilization		
Protein	*Prevotella ruminicola*	Hobson and Howard, 1969
degraders	*Ruminobacter amylophilus*	
	Clostridium bifermentans	
Urea	*Megasphaera elsdenii*	Allison,1978; Wallace,1986
hydrolysers		
Other Bacteria		
	Megasphaera elsdenii	Elsden *et al.,* 1956; Hobson *et*
	(Peptostreptococcus elsdenii)	*al* ., 1958
	Wollinella succinogenes	Wolin *et al.,* 1961
	(Vibrio succinogenes)	
	Veillonella gazogenes	Johns, 1951
Acid Utilizers	*(Veillonella alcalescens,*	
	Micrococcus lactolytica)	
	Oxalobacter formigenes	Allison *et al* .,1985
	Desulphovibrio desulphuricans	Bennink and Bryant,1973; Howard and Hungate,1976
	Desulphatomaculum ruminis	Campbell and Singleton,1986
	Succiniclasticum ruminis	Van Gylswyk,1995
Lipolytic	*Anaerovibrio lipolytica*	Hobson and Mann,1961
bacteria		
Acetogenic	*Eubacterium limosum*	Sharak-Genthner *et al*.,1981

bacteria	*Acetitomaculum ruminis*	Greening and Leedle, 1989
Tannin	*Streptococcus caprinus*	Brooker *et al*., 1994
degraders	*Eubacterium oxidoreducens*	Krumholz and Bryant,1986
Mimosine degrader	*Synergistes jonesii*	Allison *et al*., 1992
	Methanobrevibacter ruminantium	Lovely *et al*.,1984; Miller *et al* .,1986
	Methanobacterium formicicum	
Methanogenic archea	*Methanosarcina barkeri*	Kandler and Hippe, 1977; Patterson and Hespell, 1979
	Methanomicrobium mobile	Paynter and Hungate, 1968
Mycoplasma	*Anaeroplasma bactoclasticum*	Robinson and Hungate,1973
	Anaeroplasma abactoclasticum	Robinson *et al*., 1975

Archeaeal ecology

Members of the domain Archaea contribute about 0.3 to 3.3% of the microbial small subunit (16S) rRNA in the rumen (Ludwig *et al*., 2004). They are obligate anaerobes and can be unambiguously differentiated from other organisms since they all produce methane as a major catabolic product. Most species of methanogens can grow using H_2 and often formate as their energy sources and use the electrons derived from H_2 (or formate) to reduce CO_2 to CH_4. Some species can grow with methyl groups, oxidizing some to CO_2 to produce electrons that are used to reduce further methyl groups to methane. A few species can grow with acetate, effectively dissimilating acetate to CH_4 and CO_2.

The methanogens play a vital role in the rumen of scavenging molecular hydrogen generated during rumen fermentation, thereby making rumen fermentation a continuous process, but this leads to a significant loss of gross energy consumed by the animals. There is a close association between entodiniomorphid protozoa and some of the bacteria, which attach to the pellicle of protozoa. Eleven species of entodiniomorphid protozoa have been found to have adhered methanogens like *Entodinium longinucleatum, Eudiplodinium maggii, Entodinium bursa* and *Eremoplastron bovis*. The methanogens attach themselves with the ciliate protozoa to get a constant supply of hydrogen. On pumping hydrogen in the rumen, the

methanogens get detached from the protozoa (Stumm *et al*., 1982). Association of methanogenic arhaea with protozoa species has been also reported in bovine rumen (Regensbogenova *et al.,* 2004c).

Seven different species representing five genera of methanogens have been reported from the rumen of different animals, i.e. *Methanobacterium formicicum*, *Methanobacterium bryanti, Methanobrevibacter ruminantium, Methanobrevibacter smithii, Methanomicrobium mobile, Methanosarcina barkeri* and *Methanoculleus olentangyi* (Joblin *et al*., 1990; Jarvis *et al*., 2000). Methanogens are present in the rumen in large numbers varying from 10^7 to 10^9 cells/ml of rumen liquor depending upon the type of diet given to the animals, especially the fiber content in the ration.

Protozoal ecology

The rumen protozoa were first time reported to be in domestic animals as early as nineteenth century by Gruby and Delafond (1843). Not much work was done for several decades after their first report on the rumen. It was only after 1920 that the researchers paid any significant attention towards the identification, morphology and biochemical functions of protozoa in the rumen.

Microscopic observations reveal that the endosymbiotic ciliates account for about 50% of the total microbial mass in the rumen: up to 100 billion of ciliates may populate the rumen of a single cow. These ciliates play an important role in fiber digestion and the modulation of the fermentation profiles. Classical (morphological) studies have identified more than 250 species of ciliates living in the various ruminants (Williams and Coleman, 1992; Imai, 1998). The number of species in an individual host is known to be highly variable, but the average number of species has been reported about 20 in cattle, but less in sheep (Imai *et al.,* 1989) and goats (Ito *et al.,* 1995). However, as in all protists, the limited number of distinct morphological traits hampers an assessment of the ciliate diversity in the rumen. Molecular studies on rumen ciliates are rare, mainly due to difficulties in culturing these ciliates *in vitro.* Staay *et al.,* (2006) described a molecular approach to study the phylogenetic diversity of rumen protozoa. The diversity of rumen protozoa was analysed by sequencing of 18S rDNA libraries that have been created from PCR-amplified DNA of total rumen contents of cow, sheep, and a goat. These sequences were compared with the sequences of rumen ciliates obtained from GenBank Database. The

phylogenetic analysis suggests that the ciliate population of the ruminal ecosystem can be much more diverse than previously assumed.

There are hundreds of species of ciliate protozoa classified within many different genera; however, commonly identified ruminal species are primarily classified in the family's Isotrichidae and Ophryoscolecidae (Dehority, 2003). The isotrichids are completely covered by cilia whereas the Ophryoscolecids contain ciliary zones only at the anterior ends. Morphological classification was used based on cilia positioning, number of ciliary zones, as well as the presence and number of skeletal plates and caudle spines. Genus complexity was determined by nutrition and morphology, with the *entodinium* identified as the simplest genus and the subfamily Ophryoscolecinae containing the five genera, Epidinium, Epiplastron, Opisthotrichum, Ophryoscolex and Caloscolex as the most complex (Dehority, 2003).

Alternatively these can also be classified as soluble sugar utilizers, starch degraders and lignocellulose hydrolysers. The holotrich protozoa are represented by 15 different genera in the rumen of different animals. Among these genera, Isotricha, Dasytricha, Buetschlia and Charonina are some which are widely distributed in the rumen of domestic and wild ruminants and hind gut fermentors (Dehority, 1986; Eloff and Van Hoven, 1980). Several protozoa species has been reported in different ruminant (Table1.2). Some of the non-ruminant pre-gastric fermentors like camel and hippopotamus also have a large number of holotrich protozoa (Dogiel, 1928; Thurston and Grain, 1971). The enzymatic profile of holotrich protozoa indicates that they have amylase, invertase, pectin esterase and polygalacturonase in sufficiently large quantities for using starch, pectin and soluble sugars as energy source (Mould and Thomas, 1958; Abou Akkada and Howard, 1960; Bailey and Howard, 1963 and Williams, 1979).The enzymes responsible for cellulose and hemicellulose degradation have also been reported in the holotrich protozoa but the levels are very low compared to those present in the entodiniomorphid protozoa (Williams and Coleman, 1985).

Table 1.2: Protozoa diversity of the rumen microbial ecosystem of domestic and wild animals.

Protozoa	**Host**	**Reference**
Holotrich Protozoa		
	Sheep	Giesecke,1970
	Zebu cattle	Bhatia, 1936
Isotricha prostoma	Black buck	Kamra, *et al*.,1991
	Water buffalo	Dehority,1979
	Red deer	Brüggeman *et al*.,1967
	Goat	Das Gupta,1935
I. intestinalis	Cattle	Clarke,1964
	Bison	Giesecke,1970
	Mouse deer	Bhatia, 1936
	Red deer	Brüggeman *et al*., 1967
Dasytricha	Sheep	Asada *et al*., 1980
ruminantium	Black buck	Kamra *et al.,* 1991
	Water buffalo	Dehority,1979
Oligoisotricha	Water buffalo	Imai, 1981
bubali	Cattle	Dehority *et al.*, 1983
Entodinomorphid protozoa		
Entodinium bovis	Yugoslavian cattle	Wertheim, 1935
	Zebu cattle and water buffalo	Imai and Ogimoto,1984
E.bubalum	Water buffalo	Dehority,1979
E. bursa	Cattle, sheep and goat	Coleman *et al.*, 1977
E. caudatum	Cattle, sheep and goat	Coleman, 1980
E. chatterjeei	Indian goat	Das Gupta, 1935
	Water buffalo	Imai and Ogimoto,

		1984
E. longinucleatum	Cattle, water buffalo	Imai, and Ogimoto, 1984
Diplodinium dendatum	Widely distributed	Clarke, 1964
D. indicum	Indian cattle	Banerjee, 1955
Eremoplastron asiaticus	Indian cattle	Banerjee, 1955
E. bubalus	Brazillian cattle and water buffalo	Dehority, 1979
Eudiplodinium maggii	Widely distributed	Becker and Talbott,1927
Ostracodinium trivesiculatum	Cattle and water buffalo	Dehority, 1979
Polyplastron multivesiculatum	Cattle, sheep, goat, blackbuck	Kamra *et al.,* 1991; Bush and Kofoid,1948; Abou Akkada *et al*., 1969; Imai *et al*, 1978
Metadinium medium	Cattle Water buffalo African reedbuck	Imai *et al*., 1979 Dehority, 1979 Van Hoven, 1983
Epidinium caudatum	Cattle, sheep, goat	Imai *et al*., 1979
Ophryoscolex caudatus	Widely distributed	Das Gupta, 1935; Bush and Kofoid, 1948; Imai *et al.*, 1978
Caloscolex camelicus	Dromedary camel	Dogiel, 1927

The major nutritional effect of the ciliate protozoa is to change the ratio of protein to energy in the nutrients absorbed, with faunated animals having lower protein and higher energy availabilities compared with ciliate-free ruminants. Of the nutrients available for absorption, the ciliates have no consistent effect on the proportions of volatile fatty acids or amino acids. However, there is evidences that hydrogenation of lipids is increased, as is the supply of choline, and that the bioavailability of copper is reduced by the presence of ciliates.

Defaunation of young growing ruminants, that are fed high energy diets containing low levels of ruminal non degradable protein, results in increased growth rate and feed efficiency. It is unlikely, with the possible exception of wool growth, that there is other situations in which defaunation will be beneficial; and it is more likely to be detrimental to animal productivity. It remains to be determined whether manipulation of the types of ciliate protozoa in the rumen could improve animal performance.

Fungal ecology

The flagellates were observed in the rumen as early as 1910, but were believed to be flagellate protozoa (Liebetanz, 1910; Braune, 1913) and were placed in the genera *Callimastix, Sphaeromonas, Oikomonas*, etc. These flagellates were discovered as fungi for the first time in mid-seventies by Orpin (1975), who identified it as *Neocallimastix frontalis.* The flagellate zoospore grew into a mycelium which in turn transformed into a reproductive stage of rhizoids bearing zoosporangium. This was confirmed to be a true fungus by the presence of chitin in its cell wall by Orpin (1977a). The isolated organism was similar in life cycle and morphology to a chytridiomycete fungus, but this was a first report that it was a strict anaerobic fungus. Several strains of anaerobic fungi have been reported in the rumen of different ruminant species (Table 1.3). These obligate anaerobic fungi, found in the rumen and other parts of the gastro-intestinal tract of herbivorous animals, have an active and positive role to play in fiber degradation as evidenced by the presence of different enzymes involved in fiber degradation (Williams and Orpin, 1987; Paul *et al.*, 2003).

Table 1.3: Fungal diversity of the rumen microbial ecosystem of domestic and wild animals

Fungus	Source of isolation	Reference
Neocallimastix frontalis	Cow	Orpin, 1977b
N. patriciarum	Sheep	Orpin and Mann, 1986
N.hurleyensis	Cattle	Webb and Theodorou,1988
Sphaeromonas communis (caecomyces communis)	Cattle	Orpin, 1976; Wubah and Fuller, 1991
Caecomyces equi	Horse	Gold *et al*., 1988
Orpinomyces bovis	Cattle	Barr *et al*., 1989
Anaeromyces mucronatus (Ruminomyces mucronatus)	Cattle	Breton *et al*, 1990
Ruminomyces elegans	Cattle	Ho and Bauchop, 1990
Piromyces communis, Piromyce smae, Piromyces dumbonica	Horse, Elephant	Li *et al*, 1990

The enzyme profile of various fungi studied indicates that a wide variety of enzymes required for lingo-cellulose degradation are excreted. In addition it is confirmed by the scanning electron microscopic studies that these fungi prefer to get attached to the most lignified tissues of plant feed material (Akin *et al*., 1987). The fibre-based diets stimulate the fungal growth in the rumen of buffalo (Kamra *et al*., 2003) in comparison to diets rich in easily fermentable carbohydrates. Pelletted diets usually have a shorter transit time through the gastro-intestinal tract, and thereafter do not support the growth of anaerobic fungi in the rumen. Large quantities of soluble sugars inhibit the germination of zoospores on plant tissues (Roger *et al*., 1990). This might be due to lowering of pH of the rumen liquor in the presence of high sugar concentration, which inhibits the production of zoospores in the rumen (Orpin, 1977a). Because of the presence of different enzymes like proteases and esterases in addition to cellulases and hemicellulases, the fungi have an additional advantage of

better penetration of the lignocellulosic feeds over the cellulose-degrading bacteria (Fonty and Joblin, 1990).

Bacteriophage ecology

Bacteriophages are the viruses of bacteria and are reported to be present in the rumen in large numbers. They are specific for different bacteria present in the rumen. They are also considered to be obligate pathogens for the bacteria as bacteriophages are capable of lysing bacteria. These phages help in bacterial mass turnover in the rumen, which may be considered not so useful for the animals on different feeding schedules (Klieve and Swain, 1993), but by lysing the bacterial cells, the bacterial protein is easily made available to the animals as a source of amino acids. The specificity of the bacteriophages for a particular rumen bacterium may be exploited for removal or killing by lysis of unwanted rumen bacteria from the ecosystem like *Streptococcus bovis* and methanogens (Klieve *et al*., 1999; Bach *et al*., 2002). The phage population in an animal at any time is specific for that animal as the animals kept on similar diet penned together in the same shed may have diverse population of these phages. The diurnal variation in the numbers of phage particles is also very diverse. There is a drop in numbers immediately on feeding followed by a gradual increase up to 8–10 hr post feeding and then decline to reach the base level (Swain *et al*., 1996).

Application of recent DNA/RNA-based techniques in rumen ecology

Conventional culture-based methods of enumerating rumen microorganisms (bacteria, archaea, protozoa, and fungi) are being rapidly replaced by the development of nucleic acid-based techniques which can be used to characterize complex microbial communities. The foundation of these techniques is 16S/18S rDNA sequence analysis which has provided a phylogenetically based classification scheme for enumeration and identification of microbial community members. While these analyses are very informative for determining the composition of the microbial community and monitoring changes in population size, they can only infer function based on these observations. The next step in functional analysis of the ecosystem is to measure how specific and, or, predominant members of the ecosystem are operating and interacting with other groups. It is also apparent that techniques which optimize the analysis of complex microbial communities rather than the detection of single organisms will

need to address the issues of high throughput analysis using many primers/probes in a single sample. Nearly all the molecular ecological techniques are dependent upon the efficient extraction of high quality DNA/RNA representing the diversity of ruminal microbial communities. Commonly used DNA-based techniques that can be used to describe changes in the rumen microbial ecosystem are shown in Table 1.4.

Table 1.4: DNA-based techniques that can be used to describe changes in the rumen microbial ecosystem

Approach	Target	Outcome of experiment	Main limitations
16S rRNA gene sequencing	16S rRNA gene	16S rRNA gene sequence collection	Bias in Nucleic Acid extraction, PCR and cloning; laborious.
BAC vector cloning	Genomic DNA	Gene sequences	Bias in Nucleic Acid extraction and cloning; laborious
Diversity arrays	16S rRNA genes; antibiotic resistance genes	Diversity profiles	Laborious; expensive; in early stages of development
DNA microarray	mRNA	Transcriptional fingerprints	Bias in Nucleic Acid extraction and labeling; expensive
Dot-blot hybridization	16S rRNA	Relative abundance of 16S rRNA	Laborious at species level; requires 16S rRNA gene sequence data
Fingerprinting	16S rRNA gene	Diversity profiles	Bias in Nucleic Acid extraction and PCR

FISH	16S rRNA	Enumeration of bacterial populations	Laborious at species level; requires 16S rRNA gene sequence data
In situ isotope tracking	Labelled biomarkers	Identification of substrate utilizing microbes	Only suitable for simple pathways
Non-16S rRNA gene fingerprinting	Genomic DNA; cellular fatty acids	Diversity profiles	16S rRNA approaches required for identification
Probe based cell sorting	Genomic DNA, Plasmid DNA, rRNA	Sorted cells containing certain gene sequences	Dependent on sequence data
Real-Time PCR	16S rRNA gene	Relative abundance of 16S rRNA genes	expensive in early stages of development
RT-PCR	mRNA	Specific gene expression	Bias in Nucleic Acid extraction and RT-PCR
Subtractive hybridization	Genomic DNA	Unique gene sequences	Bias in Nucleic Acid extraction, sensitive for false positives

Small Sub Unit rRNA gene (Phylogenetic marker)

This tool for classifying organisms and evaluating their evolutionary relatedness was first developed by Woese (1987). Sequences of this gene are highly conserved and this gene is universally present in all organisms (Ward *et al*., 1992). The 16SrRNA gene is used for phylogenetic studies (Weisburg et al, 1991) as it is highly conserved between different species of bacteria and archaea (Coenye and Vandamme, 2003) In addition to highly conserved primer binding sites, 16S rRNA

gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. As a result, 16S rRNA gene sequencing has become prevalent in microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification. It has proved that it seems to behave as a molecular chronometer. Although the absolute rate of change in the 16S rRNA gene sequence is not known, it does mark evolutionary distance and relatedness of organisms (Harmsen and karch, 2004). It consist of both conserved and variable region, conserved region provide relatedness where as variable region provide distantness among species (specific variable regions). The gene show apparent lack of gene transfer. SSU rRNA gene just 1,542 nucleotide bases thus can be cheaply and easily copied and sequenced (O'Sullivan, 2000) The available database of rRNA sequences is now extensive, which allows detailed studies to be made on the phylogenetic position of unknown isolates. This molecular phylogeny approach has revolutionized the field of microbial ecology and has allowed meaningful phylogenetic relationships between microbes in natural ecosystems to be discerned (Olsen *et al.*, 1994). Technically, this is very feasible as the polymerase chain reaction (PCR) can be used to directly amplify gene directly from colonies using primers which are directed at universally conserved regions at both ends of the gene. GenBank, the largest databank of nucleotide sequences, has over 20 million deposited sequences, of which over 90,000 are of 16S rRNA gene (Clarridge, 2004). SSU rRNA has revolutionized the field of microbial ecology and allowed meaningful phylogenetic relationships between microbes (Olsen *et al*., 1994). Sequencing used to identify slow growing, unusual and fastidious bacteria is poorly identified by conventional method.

The small subunit (SSU) 18S rRNA gene is one of the most frequently used genes in phylogenetic studies and an important marker for random target polymerase chain reaction (PCR) in environmental biodiversity screening (Meyer *et al*., 2010). In general, rRNA gene sequences are easy to access due to highly conserved flanking regions allowing for the use of universal primers. (Meyer et al, 2010). Their repetitive arrangement within the genome provides excessive amounts of template DNA for PCR, even in smallest organisms. The 18S gene is part of the ribosomal functional core and is exposed to similar selective forces in all living beings.

CURRENT STATUS OF THE WORK

International Status

Considerable work has been carried out by various scientists on molecular ecological analysis of rumen microbiota.

Attwood *et al*., (1998) isolated cloned fragments of genomic DNA from the ruminal anaerobe *bacteroides ruminicola* subsp. *brevis* B14 and used as hybridization probes to identify closely related bacterial species. The results of this work demonstrated the feasibility of using gene probes to study changes in populations of major species of bacteria in the rumen and underline some of the difficulties, which may be encountered while attempting to introduce new bacterial strains. On the other hand, a *B. ruminicola* subsp. *brevis* specific bacteriocin may prove to be an advantage in gene transfer experiments if the toxin were to be plasmid encoded. Since producer strains are usually resistant to bacteriocin attack, if *B. ruminicola subsp. brevis B14* cells were transformed with a bacteriocin producing plasmid, expression would provide a natural positive selection system in the rumen. A vector constructed from such a plasmid may form the basis of a new genetic transformation system for ruminal bacteria.

Fibrobacter succinogenes has been considered as one of the most actively fibrolytic bacteria in the rumen; as this species possesses a variety of fibrolytic enzymes and is detected in the rumen at high density. Amann *et al*., (1992) grouped the 10 isolates of *F. succinogenes* into 4 different phylogenetic groups, based on comparative sequence analysis of 16S rRNA gene (16S rDNA). A few differences in phenotypic characters among these 4 groups have been reported. Further Koike *et al*., (2004) studied the ruminal distribution of cellulolytic bacteria. *Fibrobacter succinogenes* in relation to its phylogenetic grouping, to investigate the ecological importance of the cellulolytic bacterium *Fibrobacter succinogenes* in fiber digestion, based on competitive Polymerase Chain Reaction (cPCR), and Restriction Fragment Length Polymorphism of PCR-products (PCR-RFLP) targeted the bacterial 16S rDNA. The results of the study suggested that there could be phenotypic differences among the phylogenetic groups of *F.succinogenes* and that group I might contribute to rumen fiber digestion more than the other groups.

McSweeney *et al*., (1999) studied isolation and characterization of proteolytic bacteria from sheep and goats fed tannin rich shrub *Callindlra calothyrsus*. Tannins reduce the availability of nitrogen to ruminants but rumen bacteria that ferment proteins in presence of tannin may benefit digestion of proteins in rumen. Bacteria from sheep and goats fed Callindlra (6% condensed tannins) were isolated on proteinaceous agar medium with condensed or hydrolysable tannins. Fifteen genotypes were identified, based on 16S ribosomal DNA restriction fragment length polymorphism analysis, and all were proteolytic and fermented peptides to ammonia. A diverse growth of proteolytic and peptidolytic bacteria were present in rumen but isolates could not digest protein complexed with condensed tannin.

Asanuma and Hino, (2000) studied molecular characters, enzyme properties and transcriptional regulation of Phoshoenolpyruvate carboxy kinase (PcK) and pyruvate kinase (PyK) in *Selenomonas ruminantium.* Methanogenesis and propionate production are inversely related; hence efforts are made to decrease methane production by increasing propionate production. *S. ruminantium* is one of the propionate producers in rumen, but its ability of carbohydrate fermentation is affected by PEP carboxyl kinase (PcK) and pyruvate kinase (PyK) activities. Authors studied how synthesis of PcK and PyK in *S. ruminantium* is regulated at transcriptional level. They analyzed the gene encoding PcK and PyK with primer extension analysis. In a similar study Asanuma *et al.,* (2001), studied characterization and transcription of genes encoding enzymes involved in butyrate production in *Butyrivibrio fibrisolvens*.

Tajima *et al*., (2001a) studied diet dependent shift in the bacterial population in rumen with RT PCR. A set of PCR primers was designed for detection and quantification of different species of rumen bacteria. Using these primers and the RT PCR technique, the corresponding species in the rumen of cows from which the diet was shifted from hay to grain were quantitatively monitored. The dynamics of most of the fibrolytic species revealed significant reduction in quantity of DNA concentration.

Streptococcus bovis is considered a key lactic acid producer in rumen responsible for causing acidosis. Ghaii *et al*., (2004) studied characterization of *S. bovis* from rumen of camel and deer. The bacteria isolated and cultured on MRS agar medium were confirmed by comparative DNA sequence analysis of 16S rRNA gene

with the homologous sequence from *S. bovis* strain. The percentage similarities among the strains were > 99%.

Stahl *et al*., (1988) used phylogenetically based Hybridization Probes to study the effect of feeding of the ionophore antibiotic monensin on ruminal microbial ecology. Species- and group-specific 16S rRNA-targeted oligonucleotide hybridization probes were developed to enumerate various strains of *Bacteroides succinogenes* and *Lachnospira multiparus*-like organisms in the bovine rumen before, during, and after perturbation of that ecosystem by the addition of the ionophore antibiotic monensin. Based on probe hybridization, relative numbers of *L. multiparus*-like organisms were depressed about 2-fold during monensin addition and demonstrated a transient 5 to 10 fold increases immediately after removal of the antibiotic from the diet. The most pronounced population changes were observed among different strains of *B. succinogenes*, as evaluated by three hybridization probes. The results demonstrated the utility of rRNA-targeted hybridization to monitor complex microbial communities and are most consistent with the hypothesis that altered microbial metabolism, rather than altered population composition, is primarily responsible for changes in ruminal fermentation patterns resulting from the feeding of monensin.

Reilly *et al.,* (2002) designed 16S ribosomal DNA-directed primers in competitive PCRs to enumerate proteolytic bacteria in the rumen of cows fed four different diets. Competitive Polymerase Chain Reaction primers were designed for *Streptococcus*, *B. fibrisolvens, P. bryantii, Eubacterium sp., Prevotella*, and a universal primer for the eubacteria. DNA was extracted from rumen contents collected from eight dairy cows fed four diets: adequate nitrogen, adequate nitrogen plus carbohydrate, low nitrogen, and low nitrogen plus carbohydrate. Results revealed that the *B. fibrisolvens* was significantly higher on the adequate nitrogen plus carbohydrate and the low nitrogen plus carbohydrate diets compared with the other diets, while *P. bryantii* was significantly higher on the low nitrogen plus carbohydrate diet compared with the adequate nitrogen diet. The population of *Eubacterium sp*. was significantly lower on the adequate nitrogen plus carbohydrate and low nitrogen plus carbohydrate diets. Streptococcus populations were significantly lower on the low nitrogen plus carbohydrate diet compared with all three other diets, whereas there

were no significant differences in populations of *Prevotella* or total eubacteria on any of the diets.

Mrazek *et al.,* (2006) studied diet dependent shifts in ruminal butyrate producing *Butyrivibrio* and *Pseudobutyrivibrio* populations by competitive PCR. The goal of this experiment was to develop a simple and powerful method for the detection and enumeration of isolates belonging to the genera *Butyrivibrio* and *Pseudobutyrivibrio* in environmental samples. Specific primes were designed targeting 16S rDNA gene and competitive PCRs developed for both groups. Results (from 4 cows with different diets) suggested that high-fibre intake essentially increases the *Butyrivibrio* amounts in the rumen, whereas high energy food additives lead to its suppression. The *Pseudobutyrivibrio* concentration also changed during the experiment but without any significant relation to the host's diet.

Nelson *et al.,* (2003) studied the phylogenetic analysis of the microbial populations in the wild herbivore gastrointestinal tract of three wild African ruminant species namely eland (*Taurotragus oryx*), Thompson's gazelle (*Gazella rufifrons*) and Grant's gazelle (*Gazella granti*). The sequences were generally affiliated with four major bacterial phyla, the majority being members of the *Firmicutes* (low G+C Grampositives) related to the genera *Clostridium* and *Ruminococcus*. By contrast, with earlier studies using 16S rDNA sequences to assess biodiversity in *Bos taurus* dairy cattle, Gram-negative bacteria in the *Bacteroidales (Prevotella-Bacteroides gr*oup) were poorly represented.

McSweeney *et al.,* (2003) reported that Sheep fed 30% *Calliandra* (6% tannins) had significantly fewer primary fiber-degrading (cellulolytic) bacteria than those supplemented with 30% Lucerne when estimated by both conventional enumeration techniques and molecular techniques. Substitution of Lucerne with *Calliandra* in the diet resulted in a significant decrease in the relative abundance of populations of *Fibrobacter succinogenes* (5.9 vs 2.7%). A 16S rDNA probe to the *Bacteroides-Porphyromonas-Prevotella* group, which includes many proteolytic bacteria, also demonstrated that this population was not significantly different between animals supplemented with either *Calliandra* or Lucerne (12.9 vs 11.6%).

Koike *et al*., (2003) studied the ruminal distribution of cellulolytic bacteria *Fibrobacter succinogenes* in relation to its phylogenetic grouping to investigate the

ecological importance of the cellulolytic bacterium *F. succinogenes* in fiber digestion. This was based on competitive Polymerase Chain Reaction (cPCR) and Restriction Fragment Length Polymorphism of PCR-products (PCR-RFLP) targeted the bacterial 16S rDNA.

Koike *et al*., (2004) validated rumen bacterial species using real time PCR. They found significant diet dependent shift in bacterial ecology when fed Rice straw. Within 24 hrs of feeding the fibrolytic bacteria, *Fibrobacter succinogenes* was found to be dominant.

An *et al.,* (2005) studied prokaryote diversity in the rumen of yak (*Bos grunniens*) and Jinnan cattle (*Bos taurus*) by estimating 16S rDNA homology analysis. The sequence analysis indicated that more than half of the species harbored in yak rumen belonged to the not-yet-cultured groups at 90% 16S rDNA similarity levels with cultured species, while 36% 16S rDNA sequences amplified from the rumen of Jinnan cattle fell in these catalogues. By comparing the uncultured sequences in yak rumen with those in Jinnan cattle and cow, the former formed distinct clusters loosely related to the later, implying that yak rumen could harbor some special prokaryote phyla. 10.8% sequences retrieved in yak rumen were related to the known rumen fibrolytic bacterial species; however none was related to the known amylolysis species. While 4% and 17.8% sequences retrieved from Jinnan cattle rumen were related to cultured fibrolytic and amylolysis species, respectively.

Stevenson and Weimer, (2007) quantified several bacterial species in ruminal samples from two lactating cows. Bacterial populations showed a clear predominance of members of the genus Prevotella, which comprised 42% to 60% of the bacterial rRNA gene copies in the samples. However, only 2% to 4% of the bacterial rRNA gene copies were represented by the classical ruminal Prevotella species *Prevotella bryantii*, *Prevotella ruminicola* and *Prevotella brevis*. The proportion of rRNA gene copies attributable to *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Selenomonas ruminantium* and *Succinivibrio dextrinosolvens* were generally in the 0.5% to 1% range.

Wanapat and Cherdthon, (2009) determined the population of major ruminal bacterial species (*Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens*) in digesta and rumen fluid of swamp buffalo (*Bubalus bubalis*) fed

roughage and concentarte. It was found that feeding urea-treated rice straw solely increased these three cellulolytic bacteria numbers up to 2.65 x 10^9 and 3.54 x 10^9 copies per milliliter of *F. succinogenes*, 5.10 x 10^7 and 7.40 x 10^7 copies per millilter for *R. Flavefaciens*, and 4.00 x 10^6 and 6.00 x 10^6 copies per milliliter for *R. albus* in rumen fluid and digesta, respectively.

Gou *et al*., (2010) evaluated the microbial population in ruminal fluid using real time PCR in steers treated with virginiamycin (WM) and found that VM had selective effects on ruminal bacteria and influenced ruminal fermentation by changing a part of the specific ruminal bacteria populations.

Pitta *et al*., (2010) studied rumen bacterial diversity dynamics associated with changing from bermudagrass hay to grazed winter wheat diets using 16S based bTEFAP pyrosequencing technique in steers. Predominant genera were *Prevotella* (up to 33%) and *Rikenella*-like (up to 28%) genera on the bermudagrass diet and Prevotella (up to 56%) genus on the wheat diet irrespective of the fractions. Principle component analyses accounted for over 95% of variation in 16S estimated bacterial community composition in all three fractions and clearly differentiated communities associated with each diet. Overall, bermudagrass hay diets clustered more clearly than wheat diets.

The archaeal community compositions uncovered in the different studies varied. This may be attributable to the ruminant host or the diet, or to the DNA extraction methods and PCR primers used. Two of the studies were performed using the same DNA extraction methods and the same PCR primers (Wright *et al.,* 2004; Wright *et al.,* 2006). Both assessed archaeal diversity in the rumen of sheep. The rumen archaea of sheep held at the CSIRO (Commonwealth Scientific and Industrial Research Organisation) Yalanbee Research Station in Western Australia were dominated by members of the *Mbb. gottschalkii* (75.3%) and *Mbb. ruminantium* (19.5%) clades. In contrast, sheep in Queensland, Australia, had archaeal populations dominated by members of uncultured rumen archaea RCC clade (80.8%), and had only few members of the *Mbb. gottschalkii* clade (9.0%), no detectable members of the *Mbb. ruminantium* clade, and some members of the *Methanomicrobium* clade (7.7%). The differences in community composition could have been caused by

differences in diet, environment, health, animal genotype, and animal age (McSweeney, *et al.,* 2007).

Wright *et al.,* (2007) found that *Methanobrevibacter* spp. (50.0 to 51.9%) and members of RCC (37.8 to 50.0%) dominated in feedlot cattle in two geographic locations in Canada, while sheep in Australia and Venezuela shared very similar archaeal communities (Wright *et al.,* 2008). Whether these, and other, differences and similarities have a methodological basis, or are host-based, or are controlled by diet or animal management choices, still remains to be elucidated.

Temporal temperature gradient gel electrophoretic separation of 16S rRNA genes amplified by PCR from total rumen content of pasture fed sheep and cows, followed by sequencing prominent bands, confirmed the abundance of members of the *Mbb. ruminantium*, *Mbb. gottschalkii*, and uncultured archaea (RCC) clades (Nicholson *et al.,* 2007).

Tatsuoka, *et al.,* (2004) and Denman *et al.,* (2007) surveyed diversity of methyl coenzyme M reductase (*mcrA*) gene in the rumens of cattle. This enzyme and its gene are good markers for the presence of methanogens (Friedrich, 2005). In both studies, *mcrA* genes from *Methanobrevibacter* spp. dominated the libraries generated using primers that targeted most *mcrA* sequence types. Denman *et al.,* (2007) also detected a group of *mcr*1 A sequences belonging to an unidentified group of archaea which was also detected in landfill by Luton *et al.,* (2002). No *mcrA* genes clearly assignable to the *Methanococcales* were detected in two studies on the rumen. The methanogens that have been cultured from the rumen fall into the major clades detected in the global dataset, with the exception of *Methanosarcina* spp. (Beijer 1952; Patterson, and Hespell, 1979) and *Methanoculleus olentangyi* (Joblin, 2005). The 16S rRNA gene-based approach can be expected to detect mainly large populations. At present, the interpretation must be that *Methanosarcina* spp. and *Methanoculleus* spp. have been found in samples from the rumen, but do not appear to be numerically significant parts of the rumen archaeal community. Methodological biases against their molecular detection could also result in their absence from libraries of PCR-amplified 16S rRNA genes, but the range of different primers used in the different studies suggests that primer-bias is unlikely.

Skillman *et al.,* (2004) studied the population densities and identities of methanogens colonising new-born lambs in a grazing flock. Methanogen colonisation was found at 1–3 days after birth and population densities reached around 10^4 methanogens per gram at 1 week of age. Population densities increased in an exponential manner to a maximum of 10^8–10^9 per gram at 3 weeks of age. The genus *Methanobrevibacter* established early in young lambs and seems to be more stable than the *Methanobacterium* populations which in several instances appeared and then disappeared as the rumen developed.

In mature bovine rumen, *Methanosarcina* spp. or *Methanomicrobium* spp. were not detected in a library of cloned methanogen 16S rRNA genes (Whitford *et al.,* 2001). A study with hybridization probes found that less than 3% of the archaeal DNA from the mature bovine rumen originated from the order *Methanosarcinales* (Sharp *et al.,* 1998). Previous studies have shown that *Methanomicrobium mobile* is the predominant methanogen in the ovine rumen (Lin *et al.,* 1997; Yanagita *et al.,* 2000). By using the fluorescent in situ hybridization (FISH) method, methanogen counts were approximately 3.6% of the total rumen microflora and approximately 54% of the total methanogens were *Methanomicrobium mobile.* Skillman *et al.,* (2004) and Morvan *et al.,* (1996) also found that methanogens colonize the lamb rumen by 30 hours after birth and reach 10^6 organisms per ml by 15 days of age. The population of methanogens may be affected by the abundance of protozoa. The order *Methanbacteriales* is associated with ruminal ciliates (Sharp *et al.,* 1998; Tokura *et al.,* 1999) and the order *Methanomicrobiales* is free-living (Sharp *et al.,*1998). Because CH_4 production is affected by the quantity and composition of feedstuffs consumed, it is a reasonable assumption that the methanogen population will likewise be affected.

Approximately 20% of ruminal methanogens are associated with protozoa, and these methanogens may account for up to 25% of ruminal methanogenesis. Based on clone library analyses, majority (93.8 %) of protozoa-associated archaea can be placed in the same three genus-level groups found to dominate the total rumen archaea, the genera *Methanobrevibacter* and *Methanomicrobium* and the RCC clade. Other genus level groupings appear to be much less abundant. There were notable variations in the abundance of different groups of protozoa associated archaea in

different studies. Members of the *Mbb. ruminantium* clade were abundant (62.6%) in the study reported by Chagan *et al.,* (1999), while *Methanomicrobium* spp. dominated in the study of Regensbogenova *et al.,* (2004c) and members of the RCC clade dominated in the study of Ohene-Adjei *et al.,* (2007).

Christophersen *et al.,* (2004) suggested that in some individual animals, the free-living and protozoa-associated archaea appeared to belong to the same species, but that in other individuals they did not. Elimination of protozoa from the rumen of sheep resulted in changes in the archaeal community (Morgavi *et al.,* 2006), but it was not clear if this was due to the disappearance of uniquely protozoa-associated methanogens, or due to broader changes in rumen function as a result of defaunation that in turn affected the total archaeal community structure. The true significance of different clades of protozoa-associated archaea could be determined by careful analysis of the two groups in fractionated rumen samples, and by using rRNA targeted FISH methods to observe the localization of cells of different archaeal groups in total rumen samples.

The anaerobic ruminal protozoa have been well studied (Williams and Coleman, 1992), but much of this work is based on microscopic examination (Dehority, 1993). Difficulties in cultivating protozoa, and their polymorphic nature, have delayed effective assessment of protozoan ecology and taxonomy (Dehority, 1994).

In India, studies on rumen microbes of buffalo are limited (Jalaludin *et al*., 1992; Punia, 1992; Sahu and Kamra, 2001) and the experiments on relative microbiology of cattle and buffalo on similar diets and same environmental conditions are rare. The total number of ciliate protozoa is lower in buffalo than that in cattle, but the ciliate protozoa represented in both the species of animals included *Entodinium, Diplodinium, Eremoplastron, Eudiplodinium, Elytroplastron, Metadinium, Ostracodinium, Epidinium, Dasytricha* and *Isotricha* (Shimizu *et al*., 1983).

Dumag, (1977) studied the ciliate protozoa in the rumen of Philippine carabaos (*Bubalus bubalis*) and found 59 different species of ciliates, out of which 21 belonged to *Entodinium*, ten to *Diplodinium,* eight to *Epidinium*, four to *Ostracodinium* and *Eremoplastron*, three to *Metadinium* and *Eodinium* and one each to *Enoploplastron, Diploplastron* and *Eudiplodinium*. Three species belonged to

holotrich protozoa, e.g. *Isotricha intestinalis, Isotricha prostoma* and *Dasytricha ruminantium.* Most of these species have also been reported in the rumen of cattle (Hungate, 1966). *Metadinium medium* has been reported to be present only in the rumen of buffalo (Jalaludin *et al*., 1992). Four new species of ciliate protozoa which have been reported to be present in the rumen of buffalo are *Entodinium ogimotoi, E. bubalum, E. fujitai* and *E. tsunodai* (Ogimoto *et al*., 1983). *Oligoisotricha bubali* has been considered to be restricted only in buffalo rumen (Imai, 1981; Franzolin and Dehority, 1999), but it has also been detected in the rumen of cattle in the areas of Tennesse, USA. This protozoan has been observed in low numbers (<1% of total protozoa) to very high numbers (>70% of total protozoa) in the rumen of cattle fed different rations (Dehority *et al*., 1983). Corn silage-concentrate diet has been found to stimulate the growth of this protozoan in the rumen of cattle. The protozoa production rate, estimated by use of 35S as indicator, showed that it was lower in buffalo (25.4–27.5 mg/min) than that in the rumen of cattle calf (30.5 mg/min) (Singh *et al*., 1974). There was no difference in the numbers of ciliate protozoa in the rumen liquor of cattle and buffalo (Srivastava and Chaturvedi, 1973; Kurar *et al*., 1988).

Dehority, (1979) studied the ciliate protozoa in the rumen of Brazilian water buffalo and found that 49 species of ciliate protozoa were present, out of which eight species were described for the first time. Four new species belonged to the genus *Diplodinium (Ostracodinium), i.e. O. brazili, O. esalqum, O. nucleolobum* and *O. tiete*; three new species of *Entodinium, i.e. E. ciculum, E. spinonucleatum and E. triangulum* and one new species of *Diplodinium (Eudiplodinium), E. bubalus* have also been reported. In addition to the above, new species of *Entodinium, Diplodinium, Ostracodinium, Eudiplodinium, Elytroplastron, Epidinium, Isotricha, Dasytricha, Charonina* and *Buetschlia* have also been found in the rumen of buffalo which are commonly found in other domestic and wild ruminants.

Clarke *et al.,* (1982) estimated that masses and numbers of rumen ciliate protozoa were markedly different in individual sheep fed chaffed alfalfa hay under different feeding regimens. In their study protozoa were counted and measured in formalized samples of rumen contents from 32 sheep individually housed indoors in metabolism crates and fed chaffed alfalfa hay (*Medicago sativa*) at two levels of dry matter (DM) intake, 1,000 or 700 g. Within each intake level there were two feeding

frequencies, hourly and once daily. The results emphasized that animals must be considered individually if a detailed analysis is to be made of the interrelations within overall rumen fermentation. This means that when certain quantitative aspects of the protozoan contribution to rumen fermentation are being considered in relation to other microbial activities, as will be required for example in modeling exercises (Baldwin *et al*., 1977), ciliate numbers and the size of the individual cells in the populations in individual animals should be taken into account.

Dehority *et al.,* (1982) detected *Oligoisotricha bubali* in rumen contents of domestic cattle (*Bos taurus*) in two different areas of Tennessee which was previously observed twice in water buffalo. Concentrations ranged from <1 to 35% of the total protozoa in unweaned calves and up to 72% in older animals in feedlot. In contrast to the other genera of holotrichs, both total numbers and percent composition of *0. bubali* increased when animals were fed a corn silage-concentrate diet.

Teather *et al.,* (1983) determined the bacterial protein content and protozoal protein content of unfractionated samples from the liquid small particle phase of the rumen on the basis of direct microscopic measurement of bacteria numbers and protozoa numbers and cell volumes. Standard values of 8.7 x 10^{-11} mg of protein per bacterial cell and 5.9 x 10^{-11} mg/µm3 of protozoa cell volume, obtained from analysis of isolated cells, were used to convert the microscopic measurements to an estimate of the protein content of the rumen sample. When the correlation between bacterial and protozoal protein levels was examined within groups of animals, a highly significant negative correlation between these two parameters was found ($p < 0.001$). The variation among animals for total (bacterial plus protozoal) microbial protein was smaller than the variation among animals for bacterial or protozoal protein alone. There was also a highly significant positive correlation ($P < 0.001$) between protozoal protein level and total microbial protein level.

Imai *et al*., (1989) detected 17 species of ciliate protozoa in Malaysian water buffalo and 15 in Kedah Kelantan cattle. There was no difference in the total number of ciliates in two species of animals. *Metadinium ypsilon* and *Ostracodinium trivesculatum* have been found to be present in both the species, but other species reported in Malaysian ruminants were similar to those reported in other tropical and temperate ruminants.

Wright *et al*., (1997) reported the use of PCR methods to amplify the small subunit (ssu) ribosomal RNA genes and intergenic spacers of several morphologically distinct ruminal protozoa. However, the eukaryotic-specific primers used in their studies with axenic cultures were not suitable for digested samples because of the presence of other eukaryotic cell. They determined three complete 18S ribosomal RNA gene sequences from the rumen ciliates, *Entodinium caudatum* (1,639 bp), *Epidinium caudatum* (1,638 bp), *and Polyplastron multivesiculatum* (1,640 bp) and confirmed in the opposite direction. Trees produced using maximum parsimony and distance-matrix methods (lest squares and neighbour-joining) with strong bootstrap support, depict the rumen ciliates as a monophyletic group, *Entodinium caudatum* is the earliest branching rumen ciliate. However, *Entodinium simplex* does not pair with *E. caudatum*, but rather with *Polyplastron multivesiculatum.* Signature sequences for these rumen ciliates reveal that the published SSrRNA gene sequence from *E. simplex* is in fact a *Polyplastron* species. The free-living haptorian ciliates, *Loxophyllum, Homalozoon and Spathidium* (Subclass Hoptoria), are monophyletic and are the sister group to the rumen cilates. The litostomes (class Litostomatea), consisting of the haptorians and the rumen ciliates, are also a monophyletic group.

Eschenlauer *et al.,* (1998) constructed a λ phage cDNA expression library from washed suspensions of the rumen ciliate protozoan, *Entodinium caudatum*, which had been maintained in an isolated, monofaunated sheep. The library was screened using an anti-*E. caudatum* antiserum raised in rabbits against sonically disrupted protozoa. DNA sequences for two centrins or caltractins, a subfamily of the EF-hand Ca^{2+}-modulated proteins which are closely related, highly conserved cytoskeletal proteins, were identified and characterized. Their phylogenetic position was established relative to other centrin gene sequences. The two proteins showed homology to *Paramecium tetraurelia* centrins: 50 and 52% identities and 71 and 75% similarities in the protein sequence, over 99 and 110 amino acids fragments. Codon usage and indices revealed that *E. caudatum* follows universal codon usage, but with a restricted number of codons, and has a low G+C content.

Wright, (1999) examined internally transcribed spacer regions 1 and 2 (ITS-1 and ITS-2) and the 5.8S ribosomal RNA gene of *Isotricha prostoma* for intraspecific sequence variation. There were no differences in the ITS-1/5.8S/ITS-2 region among

cattle and sheep isolates of *I. prostoma* from Australia, Canada, and the United States, indicating that this region is 100% conserved among eight isolates from two continents.

Gocmen and Atatur, (2000) established the presence of two holotrichous ciliate genera including three species (*Isotricha prostoma, I. intestinalis* and *Dasytricha ruminantium*) and also of seven formae *of Epidinium ecaudatum* (*Epidinium ecaudatum formae ecaudatum*, *Epidinium ecaudatum formae caudatum, Epidinium ecaudatum formae bicaudatum, Epidinium ecaudatum formae tricaudatum, Epidinium ecaudatum formae quadricaudatum, Epidinium ecaudatum formae parvicaudatum* and *Epidinium ecaudatum formae cattanei*) in goats. The mean ciliate density in the surveyed goats ($34.62x10^4$cells/ml) was lower than those of domestic sheep and cattle in Turkey and of other goats previously reported from different localities. All of the ciliates determined were first records from Turkish domestic goats. Moreover, the presence of *E. e.* f. *bicaudatum, E. e.* f. *tricaudatum, E. e.* f. *quadricaudatum* and *E. e*. f. *parvicaudatum* was recorded for the first time from the rumen of goats.

Hoek *et al.,* (2000) analyzed the SSU rRNA genes of 18 heterotrichous anaerobic ciliates and their methanogenic endosymbionts in order to unravel the evolution of the mutualistic association. Anaerobic heterotrichous ciliates (Armophoridae and Clevelandellidae) possess hydrogenosomes that generate molecular hydrogen and ATP. This intracellular source of hydrogen provides the basis for a stable endosymbiotic association with methanogenic archaea. They showed that the anaerobic heterotrichous ciliates constitute at least three evolutionary lines. One group consists predominantly of gut-dwelling ciliates, and two to three, potentially four additional clades comprise ciliates that thrive in freshwater sediments. Their methanogenic endosymbionts belong to only two different taxa that are closely related to free-living methanogenic archaea from the particular ecological niches. The close phylogenetic relationships between the endosymbionts and free-living methanogenic archaea argue for multiple acquisitions from environmental sources, notwithstanding the strictly vertical transmission of the endosymbionts. Since phylogenetic analysis of the small-subunit (SSU) rRNA genes of the hydrogenosomes of these ciliates indicates a descent from the mitochondria of aerobic ciliates, it is likely that anaerobic

heterotrichous ciliates hosted endosymbiotic methanogens prior to their radiation. Therefore, the data strongly suggest multiple acquisitions and replacements of endosymbiotic methanogenic archaea during their host's adaptation to the various ecological niches.

Gurung *et al.,* (2002) surveyed rumen ciliate composition of river-type water buffalo and goat in Nepal. The result of survey revealed 13 genera representing 52 species and 20 formae of the ciliates. Of them 13 genera with 44 species and 9 formae were found from the water buffalo and 8 genera with 21 species and 12 formae from the goat. The study showed the first report of *Hsingella triciliata, Entodinium brevispinum, E. convexum, E. javanicum, E. rectangulatum E. rectangulatum*, *E. rectangulatum E. lobosospinosum, Diplodiniumm nanum, D. psittaceum, D. sinhalicum* and *Ostracodinium quadrivesiculatum* from water buffalo and *Epidinium ecaudatum E. parvicaudatum* from goat.

Staay *et al.,* (2002) investigated the ciliate population of the hindgut of mammals using a molecular approach. They extracted DNA from rumen fluid from cattle, sheep, goat, and the European red deer *Cervus elaphus*, and from feces of hindgut fermenters (horse, elephant, and zebra). 18S rDNA clone libraries from these DNAs were constructed, and randomly chosen clones were partially sequenced. Phylogenetic analysis showed that all the gut ciliates of mammals (plus marsupials) fall within a monophyletic lineage. Clones obtained from these hindgut fermenters clusters separately from the clones found from the ruminants. In contrast to the rumen ciliates from the domestic animals, where many ciliates are shared by different hosts, their study suggested that at least a significant fraction of the ciliate populations of the various hindgut fermenters might be host specific.

Cameron *et al.,* (2003) gave an expanded phylogeny of the Entodiniomorphida Ciliophora and Litostomatea. The entodiniomorphida are a diverse and morphologically complex group of ciliates which are symbiotic within the digestive tracts of herbivorous mammals. Previous phylogenies of the group have exclusively considered members of one family, the Ophryoscolecidae, which are symbiotic within ruminants. The authors improved understanding of evolution within the entodiniomorphs by expanding the range of ciliates examined to include the Cycloposthiidae and Macropodiniidae (symbionts of equids and macropodids

respectively). The entire SSU-rRNA gene was sequenced for 3 species, *Cycloposthium edentatum*, *Macropodinium ennuensis* and *M. yalanbense*, and aligned against 14 litostome species and 2 post ciliodesmatophoran outgroup species. *Cycloposthium* was consistently grouped as the sister-taxon to the Ophryoscolecidae although support for this relationship was low. This suggests that there is more evolutionary distance between the Cycloposthiidae and Ophryoscolecidae than previously inferred from studies of gross morphology, cell ontogeny or ultrastructure. In contrast, *Macropodinium* did not group with any of the entodiniomorphs, instead forming the sister group to the entire Trichostomatia (Entodiniomorphida + Vestibuliferida). This early diverging position for the macropodiniids is concordant with their morphology and ontogeny which failed to group the family with any of the entodiniomorph suborders.

Karnati *et al.*, (2003) designed a protozoa-specific primer (P-SSU- 342f) and paired with a eukaryote-specific primer to amplify a 1,360-bp fragment of DNA encoding protozoal small subunit (SSU) ribosomal RNA from ruminal fluid of cows fed a mixed forage:grain diet or alfalfa hay. Sequencing of clones showed that P-SSU-342f is specific to ruminal protozoa and, with slight modifications; the primer will be useful for ecological studies of ruminal protozoa. PCR-based techniques, in conjunction with cloning and sequence analyses can provide information on uncultured protozoa (Oakey *et al.,* 2003). The goal of study was to examine phylogenetic diversity of the protozoan community in cow rumen fluid, rumen solid, and rumen epithelium by direct retrieval and analysis of 18S rDNA sequences in a culture independent manner. The following studies were undertaken to design and validate the use of a ruminal protozoa-specific primer in combination with a eukarya-specific reverse primer for use with ruminal samples enriched with mixed protozoa but still containing plant fragments, fungi, and bacterial cells. Validation was based on the construction of libraries of DNA encoding protozoal-based SSU rRNA from cows fed different diets. The cloned rDNA sequences were associated with eight or more genera; most of the sequences derived from the cow fed the 50:50 grain: forage diets were associated with those of the genus *Entodinium*, whereas sequences derived from the alfalfa-fed cow tended to cluster with those of *Dasytricha*.

Orlandi *et al.,* (2003) targeted Single-Nucleotide Polymorphisms in the 18S rRNA gene to differentiate *Cyclospora* Species from *Eimeria* Species by Multiplex PCR. They studied *Cyclospora cayetanensis* which is a coccidian parasite that causes protracted diarrheal illness in humans. *C. cayetanensis* is the only species of this genus thus far associated with human illness, although C*yclospora* species from other primates have been named. They used a nested PCR assay to detect the parasite and amplified a 294-bp region of the small subunit rRNA gene, followed by restriction fragment length polymorphism (RFLP) or DNA sequence analysis. Since the amplicons generated from *C. cayetanensis* and *Eimeria* species are the same size, the latter step is required to distinguish between these different species. The PCR-RFLP protocol, however, cannot distinguish between *C. cayetanensis* and these new isolates. The differential identification of such pathogenic and nonpathogenic parasites is essential in assessing the risks to human health from microorganisms that may be potential contaminants in food and water sources. Therefore, to expand the utility of PCR to detect and identify these parasites in a multiplex assay, a series of genus- and species-specific forward primers were designed that are able to distinguish sites of limited sequence heterogeneity in the target gene. The most effective of these unique primers were those that identified single-nucleotide polymorphisms (SNPs) at the 3' end of the primer. Under more stringent annealing and elongation conditions, these SNP primers were able to differentiate between *C. cayetanensis*, nonhuman primate species of *Cyclospora*, and *Eimeria* species.

Regensbogenova *et al.,* (2004a) developed a rapid method for molecular identification of rumen ciliates without the need for cultivation. Total DNA was isolated from single protozoal cells by the chelex method and nearly complete protozoal 18S rRNA genes were amplified and subjected to restriction fragment length polymorphism analysis. On the basis of restriction patterns generated a molecular key was elaborated allowing identification of protozoa solely by a molecular technique without prior knowledge of morphology. No differences were observed between identical species originating from different animals or geographic locations, or between morphological variants of the same species. The ARDREA (Amplified Ribosomal DNA Restriction Analysis) provides a rapid and convenient way for identification and diversity studies of rumen protozoa.

Shin *et al.,* (2004a) suggested that rumen fluid and solid tissues contain different protozoan populations that may play specific roles in rumen function. Libraries of protozoan rDNA sequences were constructed from rumen fluid, solid tissues and epithelium. Twenty-three clones isolated from rumen fluid fell into genera identified as *Entodinium* (69.6% of clones) and *Epidinium* (31.4% of clones). Of the clones isolated from rumen fluid, a moderate number were unidentifiable (30.4%). They concluded that predominant protozoan genus identified in the whole rumen belonged to the *Entodinium* group (81%). Protozoa were not detected in the rumen epithelium. They opined that quantitative PCR techniques and a more specific set of phylogenetic probes that distinguish between protozoans species are needed to determine the significance of newly identified groups and to determine the distribution of identified protozoan clusters in rumen microbial communities. Protozoa that inhabit the rumen were detected by PCR using protozoan-specific primers. The findings suggested that rumen fluid and solid tissues contain different protozoan populations that may play specific roles in rumen function. Quantitative PCR techniques and a more specific set of phylogenetic probes that distinguish between protozoans species are needed to determine the significance of newly identified groups and to determine the distribution of identified protozoan clusters in rumen microbial communities.

Sylvester *et al.,* (2004) developed a real-time polymerase chain reaction (PCR) assay targeting the gene encoding 18S rDNA to quantify the amount of protozoal biomass in ruminal fluid and duodenal digesta. The assay included procedures for isolating and concentrating protozoal cells from the rumen for use as a standard to convert 18S rRNA gene copies to a biomass. Protozoal cells were harvested from rumen fluid and concentrated for evaluation of recovery of rDNA in samples from the rumen and the duodenum. The DNA from concentrated cells was extracted with virtually 100% efficiency both before and after column purification. After serial spiking of protozoal cells into duodenal fluid over the entire range of quantification, the recovery was highly linear and constant at 81%. After serially spiking increasing quantities of protozoal rDNA into a constant volume of duodenal samples, nonlinear regression verified constant recovery of background rDNA in duodenal samples regardless of the ratio of target: nontarget rDNA.

Wereszka *et al.,* (2004) produced an expression cDNA library from the anaerobic ciliate *Epidinium caudatum* living in the rumen of a "monofaunated" sheep. When screened in lambda ZAP Express for the ability to hydrolyze carboxymethyl cellulose (CMC) a clone with sequence similarity to glycosyl hydrolase family, 5 cellulases was isolated. After over expression in *E. coli* the recombinant protein showed an unusual pH optimum against carboxymethyl cellulose (CMC) of above 8. Since a putative N terminal signal peptide was identified suggesting that the mature protein was exported into digestive vacuoles in the ciliate's cytoplasm, it had to be concluded that this cellulase is active within the alkaline digestive vacuoles of the ciliate. Notwithstanding a distinctive codon usage pattern similar to other rumen ciliate genes, phylogenetic analysis suggested strongly that the isolated sequence was closely related to certain cellulase genes of rumen bacteria. It is concluded that this gene has been acquired by lateral gene transfer from rumen bacteria. It is postulated that this gene underwent ameliorization of its codon usage and an adaptation to an alkaline pH optimum to meet the requirements for a function in the alkaline digestive vacuoles of its host.

Skillman *et al.,* (2006b) designed PCR and real-time PCR primers for the 18S rRNA gene of rumen protozoa (*Entodinium* and *Dasytricha* spp.) and tested their specificities against a range of rumen microbes and protozoal groups. External standards were prepared from DNA extracts of a rumen matrix containing known numbers and species of protozoa. The efficiency of PCR was calculated following amplification of serial dilutions of each standard and was used to calculate the numbers of protozoa in each sample collected. Species of *Entodinium*, the most prevalent of the rumen protozoa, were enumerated in rumen samples collected from total hundred, 1-year-old merino wethers by microscopy and real-time PCR. Both the counts, developed by the real-time PCR method and microscopic counts were accurate and repeatable, with a strong correlation between them ($r2 = 0.8$), particularly when the PCR efficiency was close to optimal (i.e., two copies per cycle). *Entodinium* represented on average 98% of the total protozoa, and populations within the same sheep were relatively stable, but greater variation occurred between different sheeps (10^0 and 10^6 *Entodinia* per gram of rumen contents). With this inherent

variability, it was estimated that, to detect a statistically significant (p = 0.05) 20% change in *Entodinium* populations, 52 sheep per treatment group would be required.

Sylvester *et al.,* (2005) developed a real-time polymerase chain reaction (PCR) assay to quantify copies of the genes encoding protozoal 18S rRNA. The assay includes procedures for isolating and concentrating protozoal cells from the rumen for use as a standard to convert 18S rRNA gene copies to a biomass basis. The objectives were to 1) determine the degree of reduction of bacterial contamination in the protozoal standard, 2) determine if protozoal standards derived from ruminal fluid are appropriate for predicting duodenal flows, and 3) evaluate the assay's determined values for protozoal nitrogen (N) in the rumen and flowing to the duodenum compared with independent measurements. Their protozoal collection method reduced non associated bacterial contamination by 33-fold, the contamination of which could otherwise significantly bias RNA (microbial marker) and nitrogen percentages of concentrated protozoal fractions. Based on denaturing gradient gel electrophoresis, the use of protozoal cells isolated from ruminal fluid appears appropriate for use in quantitative assays determining protozoal N flow post-ruminall. Using real-time PCR, protozoal N was determined to be 4.8 and 12.7% of the rumen microbial N pool and 5.9 and 11.9% of the duodenal flow of microbial N on diets containing low (16%) or high (21%) forage neutral detergent fiber, respectively, which were comparable with independent measures and expectations.

David *et al.,* (2006) estimated quantitative contribution of rumen protozoa to the total nitrogen (N), conjugated linoleic acid (CLA) and vaccenic acid (VA; trans-11-18: 1) flow to the duodenum of steers fed two silage diets: control silage (CS) and silage high in water-soluble carbohydrates (HS). Protozoal duodenal flows were estimated using a real-time PCR assay to quantify the genes encoding protozoal 18S ribosomal RNA. Denaturing gradient gel electrophoresis was used to confirm that the rumen protozoa populations were similar to the protozoal population flowing to the duodenum. Estimated duodenal flow of protozoal N was 14·2 and 18·2 g/d (P>0·05) for animals fed the CS and HS diets respectively. Protozoal flow thus represented between 12 and 15% of the total N duodenal flow. In terms of fatty acid flow, protozoa accounted for between 30 and 43% of the CLA and 40% of the VA reaching the duodenum. The contribution of protozoa to 16: 0 and 18: 0 flows to the duodenum

was less than 20 and 10 %, respectively. These results show that the fatty acids within protozoa make up a significant proportion of the CLA and VA reaching the duodenum of ruminants.

Ricard *et al.,* (2006) studied the horizontal transfer of expressed genes from bacteria into ciliates which live in close contact with each other in the rumen (the foregut of ruminants) using ciliate Expressed Sequence Tags (ESTs). More than 4000 ESTs were sequenced from representatives of the two major groups of rumen Cilates: the order Entodiniomorphida (*Entodinium simplex, Entodinium caudatum, Eudiplodinium maggii, Metadinium medium, Diploplastron affine, Polyplastron multivesiculatum* and *Epidinium ecaudatum*) and the order Vestibuliferida, previously called Holotricha (*Isotricha prostoma, Isotricha intestinalis* and *Dasytricha ruminantium*). A comparison of the sequences with the completely sequenced genomes of eukaryotes and prokaryotes, followed by large-scale construction and analysis of phylogenies, identified 148 ciliate genes that specifically cluster with genes from the Bacteria and Archaea. The phylogenetic clustering with bacterial genes, coupled with the absence of close relatives of these genes in the Ciliate *Tetrahymena thermophila*, indicates that they have been acquired via Horizontal Gene Transfer (HGT) after the colonization of the gut by the rumen Ciliates. Among the HGT candidates, they found an over-representation (>75%) of genes involved in metabolism, specifically in the catabolism of complex carbohydrates, a rich food source in the rumen. They proposed that the acquisition of these genes has greatly facilitated the ciliates colonization of the rumen providing evidence for the role of HGT in the adaptation to new niches.

Samuel *et al.,* (2007) evaluated association patterns between archaea and rumen protozoa by analyzing archaeal 16S rRNA gene clone libraries from ovine rumen inoculated with different protozoa. Five protozoan inoculation treatments, fauna free (negative control), holotrich and cellulolytic protozoa, *Isotricha* and *Dasytricha* spp., *Entodinium* spp., and total fauna (type A) were tested. They used denaturing gradient gel electrophoresis, quantitative PCR, and phylogenetic analysis to evaluate the impact of the protozoan inoculants on the respective archaeal communities. Protozoan 18S ribosomal DNA clone libraries were also evaluated to monitor the protozoal population that was established by the inoculation.

Phylogenetic analysis suggested that archaeal clones associated with the fauna-free, the *Entodinium*, and the type an inoculation clustered primarily with uncultured phylotypes. *Polyplastron multivesiculatum* was the predominant protozoan strain established by the holotrich and cellulolytic protozoan treatment, and this resulted predominantly in archaeal clones affiliated with uncultured and cultured methanogenic phylotypes (*Methanosphaera stadtmanae*, *Methanobrevibacter ruminantium* and *Methanobacterium bryantii*). Furthermore, the *Isotricha* and *Dasytricha* inoculation treatment resulted primarily in archaeal clones affiliated with *Methanobrevibacter smithii*. This report provides the first assessment of the influence of protozoa on archaea within the rumen microbial community and provides evidence to suggest that different archaeal phylotypes associate with specific groups of protozoa. The observed patterns may be linked to the evolution of commensal and symbiotic relationships between archaea and protozoa in the ovine rumen environment. This report further underscores the prevalence and potential importance of a rather large group of uncultivated archaea in the ovine rumen, probably unrelated to known methanogens and undocumented in the bovine rumen

National Status

The research in rumen microbiology in India started in the seventies, but it was limited to mixed culture rumen fermentation or a very few scattered studies on pure cultures of rumen microbes were carried out (Sinha and Rangnathan, 1983; Srivastava and Chaturvedi, 1973). It was only in the nineties that pure culture studies started in a few laboratories. Even now there are not more than four or five laboratories which have full facilities for the cultivation and maintenance of rumen microbes. A few bacterial and fungal cultures have been isolated from domestic (cattle and buffalo) and wild ruminants like blackbuck, chinkara, nilgai, hog deer and spotted deer (Sahu *et al*., 2004; Paul *et al.*, 2003).

Distinctive differences in rumen ecosystem and efficiency of nutrient utilization from cellulosic feeds between cattle and buffaloes and have been documented (Sahu and Kamra, 2001). The work carried so far is on microscopic examination and counting of protozoa, bacteria and cultivation of specific rumen microbial groups. Kamra (2001) reported that there is no culture medium available, which can promote growth of all the rumen bacteria. He further stated that

classification of rumen bacteria on phenotypic characteristic and a biochemical test is not sufficient to study the diversity among the microbes. The variation in different groups of microbes can be studied by using different DNA probes homologous to some regions on bacterial 16S rRNA. He recommended isolation of microbial strains from rumen of buffalo and establishment of feed supplemented microbial strains in rumen by use of DNA probes as the area of research in ruminant nutrition.

Dighe *et al.,* (2004) compared 16S rRNA gene sequences of genus *Methanobrevibacter*. A very significant relationship was found between the 16S rRNA sequence similarity (S) and the extent of DNA hybridization (D) for the genus *Methanobrevibacter*, implying that it is possible to predict D from S with a known precision for the genus.

Chaudhary and Sirohi, (2009), studied the diversity of rumen methanogens in Murrah buffaloes (*Bubalus bubalis*) from North India, revealed *Methanomicrobium* phylotype is the most dominant phylotype of methanogens present in Murrah buffaloes (*Bubalus bubalis*).

Eight genera of ciliate protozoa have been observed in the rumen of cattle and buffalo fed on wheat straw and concentrate mixture, e.g. *Isotricha, Dasytricha, Metadinium, Diplodinium, Eudiplodinium, Ophryoscolex, Entodinium,* and *Epidinium. Oscillospira guillermondii* (Srivastava and Chaturvedi, 1973).

DEFINATION OF PROBLEM

India possesses more than 50 per cent of world buffalo population and shared >60 % milk production in India (Kumar *et al.,* 2007). Improving microbial degradation of plant cell wall polysaccharides remains one of the highest priority goals for all livestock enterprises, including the Baffloes, cattle herds and draught animals of India. Molecular characterization of rumen microflora and fauna in zebu cattle and buffalo has not been carried out yet. Majority of work is reported in exotic ruminants. Molecular characterization of rumen microbes in Indian Buffalo will help studying abundance and diversity of these microbes and their phylogenetic classification. Since our animals mainly sustain on crop residues, the microbial population is expected much different than that of exotic cattle. It will also help to study the diet dependent changes in rumen ecosystem. In the present study, we will

undertake the study on rumen microbes in buffalo and their phylogenetic classification using 16S rRNA/18S rRNA gene cloning and sequencing.

PURPOSE OF THE PRESENT STUDY

In view of the above concern, present study has been aimed to look for the diversity, abundance and dynamics of rumen microbes in buffalo. This will also help to give a detail phylogenetic classification of microbes in buffalo. The information will provide database for future research on manipulation of rumen fermentation by altering rumen microbes and genetic improvement of microbial strains for enhancing fiber-degrading ability. The study will reveal the quantification and characterization of different bacteria and protozoa in buffalo and effect of dietary changes on microbial population and diversity. It will also provide a classification scheme to predict phylogenetic relationship. The 16S rRNA of bacteria and 18S rRNA sequence of protozoa retrieved will be used to design species specific primers for detection and monitoring. To achieve the goal, the main purpose is to search for potential microbial populations occurring in the buffalo rumen ecology.

The experimental work was designed to rumen sample collection, DNA isolation from microbial communities, metagenomic PCR amplification, and classifies the organisms existing in the rumen samples. The major objectives of the study were to examine microbial makeup of rumen bacteria, archaea and protozoa. To determining density and dynamics of ruminal microflora by Real Time PCR Assay was one of the major and important aspects.

Chapter 2. Bacterial diversity in the rumen of Indian Surti buffalo (*Bubalus bubalis*), assessesed by 16S rDNA analysis

ABSTARCT

Bacterial communities in buffalo rumen were characterized using a culture-independent approach for a pooled sample of rumen fluid from 3 adult Surti buffaloes. Buffalo rumen is likely to include species of various bacterial phyla, so 16S rDNA sequences were amplified and cloned from the sample. A total of 191 clones were sequenced and similarities to known 16S rDNA sequences were examined. About 62.82% sequences (120 clones) had >90% similarity to the 16S rDNA database sequences. Furthermore, about 34.03% of the sequences (65 clones) were 85-89% similar to 16S rDNA database sequences. For the remaining 3.14%, the similarity was lower than 85%. Phylogenetic analyses were also used to infer the makeup of bacterial communities in the rumen of Surti buffalo. As a result, we distinguished 42 operational taxonomic units (OTUs) based on unique 16S r DNA sequences: 19 OTUs affiliated to an unidentified group (45.23% of total OTUs), 11 OTUs of the phylum Firmicutes, also known as the low G+C group (26.19%), 7 OTUs of the *Cytophaga-Flexibacter-Bacteroides* phylum (16.66%), 4 OTUs of Spirochaetes (9.52%), and 1 OTU of Actinobacteria (2.38%). These include 10 single-clone OTUs, so Good's coverage (94.76%) of 16S rRNA libraries indicated that sequences identified in the libraries represent the majority of bacterial diversity present in rumen.

Keywords: Surti buffalo, *Bubalus bubalis*, rumen bacteria, 16S rDNA, 16S rRNA, molecular diversity

INTRODUCTION

Livestock production in India is subsidiary to plant production. In tropical countries, the ruminants are fed on lignocellulosic agricultural by-products (like cereal straw, tree foliage, etc.) and cakes of oil seeds. Ruminants digest such plant materials by virtue of the extensive microbial community, including bacteria, fungi, and protozoa (Miron *et al*., 2001), which are found in the rumen and provide the host with nutrients, predominantly in the form of volatile fatty acids and microbial protein.

According to Woese *et al*., (1990), all microbes present in the rumen ecosystem can be classified into 3 domains: Bacteria, Archaea (methanogens), and Eucarya (protozoa and fungi). Historically, most of the knowledge of the microbial composition of rumen fluid has been derived using traditional methods, such as the roll-tube technique (Hungate 1969) or most probable number (MPN) estimates (Dehority et al. 1989). The rumen bacteria have been shown by traditional procedures to belong to some 22 predominant species (Krause and Russell, 1996). However, recent observations made it clear that prior estimation represents only a small portion of the total diverse microbial community that colonises the rumen (Krause et al. 2003). DNA-based technologies (16s RNA/ 18s RNA gene) have been used extensively to study rumen microbes both qualitatively (Sylvester *et al*., 2004) and quantitatively (Shin *et al*., 2004b). These techniques have been used to construct a library of 16S rDNA clones of rumen microbes and to demonstrate considerable diversity of rumen bacteria. Molecular research on microbial ecology of animals provides a broad perspective of application in the field of animal sciences (An *et al*., 2005). India possesses more than 50% of the world buffalo population, which produces more than 60% milk in India (Kumar *et al*., 2007). Surti is a popular breed of buffalo found in central Gujarat state. Surti buffaloes are of medium size and docile temperament, with body weight of 350-375 kg at maturity. The breed is distinguished by a fairy broad and long head with a convex shape at the top between horns. The present study was aimed to examine the diversity of rumen bacteria in Surti buffalo offered a diet of green fodder Napier bajra (*Pennisetum purpureum*), mature pasture grass (*Dichanthium annulatum*), and compound concentrate mixture. The molecular techniques now available were used to construct a library of 16S rDNA clones of rumen bacteria, and a phylogenetic tree for the clones isolated.

MATERIALS AND METHODS

Animals, diet and collection of rumen fluid

The experiment was carried out on 3 adult Surti buffaloes, at approximately 3 years of age and with a mean live weight of 201±18 kg. They were reared at the Department of Animal Nutrition, College of Veterinary Science and Animal Husbandry, Anand. The permission of the Committee for the Purpose of Control and

Supervision of Experiments on Animals (CPCSEA) was obtained prior to initiation of the study. All the animals were maintained under a uniform feeding regime (ICAR, 1998) for at least 21 days. The diet comprised green fodder Napier bajra 21 (*Pennisetum purpureum*), mature pasture grass (*Dicanthium annulatum*), and concentrate mixture (20% crude protein, 65% total digestible nutrients). The animals were offered 10 kg green, ad-lib dry grass, and 2.5 kg of concentrate mixture daily. Approximately 500 ml of rumen fluid was collected *via* a stomach tube located in the middle part of the rumen and connected to a vacuum pump at 0, 2, 4, and 6 h after feeding (Khampa *et al*., 2006). About 100 ml of rumen fluid was passed through 4 layers of cheese cloth to remove particulate matter. Remaining rumen fluid was stored at -80°C for further study. Total DNA (0, 2, 4, 6 h × 3 animals) was extracted separately by using a commercially available kit according to the manufacturer's instructions (QIAGEN Stool kit; QIAGEN, CA) and we finally pooled all the DNA samples. The total DNA mixture (pooled) was used as a template in PCR to amplify 16S rRNA. Important buffers and reagents used in present study are given in appendix A.

PCR amplification of the 16S rRNA gene

The PCR primers used to amplify 16S rDNA fragments were bacteria-specific primers (Lane, 1991): a forward primer F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and a reverse primer R1492 (5'-ATAGGYTACCTTGTTACGACT-3'). Subsequently, 16S rDNA was amplified by PCR using the metagenomic DNA and Master mix (Fermentas, UK). A total of 25µl of reaction mixture consisted of 10 pmol of each primer, 30 ng of template DNA, and 12.5µl of Master mix (Fermentas, UK). The PCR amplification was performed by Thermal Cycler (ABI, USA) using the following program: denaturing at 95°C for 5 min, followed by 30 cycles of 30 s of denaturing at 95°C, 30 s of annealing at 50°C and 2 min of elongation at 72°C, with a final extension at 72°C for 10 min for the first set. The anticipated product of approximately 1.4 kb was separated by agarose gel electrophoresis and cleaned by using a QIAquick DNA Gel Extraction Kit (QIAGEN, CA) in accordance with the directions of the manufacturer.

Cloning and sequencing

The purified PCR products were cloned in InstaTA cloning kit (Fermentas, UK) according to the instructions of the manufacturer. The recombinant plasmids were next extracted by the Mini-prep Plasmid Extraction Kit (QIAGEN, CA). Sequencing was performed for all the clones in the library with an ABI Prism 310 Genetic Analyser (Applied Biosystems Inc., CA) using BigDye Terminator (version 3.1) in the Animal Biotechnology Laboratory, AAU, Anand, Gujarat, India.

Sequence analyses and phylogenetic tree constructing

All reference sequences were obtained from the GenBank/EMBL/DDBJ/RDP (Benson *et al*., 2007). Sequences (~650 bp) from the current study were analysed by the CHECK_CHIMERA program (Maidak *et al*., 2001) to remove any chimeric rDNA clone. The similarity searches for sequences were carried out by BLAST (http://www.ncbi.nlm.nih.gov/ BLAST/Blast.cgi, Madden *et al*., 1996) and alignment was done using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html, Thompson *et al*., 1994). Ambiguously and incorrectly aligned positions were aligned manually. The distance matrix was calculated using the DNADIST program included in PHYLIP (Felsenstein 1985) and used to assign sequences in various operational taxonomic units (OTUs) or phylotypes by DOTUR (Schloss and Handelsman, 2005) and a total of 42 OTUs were distinguished, based on unique 16S r DNA sequences. Good's coverage was calculated as $[1 - (n/N)] \times 100$, where n is the number of single-clone OTUs and N is the library size, i.e. the total number of sequences (clones) for the analysed sample (Schloss and Handelsman, 2005). Phylogenetic tree was constructed by the neighbour joining method using MEGA 4.0 software (Tamura *et al*., 2007). Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies (Felsenstein 1985).

Nomenclature and nucleotide sequence accession numbers

The prefix KM was used to denote the OTUs identified. Nucleotide sequences are deposited in the GenBank database under the accession numbers EU348296-EU348106.

RESULTS AND DISCUSSIONS

The bacterial DNA was successfully extracted from rumen fluid (Figure 2.1) and PCR fragments having expected size of 1.4 kb were obtained from DNA samples (Figure 2.2)

Sequence similarity

A total of 191 16S rDNA clones of partial length were isolated from the rumen liquor of the buffaloes. All the clones were subjected to sequence analysis, followed by homology search using databases: the GenBank and the Ribosomal Database Project (RDP) database. In our library, about 62.82% clones (120 clones) had ≥90% similarity to 16S rDNA data sequences from those databases. Furthermore, about 34.03% (65 clones) of the sequences were 85-89% similar to 16S rDNA data sequences, while for the remaining 3.14 % (6 clones) the similarity was less than 85% (Table 2.1).

Phylogenetic analysis

The collection of cloned 16S rRNA gene sequences of bacteria encompassed several major bacterial lineages–Although the similarity for most of the sequences with those of known rumen bacteria was too low to identify the sequence as representing a particular species, a phylogenetic tree (Figure 2.3) was constructed to investigate their taxonomic affiliation. All 191 clone sequences were included in a phylogenetic analysis and were grouped into 42 operational taxonomy units (OTUs) affiliated to an unidentified group (19 OTUs, 45.23%), the phylum Firmicutes, also known as the low G+C group (11 OTUs, 26.19%), the *Cytophaga-Flexibacter-Bacteroides* (CFB) phylum, (7 OTUs, 16.66%), Spirochaetes (4 OTUs, 9.52%) and Actinobacteria (1 OTU, 2.38%) (Table 2.2). Thus bacterial sequences obtained from rumen formed tightly-clustered, deeply-diverging groups affiliated to the different bacterial phyla as well unclassified groups. The total includes 10 single-clone OTUs, so Good's coverage (94.76%) of 16S rRNA libraries indicated that the sequences identified in libraries represent the majority of bacterial diversity present in rumen.

Within the phylum Firmicutes, 4 OTUs showed similarity to sequences of species. These correspond to *Succiniclasticum ruminis* (KM22, 6 clones), *Acetovibrio cellulolyticus* (KM27, 4 clones), *Streptococcus* sp. (KM21, 2 clones), and *Ruminococcus callidus* (KM26, 6 clones). The remaining 7 OTUs belonged to

independent clusters, which are associated with the phylum Firmicutes. In the CFB phylum, 1 OTU was similar to *Prevotella ruminicola* (KM3, 16 clones) and another 6 OTUs constituted separate clusters that were remotely related to the *Bacteroides fragilis* group. Another 4 OTUs belonged to *Treponema* spp. (KM41, KM17, KM42, and KM12, 13 clones, Table 2.2).

None of the clones in our library were identified below the genus level, because the similarity values of our sequences were too low to assign them to particular taxa. Although there are no exact 16S rDNA similarity limits for defining specific taxa, such as genera and species, species definition in general requires sequence having greater than 98% similarity (Vandamme *et al*., 1996).Thus if a sequence has a greater than 98% similarity to a 16S rDNA of a known bacterium, it is considered to be a member of that species. In the present study, we examined rumen bacterial composition by PCR-based analysis of bacterial 16S rDNA sequences. This culture-independent method offers possibility of characterizing microbial ecosystems, independent isolation, maintenance, and propagation of bacteria under laboratory conditions. However, PCR-based methodologies are subject to certain limitations (Acinas *et al*., 2005). As discussed by Wintzingerode *et al*., (1997), care should be taken in experimental procedures and in interpretation of the results. Thus our findings reveal that rumen of the Surti buffaloes contained a diverse array of bacterial species. More than 11 OTUs (26.19%) belonged to the known phylum Firmicutes, 7 OTUs (16.66%) to the *Bacteroides fragilis* group, 4 OTUs (9.52%) to Spirochaetes, and 1 OTU (2.38%) to the Actinobacteria. About 19 OTUs (45.23%) belonged to unidentified groups (Table 2.2). The proportions of bacterial communities in Surti buffalo rumen are similar to those reported previously in other rumen ecology studies. In a metagenome analysis by pyrosequencing of rumen sample of Angus-Simmental cross steers (*Bos taurus*) fed a diet of grass-legume hay (Brulc *et al*., 2009), 62% of sequences were identified as the Firmicutes and 21% of sequences as the *Bacteroides fragilis* group. Tajima *et al*., (1999) reported that 52.4% of clones identified in the rumen liquor of Holstein cow (*Bos taurus*) fed a diet of hay belonged to the Firmicutes, and 38.1% to the CFB phylum. Edwards *et al*., (2004), summarizing the published data for rumen bacteria, reported that on average 54% of rumen bacteria were members of the Firmicutes and 40% were from the CFB phylum. Yuhei *et al*.,

(2005) found that 81.3% clones represented the Firmicutes, while 14.4% clones belonged to the Bacteroides *fragilis* group and Actinobacteria and Proteobacteria in fecal microbiota of cattle. Deng *et al*., (2007) assigned 57.1% of clones to the phylum Firmicutes, 42.2% of clones to the CFB phylum, and one clone (0.7%) to Spirochaetes in gayals (*Bos gaurus frontalis*, syn. *B. frontalis*) fed a diet composed of fresh bamboo leaves and twigs (*Sinarundinaria* sp.), with 50.5±3.16 % dry matter, 10.2± 0.40 % crude protein, 38.8±1.17 **%** crude fiber. Dowd *et al*., (2008), using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP), evaluated ubiquitous bacteria from the cattle faeces, which included *Clostridium*, *Bacteroides*, *Porpyhyromonas*, *Ruminococcus*, *Alistipes*, *Lachnospiraceae*, *Prevotella*, *Lachnospira*, *Enterococcus*, *Oscillospira*, *Cytophaga*, *Anaerotruncus*, and *Acidaminococcus* spp. Regarding cellulose degrader, *Fibrobacter*-like bacteria may not have been detected in the Surti buffaloes in the present study. The reason can be due to a small population, adherence to feed, specificity of the primers, or experimental PCR biases. The other classic fibrolytic bacteria, such as *Ruminococcus bromii* and *Butyrivibrio fibrosolvens* (Hungate 1969; Srinivas and Krishnamoorthy, 2005; Khampa *et al*., 2006) were recovered in 3 OTUs. One OTU (KM26, 6 clones) identified in the present study was related to the known fibrolytic bacterium *Ruminococcus cadillus*. The acid-utilizing bacterium *Succiniclasticum ruminis* (KM22, 6 clones), sugar-utilizing bacterium *Streptococcus* sp. (KM21, 2 clones), and *Acetovibrio cellulolyticus* (KM27, 4 clones) were also recovered from the rumen of Surti buffalo (Table 2.2). These results support the results of Koike *et al*., (2003) for a domestic rumen sample and co-culture sample. Another 7 OTUs of the phylum Firmicutes were clustered independently and their confirmation requires further investigation.

Amongst the bacteria comprising the CFB phylum, only one OTU (KM3, 16 clones) was closely related to *Prevotella runinicola*, which was identified previously as the most numerous of rumen bacteria (Stewart *et al*., 1997). The remaining 6 OTUs located in the CFB phylum were clustered loosely, and their identification requires further investigation. Overall, the present results were consistent with previous reports that CFB bacteria were less numerous in the rumen of wild animals (An *et al*., 2005), as compared to domestic cattle. Whitford *et al*., (1998)

reported that 16S rDNA sequences similar to those of *Prevotella ruminicola* prevailed in isolated material from domestic cattle. Four OTUs (13 clones) were classified as Spirochaetes. A notable finding was the presence of *Actinomyces bovis* (Figure 2.3) grouped within the Actinobacteria. The 16S rRNAs from actinobacteria are commonly found in soils (Buckley and Schmidt, 2003). Although Surti buffalo may accidentally eat soil through feed, it is unlikely that ingested actinobacteria, just passing through the gastrointestinal tract, would be detected in our analyses. *Actinomyces*-affiliated sequences have also been obtained from gastrointestinal tracts of diverse herbivores (Tajima *et al*., 1999) and humans (Eckburg *et al*., 2005). *A. bovis* were not reported in the clone libraries of bacterial 16S rDNA isolated from the gayals fed a diet of bamboo (*Sinarundinaria* sp.) leaves and twigs (Deng *et al*., 2007). It is quite possible that the variations in the species of bacteria in the rumen are affected by diet. Latham *et al*., (1972) and Tajima *et al*., (2001b) have shown that diet affects the composition of the microbial community of the rumen. However, differences between the present observations and those published previously are considered to be related, at least, with animal species. For example, *A. bovis* was retrieved from the rumen liquor of buffalo in the current study. However, this species of bacteria was not detected in bacterial 16S rDNA clone libraries of rumen fluid from gayals fed fresh bamboo leaves (Deng *et al*., 2007).

CONCLUSIONS

In spite of the possible deficiencies in analytical techniques, it is concluded that Surti buffalo has a diverse range of bacteria in the rumen. Unidentified sequences represent the majority of bacteria in the rumen of Surti. The sequences of cellulolytic bacterial groups (*Ruminococcus callidus*, *Acetovibrio cellulolyticus*, and *Streptococcus* sp.), proteolytic bacteria (*Prevotella ruminicola*), acid utilizers (*Succiniclasticum ruminis*), and pectin utilizers (*Treponema* sp.) were also recovered in the present study. The present results are consistent with the observations of Deng *et al*., (2007), An *et al*., (2005) and Koike *et al*., (2003). Definitely, further studies should be undertaken to confirm and extend the present observations.

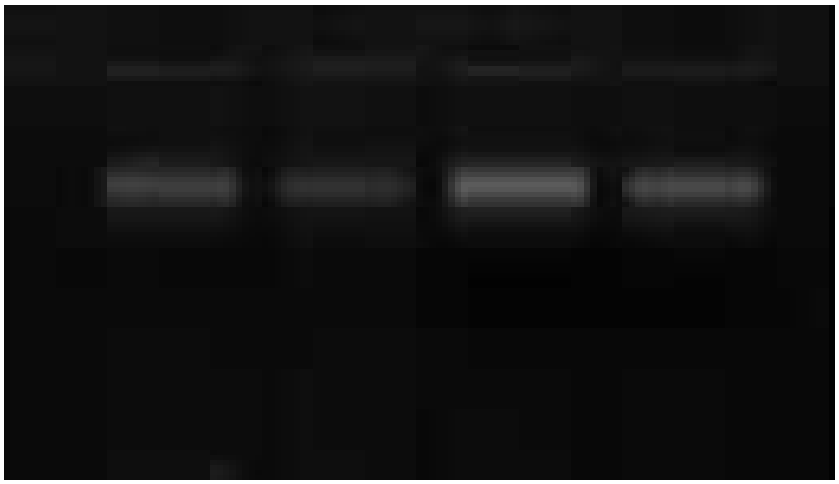


Figure 2.1. Agarose gel electrophoresis of metegenomic DNA extracted from rumen fluid of Surti buffalo

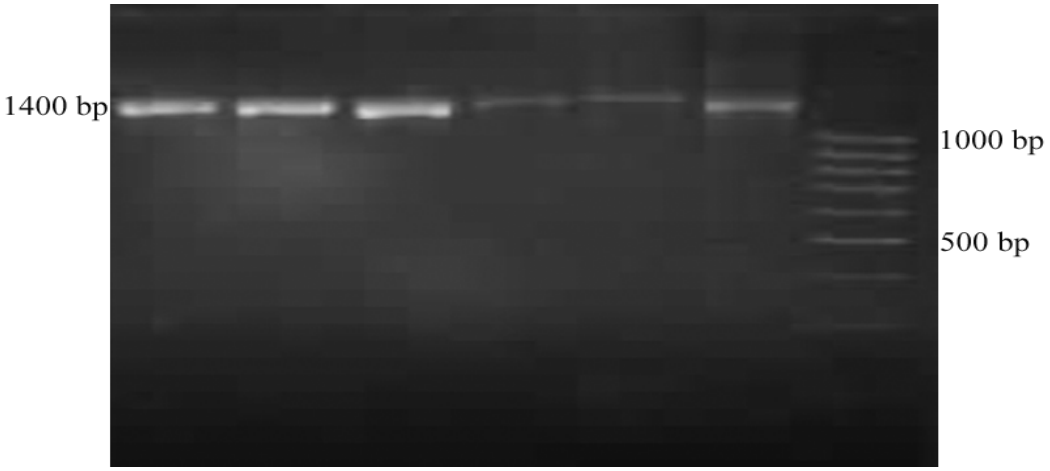


Figure 2.2. Agarose gel electrophoresis of PCR product showing amplicon of 1400 bp

Table 2.1: Similarity values of operational taxonomic units (OTUs) based on 16S rDNA sequences of bacteria (191 clones) retrieved from the rumen fluid of Indian Surti buffalo.

OTU	No. of clones	Nearest valid relative (GenBank accession no.)	Sequence identity (%)
KM1	5	URB (DQ394664)	92
KM2	13	URB (EF436391)	96
KM3	16	*Prevotella aff. ruminicola* Tc2-24 (AJ009933)	93
KM4	2	URB (EU845097)	89
KM5	24	URB (EU381899)	90
KM6	19	*Butyrivibrio fibrisolvens* isolate L8 (AY699274)	86
KM7	2	URB (EU844704)	87
KM8	6	*Selenomonas ruminantium* strain 65 (EF112197)	91
KM9	5	URB (AB270078)	90
KM10	2	URB (AB034150.)	86
KM11	5	uncultured equine intestinal *Eubacterium* sp. (AJ408101)	88
KM12	6	*Treponema* sp. (AF023049)	86
KM13	9	URB (EU381619)	90
KM14	7	URB (AB270141)	93
KM15	5	uncultured member of Lachnospiraceae (AB218344)	91
KM16	3	URB (AB185560)	87
KM17	3	URB (EU844824)	89
KM18	1	URB (AB034150)	84
KM19	3	URB (EF686622)	94
KM20	4	uncultured Firmicutes clone NI217 (FJ650689)	91
KM21	2	uncultured Firmicutes bacterium (CU925746)	83
KM22	6	*Succiniclasticum ruminis* strain DSM 9236 (NR026205)	90
KM23	2	uncultured Firmicutes bacterium (CU922655)	83

KM24	2	URB (EU259464))	95
KM25	5	uncultured *Acetivibrio* sp (EU703284)	87
KM26	6	*Butyrivibrio fibrisolvens* isolate L8 (AY699274)	87
KM27	4	URB (EF686520)	93
KM28	2	*Prevotella ruminicola* strain TC2-28 (AF218619)	87
KM29	3	rumen bacterium YS2 (AF544207)	89
KM30	1	URB (EU259402)	96
KM31	3	uncultured member of Ruminococcaceae (EU794145)	87
KM32	2	URB (EU842702)	93
KM33	2	URB (EF686519)	93
KM34	1	not available	
KM35	1	*Ruminococcus gnavus* strain A2 (EU139255)	86
KM36	1	uncultured *Prevotella* sp. (AM420039)	96
KM37	1	URB (EU719249)	87
KM38	1	URB (EF686612)	93
KM39	1	URB (AB244116)	90
KM40	1	URB (FJ983064)	85
KM41	1	*Treponema bryantii* (M57737)	87
KM42	3	URB (FJ028789)	92

URB = uncultured rumen bacteria

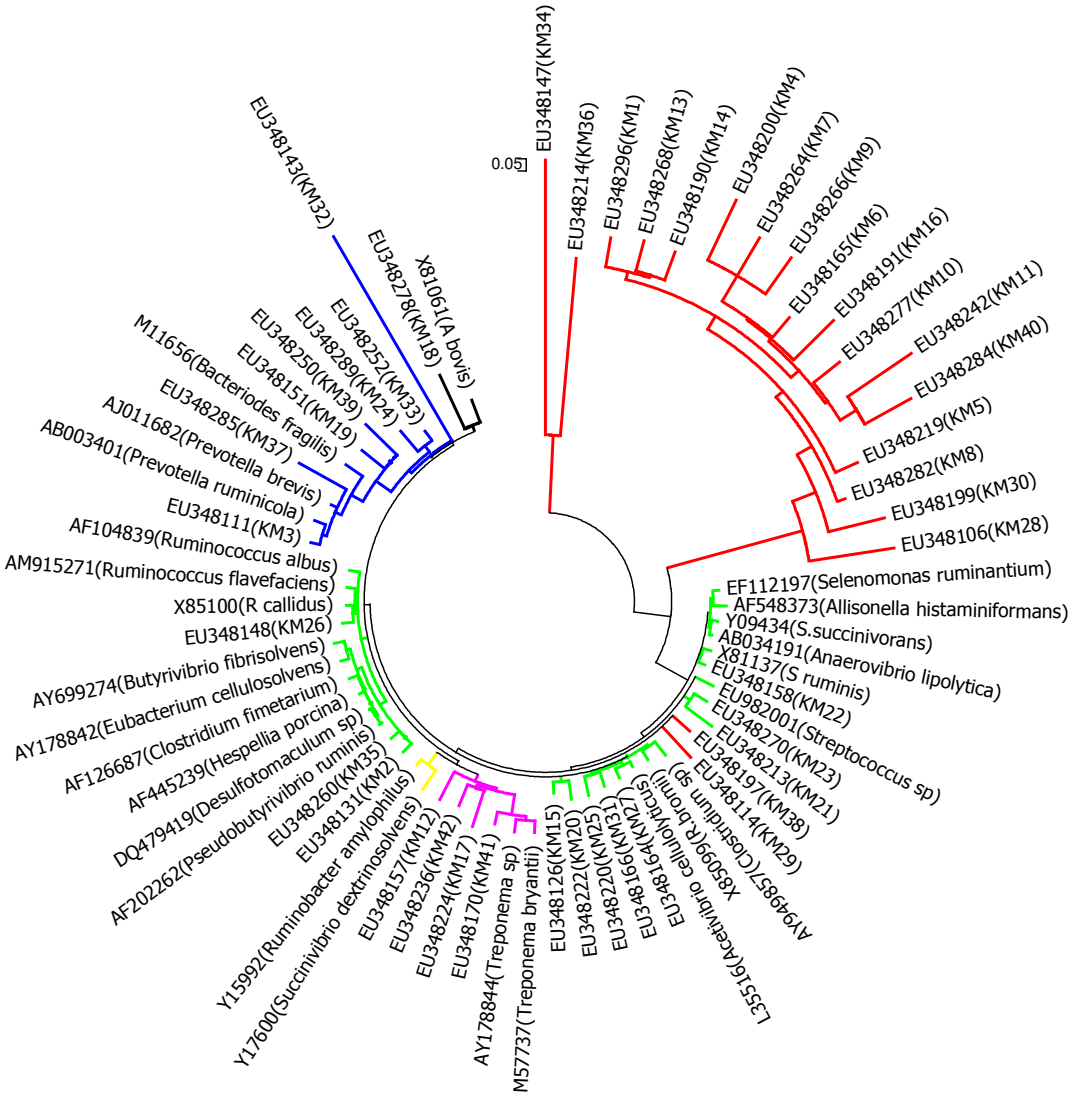


Figure 2.3. Phylogenetic relationships of partial 16S rDNA sequences of clones recovered from Surti buffalo rumen fluid samples. The unrooted tree was inferred by the neighbour joining method using the MEGA 4 software. The scale bar equals to an average of 5 nucleotide substitutions per 100 positions. Branch lines: green (Firmicutes), blue (CFB phylum), pink (Spirochaetes), yellow (Proteobacteria), black (Actinobacteria) and red (unidentified bacteria).

Table 2.2: Analysis of diversity of bacterial 16S rDNA phylotypes retrieved from the rumen of Indian Surti buffalo.

Taxon	**No. of operational taxonomic units (OTUs) and % of total OTUs**	**No. of clones**
Firmicutes (lowG+C group)	**11 OTUs (26.19%)**	**41**
a. *Succiniclasticum ruminis*	1 OTU (KM22)	6
b. *Streptococcus* sp.	1 OTU (KM21)	2
c. *Acetivibrio cellulolyticus*	1 OTU (KM27)	4
d. *Ruminococcus callidus*	1 OTU (KM26)	6
e. Unclassified	7 OTUs	23
CFB phylum	**7 OTUs (16.66%)**	**27**
a. *Prevotella ruminicola*	1 OTU (KM3)	16
b. Unclassified	6 OTUs	11
Spirochaes	**4 OTUs (9.52%)**	
Treponema spp.	(KM41, KM17, KM42, KM12)	**13**
Actinobacteria	**1 OTU (2.38%)**	**1**
Actinomyces bovis	(KM18)	
Unidentified bacteria	**19 OTUs (45.23%)**	**109**
Total	**42 OTUs**	**191**

CFB = *Cytophaga-Flexibacter-Bacteroides*

Chapter 3. Methanogen diversity in the rumen of Indian Surti buffalo (*Bubalus bubalis*), assessed by 16S rDNA analysis

ABSTARCT

The methanogenic communities in buffalo rumen were characterized using a culture-independent approach of a pooled sample of rumen fluid from three adult Surti buffaloes. Buffalo rumen is likely to include species of various methanogens, so 16S rDNA sequences were amplified and cloned from the sample. A total of 171 clones were sequenced to examine 16S rDNA sequence similarity. About 52.63% sequences (90 clones) had $\geq$90% similarity, whereas, 46.78% of the sequences (81 clones) were 75-89% similar to 16S rDNA database sequences respectively. Phylogenetic analyses were also used to infer the makeup of methanogenic communities in the rumen of Surti buffalo. As a result, we distinguished 23 operational taxonomic units (OTUs) based on unique 16S rDNA sequences: 12 OTUs (52.17%) affiliated to Methanomicrobiales order, 10 OTUs (43.47%) of the order Methanobacteriales and one OTU (4.34%) of *Methanosarcina barkeri* like clone respectively. This study has revealed the largest assortment of hydrogenotrophic methanogens phylotypes ever identified from rumen of Surti buffaloes.

Key words: methanomicrobiales, methanobacteriales, phylotypes Surti buffaloes, 16S rDNA.

INTRODUCTION

Methanogens are members of the domain Archaea, and fall within the kingdom Euryarchaeota (Woese *et al.,* 1990) Methanogens are integral to carbon cycling, catalyzing the production of methane and carbon dioxide, both potent greenhouse gases, during organic matter degradation in anaerobic soils and sediment (Chin *et al.,* 2004). Methanogens are widespread in anaerobic environments, including tundra (Raskin *et al.* 1994), freshwater lake and wetland sediments (Castro *et al.,* 2004; Earl *et al.,* 2003), estuarine and marine sediments (Banning *et al.,* 2005), acidic peatlands (Basiliko *et al.,* 2003; Galand *et al.* 2002), rice field soil (Chin *et al.,* 2004), animal guts (Rastogi *et al.,* 2008), landfills (Luton *et al.,* 2002), and anaerobic

digesters treating animal manure (Angenent *et al.,* 2002; Soliva *et al.,* 2004), food processing wastewater (Liu *et al.,* 2002), and municipal wastewater and solid waste (Zheng and Raskin, 2000). Interest in methanogens from ruminants has resulted from the role of methane in global warming and from the fact that enteric methane emission is a major source of greenhouse gas in agriculture (http://www.indiastat.com).

Currently, India possesses the world's largest livestock population of 485 million, which accounts for 13% of the global livestock population (Intergovernmental Panel on Climate Change, 2001)**.** It has 57% of the world's buffalo and 16% of the cattle population. Contribution of methane emission in India by buffalo is 42% (Chhabra *et al.,* 2009). Reducing enteric methane emissions has been identified as one way of lowering global methane emissions. However, the effectiveness of any strategy that will reduce greenhouse gas emissions and also increase production or nutritional efficiency will likely depend upon having an understanding of the numbers and/or distribution of methanogen species among ruminant livestock.

Several species of methanogens have been isolated from ruminants, but few have been consistently found in high numbers (Stewart *et al.,* 1997) and it is likely that major species of rumen methanogens are yet to be identified (Rocheleau *et al.,* 1999; Wright *et al.,* 2004). The most common species of methanogens isolated from the rumen are strains of *Methanobrevibacter*, *Methanomicrobium*, *Methanobacterium*, and *Methanosarcina* (Wright *et al.,* 2004; Jarvis *et al.,* 2000). Methanogens are difficult to study through culture-based methods, and therefore many researchers have instead used culture-independent techniques to study methanogen populations. The 16S rRNA gene is the most widely used target for gene surveys, and a number of primers and probes have been developed to target methanogen groups (Purdy *et al.,* 2003; Saengkerdsub *et al.,* 2007b; Shin *et al.,* 2004b; Tatsuoka *et al.,* 2004). Methanogens are frequently found in association with protozoa (Tajima *et al.,* 2001b; Sizova *et al.,* 2003). To date, relatively little is known of the dominant methanogens in ruminants, particularly Surti buffaloes in western India. This paper uses comparative sequence analysis of cloned 16S rRNA genes (rDNA) amplified from total DNA extracted from rumen fluid to analyse the dominant methanogens present in the rumen of Surti buffalo.

MATERIALS AND METHODS

Animals, diet, collection of rumen fluid and DNA extraction

The same as described in chapter 2

PCR primers and amplification

The primers used were 1Af (5'-TCYGKTTGATCCYGSCRGAG-3') and 1100Ar (5'-TGGGTCTCGCTCGTTG-3') (Embley *et al.,* 1992).Subsequently 16S rDNA were amplified (1100bp) by PCR using the metagenomic DNA and Master mix (Fermentas, UK). A total of 25µl of reaction mixture consisted of 10 pmol of each primer, 30 ng of template DNA, 12.5µl of Master mix (Fermentas, UK). The PCR amplification was performed by Thermal Cycler (ABI, USA) and PCR conditions were adjusted in laboratory. The anticipated product of approximately1.1 kb was was purified using Qiagen DNA Gel Extraction Kits (QIAGEN, CA) in accordance with the directions of the manufacturer.

Cloning and sequencing

The purified PCR products were cloned in PTZ57R/T vector (Fermentas, UK) as per the instructions of the manufacturer and transformed into E. coli DH-5 α competent cell. Ampicillin- and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside)-amended LB agar was used for blue-white screening of transformants. The recombinant plasmids then were extracted by the Qiagen mini-prep plasmids extraction kit (QIAGEN, CA). Plasmid inserts were amplified with primers M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') and nucleotide sequences of cloned genes were determined by sequencing with M13F/ M13R primer in ABI Prism 310 Genetic analyser (Applied Biosystems Inc., CA) using BigDye Terminator (version 3.1) at Animal Biotechnology laboratory, AAU, Anand, Gujarat, India.

Sequence analyses and phylogenetic tree constructing

All reference sequences were obtained from the Genbank/EMBL/DDBJ/RDP (Benson *et al.,* 2007). Sequences (~500 bp) from the current study were trimmed (remove low-quality base calls from the start and end of DNA sequence) manually and analysed by the CHECK_CHIMERA program (Maidak *et al.,* 2001) to remove any chimeric rDNA clone. The similarity searches for sequences were carried out by BLAST (http://www.ncbi.nlm.nih.gov) Madden *et al.,* 1996) and alignment was done

using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html (Thompson *et al.,* 1994). Ambiguously and incorrectly aligned positions were aligned manually. The distance matrix was calculated using the DNADIST program included in PHYLIP (Felsenstein 1985) and used to assign sequences in various operational taxonomic units (OTUs) or phylotypes by DOTUR (Schloss and Handelsman, 2005) with 95% confidence intervals to quantify the diversity of phylotypes and total of 23 OTUs were distinguished, based on unique 16S r DNA sequences. Good's coverage was calculated as $[1 - (n/N)] \times 100$, where n is the number of single-clone OTUs and N is the library size, i.e. the total number of sequences (clones) for the analysed sample (Schloss and Handelsman, 2005). Phylogenetic tree was constructed by the neighbour joining method using MEGA 4.0 (Tamura *et al.,* 2007). Bootstrap re-sampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies.

The prefix meth was used to denote OTUs identified and nucleotide sequences have been deposited in the Genbank database under the accession numbers GQ120718- GQ120889.

RESULTS AND DISCUSSIONS

The bacterial DNA was successfully extracted from rumen fluid (Figure 3.1) and PCR fragments having expected size of 1.1 kb were obtained from DNA samples (Figure 3.2)

One hundred seventy one 16S rDNA sequences were analyzed. On the basis of sequence similarity, all of the sequences were related to methanogens. In our library, about 52.63% sequences (90 clones) had >90% similarity to the 16S rDNA database sequences. Furthermore, about 46.78% of the sequences (81 clones) were 75-89% similar to 16S rDNA database sequences (Table 3.1). The sample preparation technique, centrifugation before DNA extraction, allowed us to preferentially examine methanogens isolated from the fluid fraction of rumen contents. Two distinct clusters were generated by Maximum Parsimony method analysis of sequences (Figure 3.3). The first largest cluster contained 12 OTUs (83 clones) grouped with order Methanomicrobials forming two distinct subclusters that were supported by high bootstrap values (Figure 3.3). Subcluster Methanomicrobials I consisted of 51 clones

(7 OTUs) identical or nearly identical sequences (similarity values ranged from 85 to 96 %) that were similar to *Methanomicrobium mobile*-like clones. The second subcluster, belonged *Methanomicrobium mobile*-like clones (Table3.2). Phylotypes within the Methanobacteriales represented 48.5% (83 clones) of total clones which spanned 10 OTUs. Within this cluster, the cloned sequences also formed two subclusters. It should be noted that the significance of the subclusters is not supported by high bootstrap values (Figure 3.3). Although, the rDNA sequences may represent species of *Methanobacterium bryantii.* A total of one OTUs representing 4.34% of total OTU were closely related to cultured species belonging to the *Methanosarcina barkeri* like clone. Thus methanogens sequences obtained from rumen formed culsterd affiliated to different methanogens as well as unidentified group. The total includes seven single-clone OTUs, so Good's coverage (96%) of 16S r DNA libraries indicated that the sequences identified in libraries present the majority of methanogens diversity present in rumen.

The results of the present study show that Surti rumen 16S rRNA library consist about 97% clones belonged to the hydrogenotrophic methanogens (Methanomicrobials and Methanobacterials), while the acetoclastic methanogens represented merely 3% of the total clone diversity. Earlier phylogenetic studies based on the 16S rRNA and *mcrA* genes also revealed that majority of the sequences retrieved from bovine rumens and cattle dung were affiliated to hydrogenotrophic methanogens belonging to Methanomicrobiales and Methanobacteriales (Tatsuoka *et al.,* 2004). The methanogen community of buffaloes rumen should reflect the same trend as observed in a typical cattle rumen such as the greater abundance of hydrogenotrophic methanogens than the acetoclastic methanogens.

Boone *et al.,* (1993) considered that a sequence similarity of 98 % or less was evidence for separate species within the methanogens. Based on this, Methanomicrobials would be considered as *Methanomicrobium mobile*-like strains. However, as pointed out by Martinez-Murcia *et al.* (1994), 16S rRNA sequence similarity values, recommended to define a species, provide a working definition that has been empirically derived, and values should not be treated as absolute or fixed. For example, 16S rRNA sequences from strains of *Methanobacterium thermoformicicum* and *M. thermoautotrophicum* used in their study were greater than

98% similar, yet these organisms were considered distinct species. Similarly, Shin *et al.* (2004b) reported that 85% (89 of 104 clones) of the total clones from the bovine rumen belonged to the order Methanomicrobiales, with 61 clones resembling *Methanomicrobium mobile.* Interestingly, *Methanomicrobium mobile* was not detected in sheep from Western Australia (Wright *et al.,* 2004).

Zinder (1993) showed that in a typical cattle rumen, the approximate steady-state amounts of volatile fatty acids (VFA) are 63% acetate, 21% propionate, and 16% butyrate and other higher fatty acids. VFA are generally absorbed by the rumen epithelium and subsequently converted to animal proteins, and therefore not available for utilization as a carbon source by acetoclastic methanogens (Methanosarcinales) residing in rumen. Therefore, acetoclastic methanogens make up only a small percentage of total methanogen community in cattle rumen. The only carbon source available in plenty for methanogens is H_2/CO_2, thus hydrogenotrophic methanogens (Methanomicrobiales and Methanobacteriales) that are capable of using H_2/CO_2 can multiply easily and are observed in high abundance in rumen (Zinder 1993).

Previous culture based studies have isolated methanogens of the genus *Methanomicrobium* and *Methanobacterium* from the bovine rumen (Jarvis *et al.*, 2000), although *Methanobrevibacter* and *Methanosarcina* tend to be isolated at higher population levels (Purdy *et al.,* 2003). While, *Methanobrevibacter*-like clone could not detect in the present study. This may be due to differences in sample preparation, animal diet or geographic region. The results of the present study also corroborate with earlier observations of Rastogi *et al.,* (2008), where in the fresh cow dung *mcrA* library 93.5% clones belonged to the hydrogenotrophic methanogens, while the acetoclastic methanogens (Methanosarcinales) represented merely 6.5% of the total clone diversity, in the 8-month-old dung, which included 80% of clones belonging to hydrogenotrophic methanogens, while acetoclastic methanogens constituted only 20% of the total clone diversity.

Although some feeding strategies reduce ruminal methane emissions, the amount of CH_4 produced during ruminal fermentation is dependent upon the nature of the substrate being fermented. In general, methanogenic potential of the ruminal microflora is greatest for the fermentation of structural carbohydrates compared to that of non-structural carbohydrates (Boadi *et al.,* 2004).The addition of fat or

individual fatty acids to ruminal cultures decrease CH_4 production (Soliva *et al.*, 2004). Ruminal methane is formed by the action of methanogenic archaea typified by hydrogenotrophic methanogens, which is present in ruminants fed upon a wide variety of diets worldwide. Genome sequences would provide new insights into the lifestyle and cellular processes of this important rumen hydrogenotrophic methanogens (Methanomicrobiales and Methanobacteriales) under control feeding regime. It would also define vaccine and chemogenomic targets for broad inhibition of rumen methanogens and represents a significant contribution to worldwide efforts to mitigate ruminant methane emissions and reduce production of anthropogenic greenhouse gases.

CONCLUSIONS

Over all more studies are needed on the effects of diets composition and animal species on the diversity of methanogens and enteric methane emission in the rumen. This study has revealed the largest assortment of hydrogenotrophic methanogens phylotypes ever identified from rumen of Surti buffaloes and the need to better understand the factors influencing methanogen diversity with methane emission. Further studies are needed to examine methanogen diversity in goat, sheep and dairy cattle located in the Gujarat state. Such studies would significantly enhance our knowledge and ability to use novel methods to manipulate the rumen methanogen populations to reduce methane production from ruminant animals. Reducing enteric methane emissions is likely to be one of the key mitigation strategies for the reduction of greenhouse gas emissions in the agricultural sector.

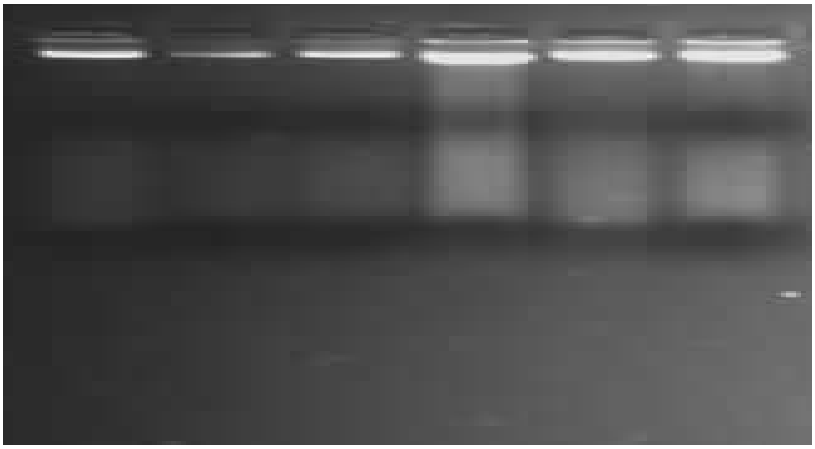


Figure 3.1. Agarose gel electrophoresis of metegenomic DNA extracted from rumen fluid of Surti buffalo

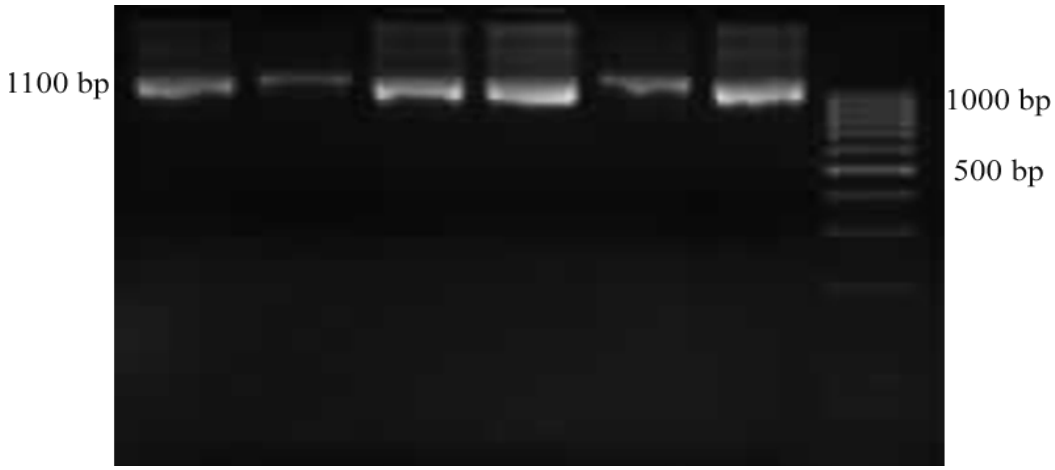


Figure 3.2. Agarose gel electrophoresis of PCR product showing amplicon of 1100 bp

Table 3.1: Similarity values of operational taxonomic units (OTUs) based on 16S rDNA sequences of methanogens (171 clones) retrieved from the rumen fluid of Indian Surti buffalo.

OTU	No of Clone	Nearest valid relative	Accession No	Similarity (%)
Meth1	37	*Methanomicrobium mobile*	M59142	94
Meth 2	29	*Methanomicrobium mobile*	AY196679	88
Meth 3	24	*Methanomicrobium sp*	X99139	90
Meth 4	5	Uncultured *Methanobrevibacter sp.*	FJ938102	89
Meth 5	1	Uncultured methanogenic archaeon	EU794805	76
Meth 6	3	Uncultured Methanobacteriales archaeon	DQ402018	93
Meth 7	4	*Methanomicrobium mobile*	AY196679	85
Meth 8	2	*Methanomicrobium mobile*	AY196679	89
Meth 9	2	*Methanomicrobium mobile*	AY196679	86
Meth 10	1	Uncultured Methanobacteriales archaeon	DQ402018	75
Meth 11	3	*Methanomicrobium mobile*	AY196679	86
Meth 12	22	Uncultured rumen archaeon	FJ586387	88
Meth 13	1	*Methanomicrobium mobile*	AY196679	91
Meth 14	21	Uncultured archaeon clone	EU487521	96
Meth 15	2	*Methanomicrobium mobile*	AY196679	87
Meth 16	2	*Methanomicrobium mobile*	AY196679	90
Meth 17	1	Not available	-	-
Meth 18	4	*Methanomicrobium mobile*	AY196679	85
Meth 19	1	Uncultured compost archaeon	DQ365171	91
Meth 20	1	Uncultured archaeon clone	EU487521	90
Meth 21	1	Uncultured rumen archaeon	FJ586416	84
Meth 22	2	*Methanomicrobium mobile*	AY196679	86
Meth 23	2	*Methanomicrobium mobile*	AY196679	85

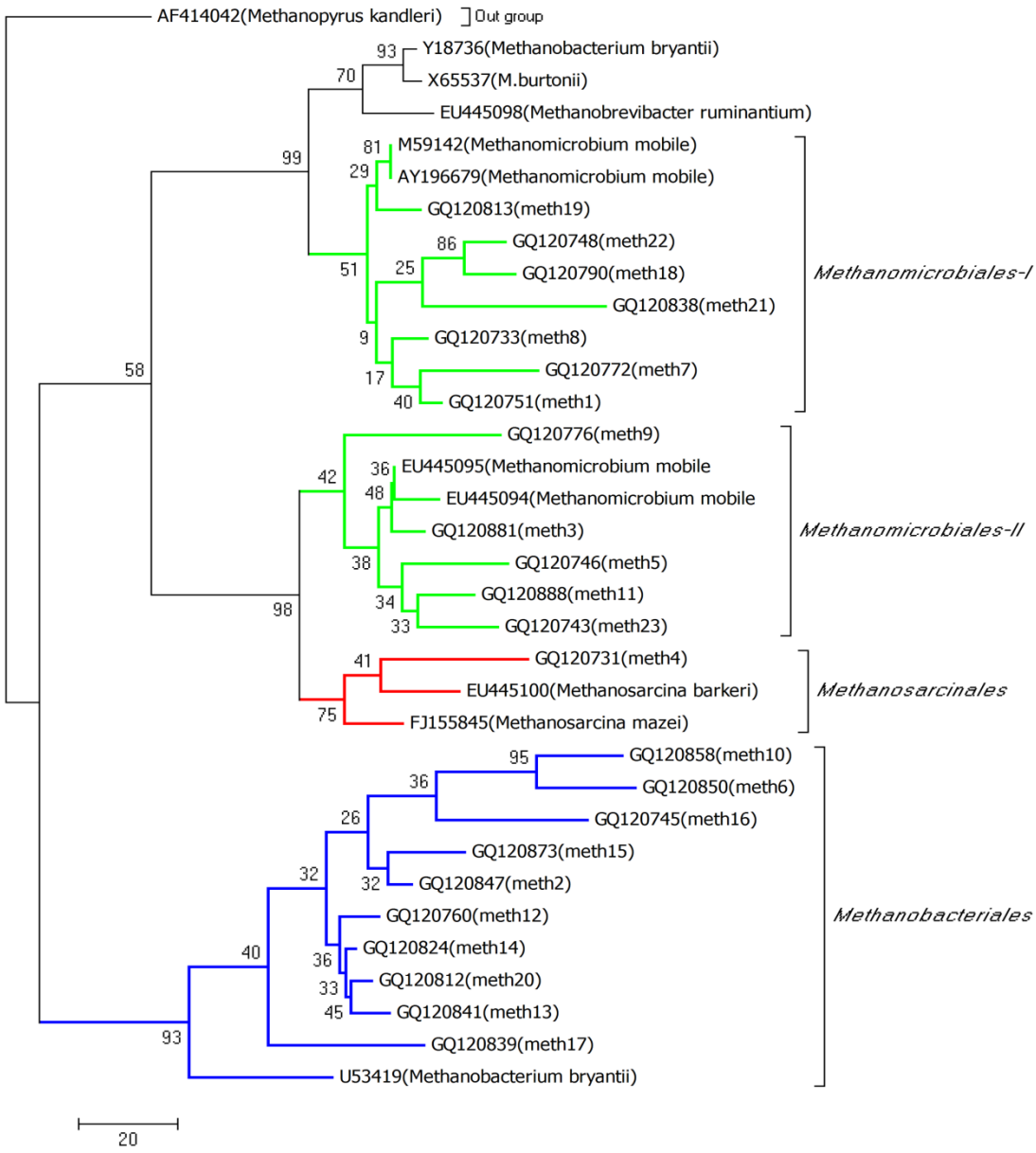


Figure 3.3. Phylogenetic relationships of partial 16S r DNA sequences of clones recovered from Surti rumen samples. The rooted tree was constructed as a maximum parsimony tree using close-neighbor interchange level 1 and bootstrapped with 1,000 trials, using the MEGA 4 software tree building program. All positions containing gaps and missing data were eliminated from the data set .The *Methanopyrus kanderi* (AF414042) are used as the out-group for rooting the tree. The scale bar represents the number of changes over the whole sequence.

Table 3.2: Analysis of diversity of methanogen16S rDNA phylotypes retrieved from the rumen of Indian Surti buffalo.

Taxon	**No. of operational taxonomic units (OTUs) and % of total OTUs**	**No. of clones**
Methanomicrobiales	**12 OTUs (52.17%)**	**83**
a) Methanomicrobials I	7 OTUs	51
b) Methanomicrobials II	5 OTUs	32
Methanobacteriales	**10 OTUs (43.47%)**	**83**
Methanosarcinales (*Methanosarcina barkeri*)	**1 OTU (4.34%)**	**5**
Total	**23 OTUs**	**171**

Chapter 4. Methanogenic diversity within the rumen of Surti buffalo by methyl coenzyme M reductase A (mcrA) genes, point methanobacteriales

ABSTRACT

Methane emissions from ruminant livestock are considered to be one of the more potent forms of greenhouses gases contributing to global warming. Many strategies to reduce emissions are targeting the methanogens that inhabit the rumen, but such an approach can only be successful if it targets all the major groups of ruminant methanogens. Therefore, a basic knowledge of the diversity of these microbes in breeds of buffalo is required. Therefore, methanogenic community in rumen of Surti buffaloes was analyzed by PCR amplification, cloning, and sequencing of methyl coenzyme M reductase (mcrA) gene. A total of 76 clones were identified revealing 14 different sequences (OTUs). All 14 sequences were similar to methanogens belonging to the orders Methanobacteriales. Within methanobacteriales, six OTUs (12 clones) were similar to *Methanosphaera stadtmanae*, seven OTUs (63 clones) were similar to unclassified methanobacteriales and the remaining one OTU (1clone) was belonged to unknown methanogen. Over all members of the Methanobacteriales dominated the mcrA clone library in the rumen of Surti buffalo. Further studies and effective strategies can be made to inhibit the growth of Methanobacteriales to reduce the methane emission from rumen which would help in preventing global warming.

Key words: methanomicrobiales, methanobacteriales, mcrA, OTUs, Surti buffaloes 16S rDNA.

INTRODUCTION

The rumen is characterized by its high microbial population density and complexity of micro-ecological interactions. Methane is biologically produced by the metabolism of the diverse group of methanogenic micro-organisms, methanogens, which are phylogenetically placed exclusively as members of the archaea domain.

They inhabit typical anaerobic environments, such as wetlands, sediments, geothermal springs and the digestive tracts of mammals (Garcia *et al.,* 2000). Methane is an important greenhouse gas which significantly contributes to global warming. Livestock is a major anthropogenic source of methane emission from agriculture and contributes about 18% of the global greenhouse gas (GHG) emissions, and as much as 37% of anthropogenic methane, mostly from enteric fermentation by ruminants (FAO, 2006)**.** Livestock rearing has been an integral part of the agricultural system in India. Currently, India possesses the world's largest livestock population of 485 million, which accounts for 13% of the global livestock population (MOA, 2003)**.** It has 57% of the world's buffalo and 16% of the cattle population. Contribution of methane emission in India by buffalo is 42% (Chabara *et al*., 2009).

Several groups have reported the monitoring of methanogen populations from environmental samples through targeting of the 16S ribosomal gene (Yu *et al*., 2005; Stewart *et al*., 2006 and Wright *et al.,* 2007). While researchers have traditionally used the 16S rRNA gene for phylogenetic diversity, many researches are now addressing the diversity of the methanogenic archaea by studying sequence divergence within the methyl coenzyme- M reductase subunit A (mcrA) gene (Lueders *et al*., 2001; Luton *et al*., 2002; Hallam *et al*., 2003, Tatsuoka *et al*., 2004, Rastogi *et al.,* 2008). Methyl coenzyme-M reductase is ubiquitous to methanogens and is crucial to the terminal step of methanogenesis where it is involved in the reduction of the methyl group bound to coenzyme-M. There is no report concerning to mcrA genes from the buffalo rumen, therefore, we examine the community of methanogens using comparative sequence analysis of the mcrA amplified from total DNA extracted from rumen fluid of Surti buffalo.

MATERIALS AND METHODS

Animals, diet, collection of rumen fluid and DNA extraction

The same as described in chapter 2

Total DNA (each hrs) was extracted separately by using a commercially available kit according to the manufacturer's instructions (QIAGEN Stool kit; QIAGEN, CA). The individual DNA was used as a template in PCR to amplify *mcrA* gene.

PCR primers and amplification

The mcrA primers used were ME1 (5'-AGCMATGCARATHGGWATGTC-3-') and ME2 (5'-ATCATKGCRTAGTTDGGRTAGT-3') (Hales *et al*., 1996), subsequently mcrA gene were amplified (760bp) by PCR using the metagenomic DNA and Master mix (Fermentas, USA). A total of 25µl of reaction mixture consisted of 10 pmol of each primer, 30 ng of template DNA, 12.5µl of Master mix (Fermentas, USA). The PCR amplification was carried out as follows: 1 cycle at 95^{o}C for 3 min, 35 cycles of 95^{o}C for 30 s, 60^{o}C for 1 min, 72^{o}C for 1 min and a final elongation at 72^{o} for 10 min by using thermal Cycler (ABI, USA). The anticipated product of approximately 760 bp was cleaned separately using a Qiagen DNA Gel Extraction Kits (QIAGEN, CA) in accordance with the directions of the manufacturer and pooled the purified PCR products in equimolar concentration.

Cloning and sequencing

The purified PCR products were cloned in InstaTA cloning kit (Fermentas, USA) as per the instructions of the manufacturer. The recombinant plasmids then were extracted by the Qiagen mini-prep plasmids extraction kit (QIAGEN, CA). Sequencing was performed for all the clones in the library with an ABI Prism 310 Genetic analyser (Applied Biosystems Inc., CA) using BigDye Terminator (version 3.1) at Animal Biotechnology laboratory, AAU, Anand, Gujarat, India.

Sequence analyses and phylogenetic tree constructing

All reference sequences were obtained from the Genbank/EMBL/DDBJ (Benson *et al*., 2007. Sequences (~600 bp) from the current study were analysed by the CHECK_CHIMERA program (Maidak *et al*., 2001) to remove any chimeric clone. The similarity searches for sequences were carried out by BLAST (http://www.ncbi.nlm.nih.gov/ BLAST/Blast.cgi (Madden *et al*., 1996) and alignment was done using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html (Thompson *et al*., 1994). Ambiguously and incorrectly aligned positions were aligned manually. The distance matrix was calculated using the PRODIST program included in PHYLIP (Felsenstein, 1985) and used to assign sequences in various operational taxonomic units (OTU) or phylotypes by DOTUR (Schloss and Handelsman, 2005) and total of 14 OTUs were distinguished, based on unique MCR sequences. The percentage of good coverage was calculated as [1 - (n/N)] X 100, where 'n' is the

number of single clone OTUs and 'N' is the library size. Phylogenetic tree was constructed by the neighbour joining method using MEGA 4.0 (Tamura *et al*., 2007). Bootstrap re-sampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies. The prefix mcrA was used to denote OTU identified and nucleotide sequences have been deposited in the Genbank database under the accession numbers GQ120890-GQ120965.

RESULTS AND DISCUSSIONS

The metagenomic DNA was successfully extracted from rumen fluid (Figure 4.1) and PCR fragments having expected size of 0.670 kb were obtained from DNA samples (Figure 4.2).

Methanogen-specific DNA fragments were amplified from DNA extracted from the Surti rumen fluid by PCR with primers targeting mcrA genes. The amplified fragments from three rumen fluids, which were approx. 760 bp, are shown in Figure 4.3. All the clones were subjected to sequence analysis followed by online homology search, Genbank which implements the BLAST algorithm (Madden *et al*., 1996). None of clone (76) from our library was assigned any genera/ species. Because the similarity values of our sequences were too low to assign them to particular taxa with a reasonable degree of confidence. In our library 14 sequences (OTUs) were identified. Phylogenetic analysis was performed to clarify their taxonomic position. The sequences obtained in the present study were placed in the single cluster Methanobacteriales. Within the methanobacteriales, 63 clones (7 OTUs) belonged to the unidentified methanobacteriales and 12clones (6 OTUs) sequences were found to be the closest to *Methanosphaera stadtmanae*. Of the clones isolated from rumen sample, one clone was unknown methanogen (1 OTU) (Table 4.1). Thus MCR sequences obtained from rumen formed tightly-culustered affiliated to the different orders as well as unclassified group. The total four single -clone OTUs, so Good's coverage (94.73%) of MCR libraries indicated that the sequences identified in libraries represent the majority of methanogens diversity present in rumen.

Of 14 OTUs, 13 sequences (OTUs) of the DNA have been obtained from the 76 clones in the present study, these sequences were placed in the same cluster, which were relatively close to unidentified methanobacteriales (63 clone) and *M. stadtmanae*

(12 clones), in phylogenetical placements (Figure 4.3). Whitford et al. (2001) also found several sequences that were related to *Methanosphaera stadtmanae*. Wright *et al*., (2007) also found a sequence (ON-CAN.13) in cattle from Ontario that was 95.8% similar to that of Methanosphaera stadtmanae and 99.8% similar to that of their clone ARC29. Methanogens similar to *Methanosphaera stadtmanae* have also been reported in pasture-fed dairy cattle (Skillman *et al*., 2006a) The DNA sequences of mcrA genes, isolated in this study, showed similarities with unidentified methanobacteriales and to *M. stadtmanae*. It has been known that mcrA genes could be used as phylogenetic tool for the specific detection and the identification of methanogenes, because the phylogeny of the mcrA genes and 16S rDNA from the recognized orders of methanogens clearly had a strong similarity (Luton *et al*., 2002).

CONCLUSIONS

Our results show that rumen of Surti buffaloes contain one of the essential and diagnostic genes of the methanogenic pathway. The identification of these genes provides a means to identify cluster/ group on the basis of mcrA sequence. Moreover, identification of rumen associated mcrA groups defines a functional genomic link between methanogenic and putative reverse methanogenic archaea. Specific questions relating to methanogenic protein function in buffalo rumen await further genomic, biochemical, structural, and proteomic analysis.

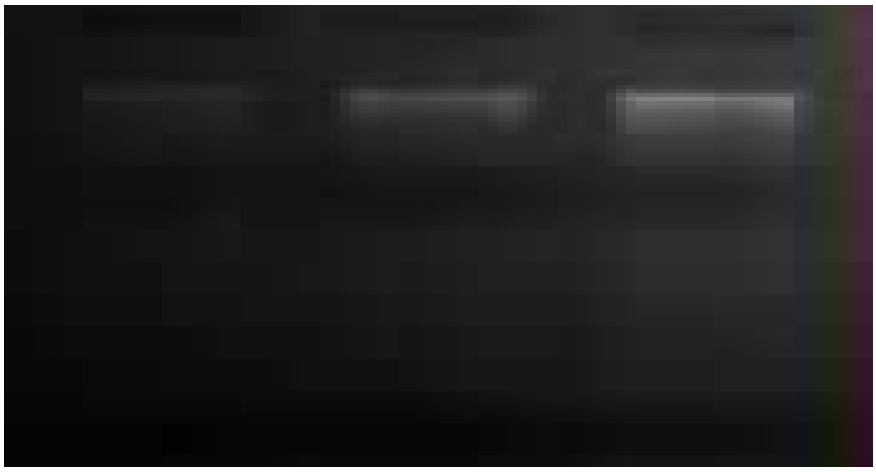


Figure 4.1. Agarose gel electrophoresis of metegenomic DNA extracted from rumen fluid of Surti buffalo

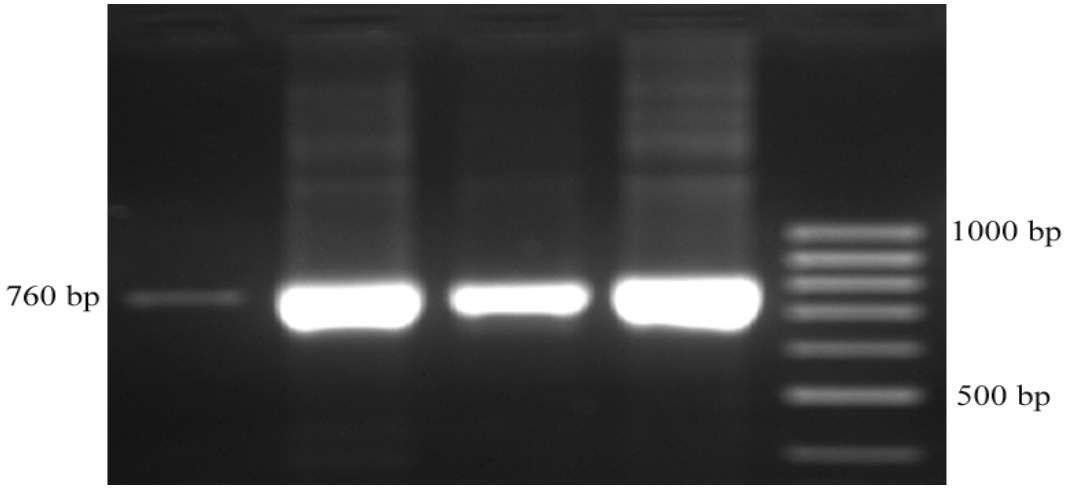


Figure 4.2. Agarose gel electrophoresis of PCR product showing amplicon of 760 bp

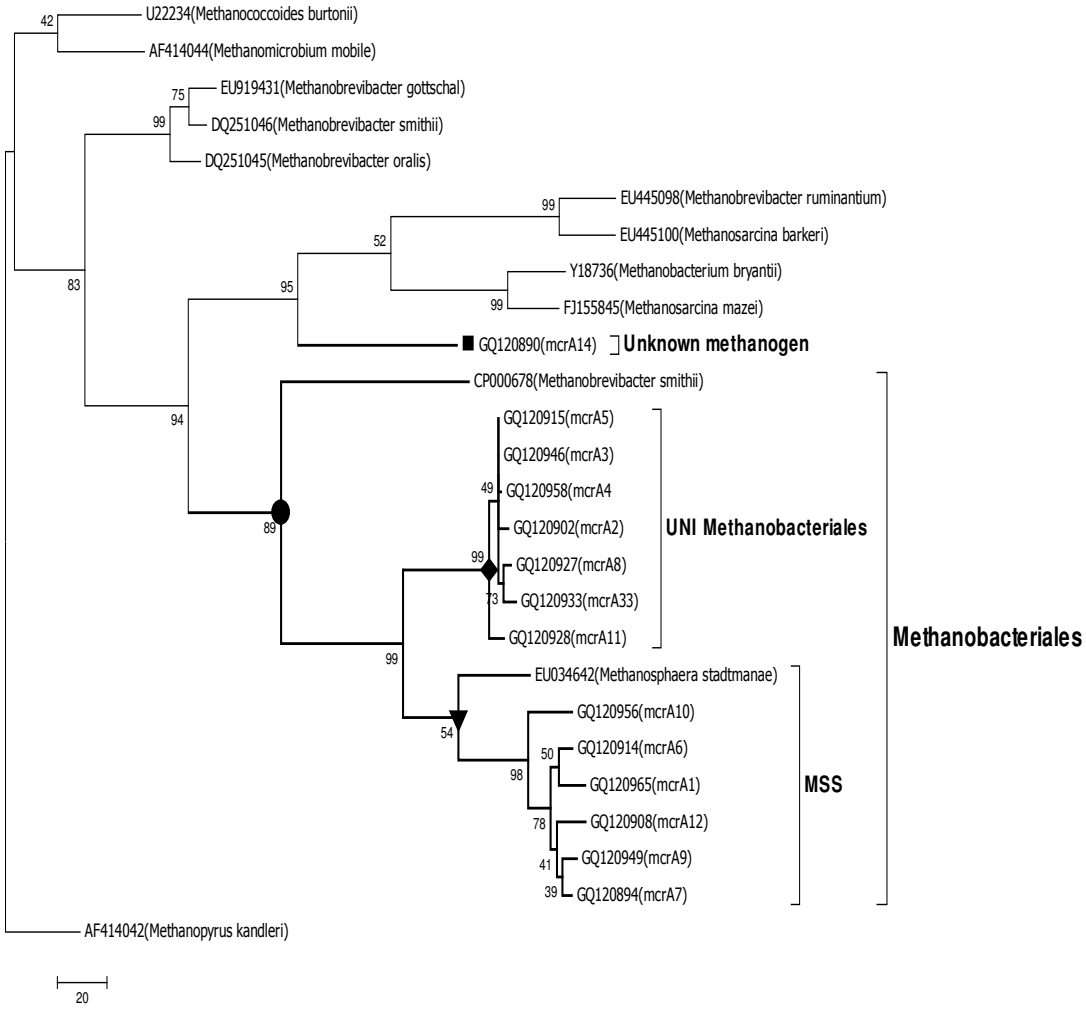


Figure 4.3. The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Phylogenetic analyses were conducted in MEGA4. (UNI= unidentified and MSS= *Methanosphaera stadtmanae*).

Table 4.1: Analysis of mcrA gene phylotypes diversity retrieved from the rumen fluid of Surti buffaloes

Items	**16S r DNA library**
Library size [a] (N)	76
OTUs [b]	14
Single clone OTU [c] (n)	4
Good's coverage [d] (%)	94.73
Clone distribution	
(i) Methanobacteriales	**14 OTUs** (76 clones)
a. *Methanosphaera stadtmanae*	**6 OTUs** (12 clones)
b. *Unidentified Methanobactriales*	**7 OTUs** (63 clones)
Unknown methanogen	**1 OTU** (01 clone)
Total	**14 OTUs (76 Clones)**

[a] Number of clones analyzed from library

[b] OTUs based on mcrA gene sequences

[c] OTUs containing only single clone

[d] The higher percentage coverage means more diversity is captured

Chapter 5. Assessment of protozoa in Surti buffalo (*Bubalus bubalis*). rumen based on the 18S rDNA Sequences

ABSTRACT

The molecular diversity of rumen protozoa in Surti buffalo *(Bubalus bubalis*) was investigated using 18S rRNA gene librariy prepared from the pooled sample of rumen fluid from three adult animals. A total of 172 clones were sequenced and similarities to known 18S rDNA sequences were examined. About 12 OTUs had ≥91% similarity to18S r DNA data sequences. Furthermore, about 27 OTUs of the sequences were 86-90% similar to 18S r DNA database sequences and remaining 14 OTUs, the similarity was less than 85 %. Phylogenetic analyses were also used to infer the makeup of protozoal communities in the rumen of Surti buffalo. As a result, 40 OTUs (123 of 172 clones) belonged to entodiniomorphid protozoa, indicating that this group is most dominant component of protozoal populations in Surti buffaloes (*Bubalus bubalis*). 12 OTUs (45 clones) belonged to the holotrich protozoa. Among holotrich, 12 clones isolated from rumen fluid fell into two species identified as *Dasytricha ruminantium*-like clone (7clones) and *Isotricha prostoma*-like clone (5 clones). One OTU (4clones) belonged to the Haptorida protozoa.

Key words: Buffalo Rumen protozoa, 18S rDNA, Phylogenetic analysis, protozoal diversity**.**

INTRODUCTION

Protozoa are unicellular eukaryotic microorganisms which are ubiquitous in nature and anthropogenic environments. The rumen ciliates are potentially an agriculturally important group of protozoa found in domestic and wild ruminants (Williams and Coleman, 1992). Several factors seem to influence the composition of the protozoan population in the rumen. These include type and amount of feed consume, pH, turnover rate and feed level. Rumen protozoa utilize hydrogen-generating fermentation to provide substrates for methanogens. The protozoa, in turn, benefit from hydrogen removal by the methanogens because hydrogen is inhibitory to protozoan metabolism (Wolin 1974). Earlier reports have provided evidence of strong

relationships such as endosymbiosis between ruminal protozoa and methanogens (Finlay *et al*., 1994; Ushida *et al.,* 1997). Ciliate protozoa play a diverse role in the ruminal metabolism of nutrients. To improve the efficiency of feed crude protien utilization, considerable effort has been made to find a means of total elimination of protozoa from the rumen (defaunation) and a massive reduction in the rumen protozoa population (reduced fauna). By chemical drenching of experimental animals has been found to improve milk production. Detection and identification of protozoa have commonly been achieved through microscopic examination of morphological features. It remains difficult and time-consuming to reliably detect or identify many protozoan species by these methods, as protozoa may be fragile and inconspicuous and as it may be difficult to determine whether a given morphological feature can be regarded as distinct or not (Fried *et al.,* 2002; Sims *et al*., 2002 ; Caron *et al*., 2004). The anaerobic ruminal protozoa have been well studied (Williams and Coleman 1997), but much of this work is based on microscopic examination (Dehority 1993). Difficulties in cultivating protozoa, and their polymorphic nature, have delayed effective assessment of protozoan ecology and taxonomy (Dehority, 1994). The small subunit ribosomal RNA (SSU-rRNA) gene called 16S rRNA in prokaryotes and 18S rRNA in eukaryotes is widely used as molecular marker to identify morphologically indistinguishable species, to infer their phylogenetic relationships, and to elucidate diversity. As molecular-based approaches to assess protozoal population for quantitative purposes. PCR-sequencing methods have been extensively used to examine protozoal diversity in rumen samples (Karnati *et al*., 2003; Sylvester *et al*., 2004). PCR-DGGE has also been used in the profiling of protozoal communities in the rumen (Regensbogenova *et al*., 2004b; Sylvester *et al*., 2005).

India possesses more than 50 % of world's buffalo population; Indian buffaloes produce more than 60% milk in India (Kumar *et al*., 2007). Surti is a popular breed of buffalo found in central Gujarat state. The Surti buffaloes are of medium size and docile temperament and body weight 350 to 375kg at maturity. Since our animals mainly sustain on crop residues, the protozoa population is expected much different than that of exotic cattle. The present study was conducted to examine the diversity of rumen protozoa in Surti buffalo offered diet, green fodder bajra (*Pennisetum purpureum*), mature pasture grass (*Dicanthium annulatum*) and

compound concentrate mixture. The molecular techniques were used to construct a library of 18S rDNA clones of rumen protozoa, and a phylogenetic tree for the clones isolated.

MATERIALS AND METHODS

Animals, diet, collection of rumen fluid and DNA extraction

The same as described in chapter 2

The total DNA mixture was used as a template in PCR to amplify 18S r DNA.

PCR primers and amplification

A protozoa specific forward primer P SSU 342f (5′ACTTTCGATGGTAGTGTATTGGACTAC-3′) corresponding to 316 to 342 bp (*Saccharomyces cerevisiae* numbering) was used with a Eukarya-specific reverse primer Medlin B (5′-ATGATCCTTCTGCAGGTTCACCTAC-3′) (Medlin *et al*., 1988). Subsequently 18S r DNA fragment were amplified (1360bp; Figure 5.2) by PCR using the metagenomic DNA and Master mix (Fermentas, UK). A total of 25µl of reaction mixture consisted of 10 pM of each primer, 30 ng of template DNA, 12.5µl of Master mix (Fermentas, UK). The PCR amplification was performed by Thermal Cycler (ABI, USA) and the PCR conditions were as follows: initial denaturation at 95°C for 5 minutes, 30 cycles of amplification consisting of a 1-minute denaturation step at 94°C, a 1-minute annealing step at 37°C, a 3-minutes extension step at 72°C, and a final 10-minutes extension at 72°C. The anticipated product of approximately 1.36 kb was cleaned using a Qiagen DNA Gel Extraction Kits (QIAGEN, CA) in accordance with the directions of the manufacturer.

Cloning and sequencing

The purified PCR products were cloned in InstaTA cloning kit (Fermentas, UK) as per the instructions of the manufacturer. The recombinant plasmids then were extracted by the Qiagen mini-prep plasmids extraction kit (QIAGEN, CA). Plasmid inserts were amplified with primers M13F (5'-GTAAAACGAC GGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') and nucleotide sequences of cloned genes were determined by sequencing with M13F/ M13R primer in ABI Prism 310 Genetic analyser (Applied Biosystems Inc., CA) using BigDye Terminator (version 3.1) in the Animal Biotechnology Laboratory, AAU, Anand, Gujarat, India.

Sequence analyses and phylogenetic tree analysis

All reference sequences were obtained from the Genbank/EMBL/DDBJ/ (Benson *et al*., 2007). Sequences (~550 bp) from the current study were analysed by the CHECK_CHIMERA program (Maidak *et al*., 2001) to remove any chimeric rDNA clone. The similarity searches for sequences were carried out by BLAST (http://www.ncbi.nlm.nih.gov/ BLAST/Blast.cgi (Madden *et al*., 1996) and alignment was done using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html (Thompson *et al*., 1994). Ambiguously and incorrectly aligned positions were aligned manually. The distance matrix was calculated using the DNADIST program included in PHYLIP (Felsenstein, 1985) and used to assign sequences in various operational taxonomic units (OTUs) or phylotypes by DOTUR (Schloss and Handelsman, 2005) with 95% confidence intervals to quantify the diversity of phylotypes and total of 53 OTUs was identified, based on unique 18S rDNA sequences. The percentage of good coverage was calculated as [1 - (n/N)] X 100, where 'n' is the number of single clone OTUs and 'N' is the library size. Phylogenetic tree was constructed by the eighbor joining method using MEGA 4.0 software (Tamura *et al*., 2007). Bootstrap re-sampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies (Felsenstein, 1985).

The prefix IBRB was used to denote OTU identified and nucleotide sequences have been deposited in the Genbank database under the accession numbers EU345005-EU345176.

RESULTS AND DISCUSSIONS

The metagenomic DNA was successfully extracted from rumen fluid (Figure 5.1) and PCR fragments having expected size of 1.360 kb were obtained from DNA samples (Figure 5.2)**.**

The molecular inventory of protozoa revealed in present study showed the occurrence of complex protozoal communities in buffalo rumen ecology. The number and distribution of phylotypes indicates the protozoa diversity in rumen of Surti buffalo. The composition of 18S rDNA sequences and the phylogenetic ascription of clones to cultured protozoa with known physiology and substrate spectrum suggest a likely role of these species in rumen function. Nevertheless, considering the

probability of various PCR induced anomalies, gene copy number, and other methodological experimental biases (Acinas *et al*., 2005), it may be erroneous to assume that the obtained phylotypes distribution would be the actual species distribution in samples.

Sequence similarity with database sequences

All the clones were subjected to by online homology search, in Genbank which implements the BLAST algorithm (Madden *et al*., 1996). Although the similarity for most of the sequences with those of known rumen protozoa was too low to identify the sequences as representing a particular species (Table 5.1). In our library 12 OTUs had ≥91% similarity to18S r DNA database sequences. Furthermore, about 27 OTUs of the sequences were 86-90% similar to 18S r DNA database sequences and remaining 14 OTUs, the similarity was less than 85 % (Table 5.1). Phylogenetic analysis was performed to clarify their taxonomic position.

Phylogenetic placement of sequences

The results of phylogenetic analysis of sequences from the rumen fluid library are shown in Figure 5.3. In this library, majority of sequences (40 OTUs) were *entodiniomorphid* protozoa (123 clones). About 12 OTUs (45 clones) belonged to the *Holotrich* protozoa. Only one OTU (IBRP19; 04 clones) belonged to the group *Haptorida.* This may reflect its functional importance in the rumen and may represent dietary transient. Thus 18S r DNA sequences obtained from rumen formed tightly-culustered affiliated to the different group. The total eleven single-clone OTUs, so Good's coverage (93.75%) of 18S rDNA libraries indicated that the sequences identified in libraries represent the majority of protozoa diversity present in rumen.

Compared to other ecosystems, little information is available on protozoa in buffalo rumen. The relative lack of information on ruminal protozoa may be due to difficulties with isolation, culture, or maintenance. Rumen isolates often lose viability for unknown reasons during purification or sub culturing of pure isolates. More than 42 genera of ruminal protozoa have been described based on cultivation-based and morphological studies (Williams, 1986; Dehority, 1993). Although sequences of few genera are available in sequence databases. Most genera are representatives of typical bovine rumen populations viz *Entodinium, Diplodinium, Eudiplodinium, Ostracodinium, Metadinium, Enoploplastron, Polyplastron, Epidinium,*

Ophryoscolex, Isotricha and *Dasytricha* (Williams and Coleman, 1992; Karnati *et al*., 2003). The present study revealed the phylogenetic diversity of the protozoan community in the rumen fluid of Surti buffalo by analyzing protozoan 18S rDNA sequences. BLASTn searches showed that sequenced clones shared similarity (79 to 96%) with ruminal protozoan sequences with Genbank database. The rumen fluid library was classified into three phylogenetic groups. The largest group was affiliated with the *entodiniomorphid* protozoa (40 OTUs. 123 clones), second group affiliated with the *Holotrich* protozoa (12 OTUs, 45 clones) and third the group affiliated with the *Haptorida* protozoa (01 OTU, 04 clones) (Table 5.2). Within holotrich, two species was identified as *Dasytricha ruminantium*-like clone (7 clones) and *Isotricha prostoma*-like clone (5 clones). The predominant protozoa identified in this study were the *Entodiniomorphid* group (about 75%). These results agree with a recent report of high numbers of *Entodinium* in the bovine rumen (Leng *et al*., 2010; Karnati *et al.,* 2003; Shin *et al*., 2004a). Similar result has been reported (Akbar *et al*., 2009) in Ghizel Sheep fed in pasture and nourished by dried grape by-product. Karnati *et al*., (2003) reported that their protozoan specific primers (used here) had a single mismatch with the 18S rDNA of *Entodinium sp*, but matched exactly with the 18S rDNA sequences of other protozoan species. They suggested that a PCR primer degenerate at the mismatched position would help minimize PCR bias, allowing for more representative retrieval of ruminal protozoan 18S rDNA from complex ruminal samples.

However, *Isotricha sp*, *Polyplastron sp* and *Ophryoscolex sp*. were identified in the washed ciliate suspension and many methanogens were detected on ciliate cells by F420 auto fluorescence (Tokura *et al*., 1999). *Dasytricha ruminantium*-like clones and *Isotricha prostoma*-like clones were also observed in our studies. Biochemical differences between *Isotricha* and *Dasytricha* (Gutierrez, 1955) have been examined by (Howard, 1959). *Dasytricha* is more versatile than *Isotricha*, fermenting cellobiose, galactose, and maltose. Fermentation products from galactose were the same as those formed from glucose. From glucose, the holotrich produced lactic, acetic, and butyric acids, carbon dioxide, hydrogen, and traces of propionic acid (Howard, 1959). A single OTU (IBRP19) located within the haptorida protozoa and may represent a dietary transient.

All entodiniomorphid protozoa engulf and utilize starch grains for energy (Williams and Coleman, 1997); however, the rate and amount of uptake can be different between species (Williams and Coleman, 1997). Smaller protozoal species (e.g., *En. simplex* and *En. caudatum*) engulf starch grains faster than larger protozoa but larger protozoa, engulf starch at a constant rate for several hours (Williams and Coleman, 1997). Cellulose is not universally used as is starch and only a few genera have been identified as cellulolytic protozoa. *Eudiplodinium maggii* was identified by Coleman (1979) as a protozoon capable of digesting cellulose at a faster rate than starch. Although only a few protozoal species are classified as truly cellulolytic, all entodiniomorphid protozoa except *Entodinium* species contain cellulase and are thought to contribute to ruminal fiber digestion.

CONCLUSIONS

We conclude that the majority of sequences were *entodiniomorphid* protozoa. In interpreting the results of the present study some difficulties were encountered because of possible flows in the analytical techniques used. As discussed previously (Wintzingerode *et al*., 1997) care must be taken in PCR-based analysis with respect to both experimental procedures and analysis of results. Genome size and the copies of *rrn* genes also affect the amount of PCR amplicon (Farrelly *et al*., 1995). Metabolic products of the microbial community heavily influence host animal nutrition. Therefore, microbial community and its collective activities also determine host nutrition and production (Sharp *et al*., 1998). An advanced set of protozoan-specific phylogenetic probes and quantitative real time PCR assay are needed to their distribution throughout rumen microbial communities. Future studies to understand the effects of varying rumen protozoa on different animal feeding habit and methanogenesis will also be important.

Figure 5.1. Agarose gel electrophoresis of metegenomic DNA extracted from rumen fluid of Surti buffalo

Figure 5.2. Agarose gel electrophoresis of PCR product showing amplicon of 1360 bp

Table 5.1: Similarity values of operational taxonomic units (OTUs) based on 18S rDNA sequences of 172 clones retrieved from the rumen fluid of Indian Surti buffalo.

OTU	No. of Clone	Nearest relative	Accession No	Similarity (%)
IBRP1	02	*Ostracodinium gracile*	AM158468	87
IBRP2	04	Uncultured Rumen Protozoa	AF502929	89
IBRP3	05	*Ostracodinium gracile*	AM158468	89
IBRP4	12	Uncultured Canadian Arcott wether rumen protozoa	DQ832560	88
IBRP5	04	*Ostracodinium gracile*	AM158468	90
IBRP6	03	Uncultured Canadian Arcott wether rumen protozoa	DQ832560	87
IBRP7	03	*Cycloposthium ishikawai*	EF632076	86
IBRP8	04	*Cycloposthium edentatum*	EF632077	86
IBRP9	04	Uncultured rumen protozoa clone YCRPB55	EU163779	87
IBRP10	03	*Isotricha prostoma*	AM158455	88
IBRP11	05	Uncultured Canadian Arcott wether rumen protozoa	DQ832560	94
IBRP12	06	Uncultured Canadian Arcott wether rumen protozoa	DQ832564	92
IBRP13	01	Uncultured ciliate	AM158846	86
IBRP14	03	*Ostracodinium gracile*	AM158468	88
IBRP15	05	*Isotricha prostoma*	AM158455	92
IBRP16	02	Uncultured rumen protozoa	EU163783	81
IBRP17	02	*Ostracodinium gracile*	AM158468	89
IBRP18	10	*Isotricha prostoma*	AM158455	91
IBRP19	04	*Troglodytella abrassarti*	AB437347	86
IBRP20	02	Uncultured Canadian Arcott wether rumen protozoa	DQ832565	84

IBRP21	02	Uncultured rumen protozoa clone CRA9	AF502927	86
IBRP22	06	*Teuthophrys trisulca africana*	DQ411863	85
IBRP23	08	Uncultured rumen protozoa clone YCRPB55	EU163779	89
IBRP24	11	*Dasytricha ruminantium*	AM158463	91
IBRP25	02	Uncultured rumen protozoa	EU163779	84
IBRP26	02	Uncultured rumen protozoa clone CRA5	AF502923	83
IBRP27	05	Uncultured rumen protozoa clone YCRPB55	EU163779	89
IBRP28	02	Uncultured ciliate	AM158846	89
IBRP29	02	Uncultured ciliate	AM158873	91
IBRP30	07	*Dasytricha ruminantium*	AM158463	86
IBRP31	02	*Ostracodinium gracile*	AM158468	87
IBRP32	04	*Ostracodinium gracile*	AM158468	89
IBRP33	01	*Isotricha prostoma*	AM158454	84
IBRP34	02	Uncultured rumen protozoa clone YCRPB59	EU163783	85
IBRP35	04	*Dasytricha ruminantium*	AM158463	87
IBRP36	01	Uncultured rumen protozoa	EU163779	93
IBRP37	01	Uncultured rumen protozoa clone YCRPB55	EU163779	86
IBRP38	02	Uncultured rumen protozoa clone YCRPB55	EU163779	91
IBRP39	01	*Polyplastron multivesiculatum*	AM158458	92
IBRP40	03	Uncultured rumen protozoa clone YCRPB59	EU163783	91
IBRP41	01	*Ostracodinium gracile*	AM158468	89
IBRP42	02	Uncultured Canadian Arcott wether rumen protozoa	DQ832560	83

IBRP43	01	*Dasytricha ruminantium*	AM158463	86
IBRP44	01	*Isotricha prostoma partial*	AM158456	84
IBRP45	02	*Cycloposthium ishikawai*	EF632076	89
IBRP46	02	Uncultured rumen protozoa clone YCRPB59	EU163783	82
IBRP47	01	Uncultured rumen protozoa clone YCRPB1	EU163725	91
IBRP48	02	Uncultured ciliate	AM158846	96
IBRP49	01	*Ostracodinium gracile*	AM158468	80
IBRP50	01	Uncultured rumen protozoa clone YCRPB65	EU163789	79
IBRP51	01	*Isotricha prostoma*	AM158454	86
IBRP52	01	*Ostracodinium gracile*	AM158468	80
IBRP53	01	Uncultured rumen protozoa clone YCRPB59	EU163783	82

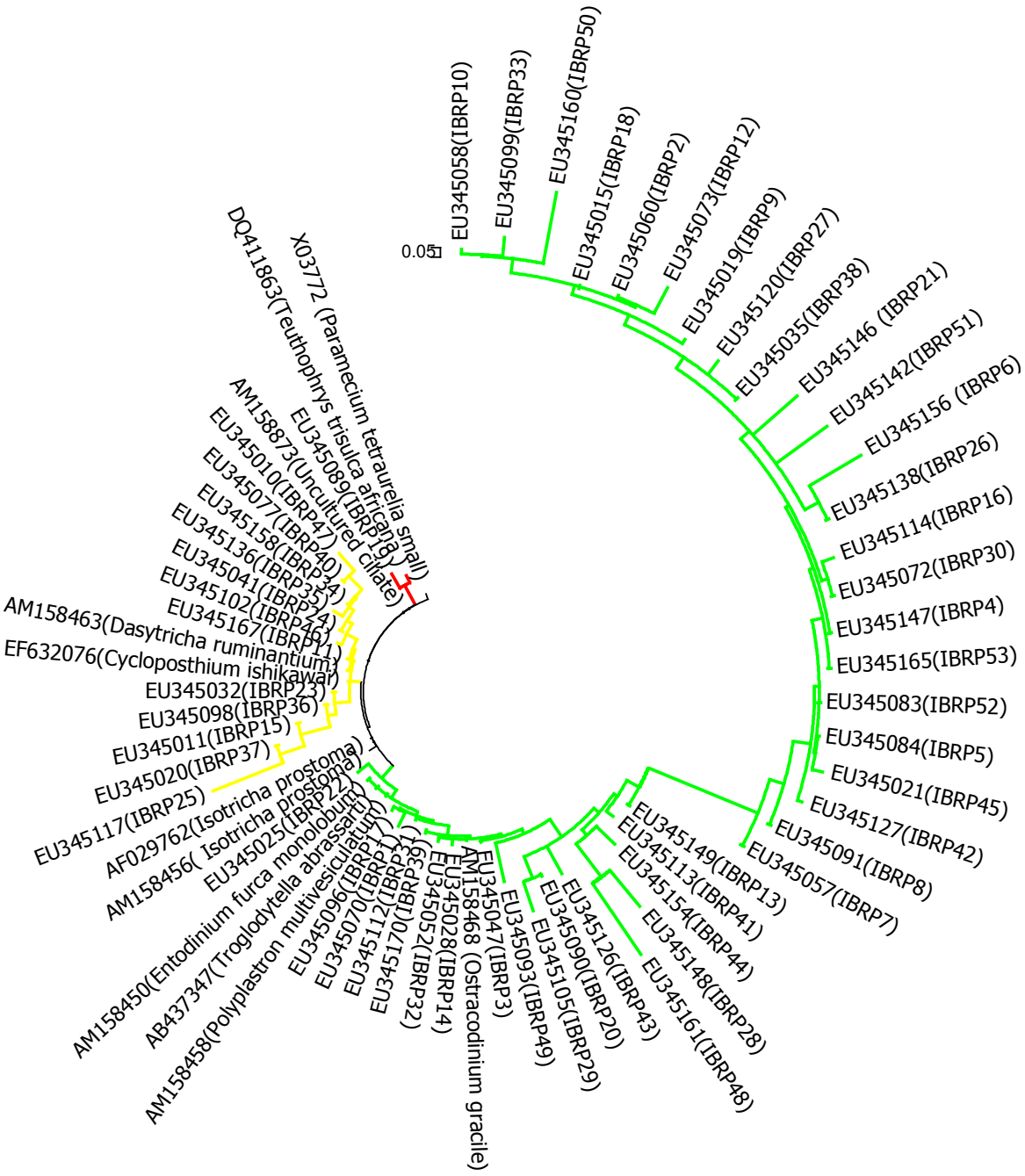


Figure 5.3. Phylogenetic relationships of partial 18S rDNA sequences of clones recovered from Surti rumen samples. The rooted tree was inferred by the neighbour joining method with 1,000 bootstrap replicates using the MEGA 4 software. The *Paramecium tetraurelia* (X03772) are used as the out-group for rooting the tree. The scale bar represents 5% sequence divergence. Branch lines: green (Entodiniomorphid protozoa), yellow (Holotrich Protozoa) and red (Haptorida protozoa).

Table 5.2: Analysis of diversity of 18S rDNA phylotypes retrieved from the rumen of Indian Surti buffalo.

Items	**18S r DNA libraries**
Library size [a] (N)	172
OTUs [b]	53
Single clone OTU [c] (n)	11
Good's coverage [d] (%)	93.75
Clone distribution	
(i) **Entodiniomorphid protozoa**	**40 OTUs (123 clones)**
(ii) **Holotrich Protozoa**	**12 OTUs (45 clones)**
a. *Dasytricha ruminantium*-like clone	07 OTUs (07 clones)
b. *Isotricha prostoma*-like clone	05 OTUs (05 clones)
(iii) **Haptorida protozoa**	**01 OTU (04 clones)**
Total	**53 OTUs (172 Clones)**

[a] Number of clones analyzed from library

[b] OTUs based on 18S r DNA sequences

[c] OTUs containing only single clone

[d] The higher percentage coverage means more diversity is captured

Chapter 6. Assessment of microbial population size in the rumen of Surti buffalo (*Bubalus bubalis*): a quantitative real time PCR approach

ABSTRACT

Traditional methods for enumerating and identifying microbial populations within the Surti rumen can be time consuming and cumbersome. In the present study, a real-time PCR SYBR Green assay, using PCR primers to target total rumen microbiome of Surti buffalo (*Bubalus bubalis*) has been described. The primer sets used, were found to be target specific with no detectable cross-reactivity. Subsequently a real-time PCR approach was used to determine the population of major ruminal microbial species (Fibrolytic bacteria, Non fibrolytic bacteria, Protozoa and Methanogens) in rumen fluid of *Bubalus bubalis* fed green fodder, dry roughage and compound concentrate mixture. Among the monitored fibrolytic species, *Ruminoccucus albus* ($1.37x10^8$copies/ ml of rumen fluid) was found to be the dominant, accounting for 5.66 % of total bacteria after 24 hrs feeding. *Streptococcus bovis* and *Selenomonas ruminantium* in non-fibrolytics, were detected 0.11% and 0.025% of total bacteria, respectively. Such levels of non-fibrolytics in Surti buffalo rumen suggest a synergistic relationship between fibrolytics and non-fibrolytics. Among ciliate protozoa, *Dasytricha ruminantium* was most prevalent in the rumen, accounting 0.049 % of the total ciliate protozoa. Out of three orders of methanogens viz *Methanomicrobials, Methanobacterials* and *Methanococcales,* the population of *Methanomicrobials* and *Methanobacterials* was higher than *Methanococcales*, accounting 4.0% and 2.17 % respectively of total archaea.

Key words: Fibrolytic and non-fibrolytic bacteria, ciliate protozoa, methanogens, 16S gene, Real-time PCR.

INTRODUCTION

The digestion of plant material and subsequent conversion for energy requirements to the host ruminant are performed through a complex symbiotic relationship of microbiota within the rumen (Mackie, 1997). The composition and

proportion of microorganisms are influenced by external factors, such as diet, feeding frequency, age, geographical location and ruminant–host interaction (Hungate, 1966). Bacteria are considered to be the most important for the biological degradation of dietary fibers due to their fibrolytic activity and biomass in the rumen. Although fibrolytic species such as *Fibrobacter succinogenes, Ruminococcus albus* and *Ruminococcus flavefaciens* play a key role in plant fiber degradation (Forsberg *et al*., 1997), positive interactions between them and non-fibrolytic bacteria such as *Selenomonas ruminantium* and *Treponema bryantii* have been reported (Kudo *et al*., 1987). In an early study, the synergism between *R. flavefaciens* and *S. ruminantium* was suggested as enhancing propionate production (Scheifinger and Wolin, 1973). Sawanon and Kobayashi, (2006) reported that fiber digestibility and propionate production significantly increased in coculture of *R. flavefaciens* and *S. ruminantium* compared to mono-culture of *R. flavefaciens*. These findings indicate that non-fibrolytic bacteria may also be important to facilitate plant fiber degradation in the rumen. Therefore, non-fibrolytic bacteria as well as fibrolytic bacteria should be monitored in order to estimate overall contribution of bacteria to ruminal fiber digestion.

Interest in methanogens from ruminants has resulted from the role of methane in global warming and from the fact that enteric methane emission is a major source of greenhouse gas in agriculture. India possesses the world's largest livestock population of 485 million, which accounts for 13% of the global livestock population**.** It has 57% of the world's buffalo and 16% of the cattle population. Contribution of methane emission in India by buffalo is 42% (Chhabra *et al*., 2009). Reducing enteric methane emissions has been identified as one way of lowering global methane emissions. Methane production from enteric fermentation can be affected by a number of factors. Level of feed intake, forage processing, type of carbohydrate, addition of lipids and ionophores to the diet, changes in rumen microorganisms and level of animal productivity have been identified (McAllister *et al*., 1996).

Ciliates are the most abundant protozoa found in the rumen of both domesticated and wild ruminants. Rumen ciliates are involved in host metabolism and digestion of plant material (Williams and Coleman, 1992) and play an important role in the rumen microbial ecosystem by producing hydrogen as a by-product of plant

digestion. The hydrogen is then used by methanogenic archaea to reduce carbon dioxide to methane, a potent greenhouse gas. Removal of protozoa from the rumen (i.e., defaunation) has been shown to reduce methane emission by an average of 13% (Hegarty, 1999). A more efficient use of nutrients in ciliate-free animals, especially when given poor diets that limit animal production, has also been reported (Eugene *et al*, 2004). Because of the current interest in methane mitigation (Howden and Reyenga, 1999), it is likely that methods to accurately quantify protozoa in the rumen will become increasingly important.

Real-time PCR is a powerful tool that allows for the rapid quantification of a target DNA sequence through the design of specific primer sets. Researchers have shown that this technique can be used successfully on samples extracted from rumen contents to monitor microbial populations in the rumen (Koike *et al*., 2007; Wanapat and Cherdthong, 2009). Real-time PCR is an approach that allows continuous monitoring of PCR product formation, and techniques vary according to the method of fluorescence generation. Real-time PCR has the ability to enumerate targeted microbes with high sensitivity (Zimmerman and Mannhalter, 1996) and has been used to analyze various ecosystems, such as water (Leser *et al*., 1995) and rumen ecology (Reilly and Attwood, 1998).

Increased knowledge concerning the rumen fibrolytic and non fibrolytic bacterial population will allow insight into the fiber-digestion capabilities of ruminant animals as well as protozoan and archaea population. However, very limited research has been conducted in Indian buffalo (Surti) with regard to the ruminal microbial population using molecular techniques. Therefore, this study was conducted to determine the population size of rumen microbes in surti buffalo by real-time PCR assays fed green fodder Napier bajra (*Pennisetum purpureum*), mature pasture grass (*Dichanthium annulatum*), and compound concentrate mixture.

MATERIALS AND METHODS

Animals, diet and collection of rumen fluid

The same as described in chapter 2

Samples of Rumen liquor (about 500 ml) were collected from three buffaloes at 4 hrs 8 hrs and 24 hrs after feeding by a suction pump using a flexible stomach tube

(Khampa *et al*., 2006). Finally pooled ruminal fluid collected from all three animals among 4 hrs, 8 hrs and 24 hrs respectively.

DNA extraction and PCR amplification of 16S rRNA and 18S rRNA genes

Total genomic DNA was isolated from 1 ml aliquot of pooled ruminal fluid (1500 ml) by using Qiagen stool kit as per manufacturer instruction with little modification. The sample was taken using a wide-bore pipette, so as to ensure that a homogeneous sample containing plant particles and liquid. Species specific PCR primers, used for the amplification of target region of the 16S rRNA and 18S rRNA gene (target DNA), were chosen from the literatures (Table 6.1). The target DNA of total bacteria, bovine bacteroides, all fibrolytic and non fibrolytic bacteria were amplified from the metagenomic DNA with respective primer sets, as described previously by (Muyzer *et al*, 1993; Layton *et al*., 2006; Tajima *et al*, 2001a; Koike and Kobayashi, 2001). The target DNA of ciliate protozoa, *Dasytricha ruminatium* and *Entodinium sp.* were amplified from the metagenomic DNA with respective primer sets, as described previously by Skillman *et al*., (2006b). Total archaea and Order-specific PCR primers (*Methanomicrobials, Methanobacterials* and *Methanococcales*) were also used to amplify partial 16S rDNA regions (target DNA) from the metagenomic DNA with respective primer sets, as described by Yu *et al*., (2004). All the primers sequences of all targets are given in Table 6.1. All quantitative Real-time PCR amplification and detection were performed using ABI7500 system software SDSv1.3 (ABI7500, USA). The reaction was conducted in a final volume of 25.0µl containing the following: 12.5µl Qiagen DNA Master SYBR Green I, 10.0 pmol each of forward primer and reverse primer respectively, 7.5µl distilled water, and 3.0ul of DNA solution of unknown concentration. Amplicon specificity was performed via dissociation curve analysis of PCR products by increasing the temperature at a rate of 1^0C every 30s from 60^0C to 95^0C. All PCRs were performed in duplicate. Before starting the real time PCR assay, conventional PCRs for the validation of the specificity of the primers against target genes were performed and reactions were done using minicycler (MJ Research, USA) under the respective PCR conditions (Table 6.1). The PCR products were analyzed by running on 1.5% agarose gels containing ethidium bromide and visualized for a single specific band and the absence of primer dimmer products.

Preparation of standard plasmid for real-time PCR assays

Plasmid DNA containing the respective target gene sequence, used as the standard DNA in real-time PCR, was obtained by PCR cloning using the species-specific primer sets. After the confirmation of a single band of the correct size with respective pair of primers (Table 6.1) on an agarose gel, the PCR products were excised from the gel. The PCR products were purified using the Qiagen gel Purification Kit (Qiagen, CA), and then ligated into pTZR57 T/A cloning vector (Fermetas, UK). The ligated products were transformed to competent *Escherichia coli* DH5 alpha cells by heat shock. Plasmids were purified from positive clones using a QIAprep spin miniprep kit (Qiagen, Valencia, CA, USA), and the plasmids containing the correct insert were screened out by PCR amplification with respective primer sets. Prior to preparation of the standard, DNA sequences of each cloned target DNA were confirmed by sequencing (ABI 310, USA). The concentration of the plasmid was determined with a Nanodrop spectrophotmeter. Copy number of each standard plasmid was calculated using formula; Copy No/ µl = Concentration of plasmids (gm/µl) x 6.022 x 10^{23} / length of recombinant plasmid (bp) x 660, (660= Molecular weight of nucleotide base, 6.022 x 10^{23} = Avogadro's number). Ten-fold dilution series ranging from 10^{9} to 10 copies were prepared for each target. Real-time PCR was performed with ABI system (ABI 7500). The Qiagen DNA Master SYBR Green I was used for PCR reaction. The optimal amplification conditions for each primer set were obtained with 10 pM each primer with the combination of annealing temperature and extension time are shown in Table 6.1. The 10-fold dilution series of the standard plasmid for the respective target was run along with the samples. Amplification of each sample was performed in duplicate. Quantification was made using standard curves obtained from the amplification profile of known concentrations of the standard plasmid for the respective target.

Statistical analysis

Statistical analysis of data was performed by using software of Sigma Stat 32 (SPSS Inc., Chicago, Illinois). The differences determined by the method of least significant differences at the 5% level ($P<0.05$) for rumen fluid at 4 hrs, 8 hrs and 24 hrs after feeding (One way ANOVA).

RESULTS AND DISCUSSIONS

The metagenomic DNA was successfully extracted from rumen fluid. Qualitative PCR detection of fibrolytic bacteria, Non fibrolytic bacteria, methanogen and protozoa are shown in Figure 6.1 and 6.2.

Real-time PCR-based quantification of representative rumen bacteria was reported in a few studies (Ozutsumi *et al*., 2006). In the present study, we assessed the rumen bacterial species, methaogens and protozoa species using real-time PCR assays. In the reaction for all standard, nearly perfect linear regressions (r^2 = 0.9930 to 0.9995), intercept (31.26 to 44.78) and slope (-3.2 to -4) were obtained between threshold cycle and quantities of standard. The graphs of standard curve, amplification curve and dissociation curve are given in Plate 6.1 to 6.19.

Quantitation of total bacteria, fibrolytic and non fibrolytic bacteria

Figure 6.3 shows the changes of population sizes of total bacteria and the target species in Surti buffalo rumen. Total bacteria and bovine bacteroides were detected at 2.7x 10^9 copies and 3.1 x 10^8 per ml ruminal fluid after 4 hrs incubation in the rumen and increased to 5.9x10^9 and 3.2 x 10^8 copies per ml ruminal fluid at 24 hrs respectively (Figure 6.3). With the increase of total bacterial number in rumen from 4 hrs to 24 hrs, the target bacteria, fibrolytics and non-fibrolytics, remarkably increased except *Ruminobacter amylophilus* and *Treponema bryantii* (Figure 6.3). Among the fibrolytic bacteria, *R. albus* was most abundantly detected (1.37x10^8 copies/ ml of rumen fluid corresponding to 5.66% of total bacteria after 24 hrs incubation) followed by *R. flavifaciens* (2.5x10^8 copies/ ml of rumen fluid, 4.24% of total bacteria), *F. succinogenes* (1x10^8 copies/ ml of rumen fluid, 1.72% of total bacteria) and *P. ruminicola* (5.7x10^7 copies/ ml of rumen fluid, 0.97% of total bacteria) detect (Figure 6.3; Table 6.2). In non fibrolytic bacteria, *Streptococcus bovis* (5.3 x10^6 copies/ ml of rumen fluid) and *Selenomonas ruminantium* (1.1 x10^6 copies/ ml of rumen fluid) was detected, accounting 0.11% and 0.025% of total bacteria, respectively at 24 hrs feeding (Figure 6.3). The population size of all fibrolytic and nonfibrolytic bacterial species were significantly higher ($P < 0.05$) in 24 hrs after feeding except *S. ruminantium, R. amylophilus* and *T. bryantii*.

Quantitation of total archaea and methanognes

External standards for real-time PCR were prepared as described above. Several groups have reported the monitoring of methanogen populations from environmental samples through targeting of the 16S ribosomal gene (Yu *et al*., 2004; Stewart *et al*., 2006; Wright *et al.,* 2007; Denman *et al*., 2007) have been reported to monitor the methanogen populations from environmental samples through targeting of the 16S ribosomal gene. Total archaea was detected at 7.23 x 10^7, 6.9 x 10^7 and 6.0 x 10^7 copies / ml ruminal fluid at 4hrs, 8 hrs and 24 hrs respectively (Figure 6.3). The number of 16S rRNA gene copies of *Methanomicrobiales* (1.4x10^6, 2.5x10^5 and 2.4x10^6 copies / ml), *Methanobacteriales* (5.2x10^5, 6.7x10^6 and 1.3x10^6 copies / ml) and *Methanococcales* (3.4x10^5, 1.2x10^5 and 3.4 x10^5 copies / ml) accounting 4.0 % , 2.17 % and 0.53 % of total arcahea at 24 hrs respectively (Table 6.3). However, at 8 hrs feeding the population size of *Methanobacteriales* was 6.7 x10^6 copies / ml (Figure 6.4). The population size of *Methanomicrobiales* was significantly higher ($P < 0.05$) in 24 hrs after feeding.

Quantitation of ciliate protozoa and target species

External standards for real-time PCR were prepared as described above from a simulated rumen matrix. For each standard, a linear regression ($r^2 = 0.990$) derived from the Ct of each DNA dilution versus the log dilution enabled the PCR efficiency. Ciliate protozoa was detected at 3.5x10^8 copies per ml ruminal fluid after 4hrs feeding and increased to 4.1x10^8 copies per ml ruminal fluid at 24 hrs feeding (Figure 6.5). The number of 18S rRNA gene copies of *Dasytricha ruminantium* (2.0x10^5) at 24 hrs was higher than *Entodinium sp.* (1.3x10^4) and accounting 0.049 % of ciliate protozoa (Table 6.4). The population size of *Dasytricha ruminantium* was significantly higher ($P < 0.05$) in 8 hrs after feeding.

To the best of our knowledge, no previous study has reported the population size of rumen microbes of Indian buffalo, particular in the Surti rumen. In the present study, our observation indicates that a large number of bacteria were present in the ruminal fluid. Because each bacterial species has a different copy number of 16S rRNA gene (ranging from 1 to 15; Klappenbach *et al*., 2001), this is comparable to the level of rumen bacterial count reported in the literature following microscopy analysis (Hungate, 1966).

The present results among cellulotyic bacteria revealed that *R. albus*, *R. flavifaciens* and *F. succinogenes* are core member of fiber attaching bacteria, playing a significant and impartant role in the digestion of fiber rich diet. *R. albus* was the most dominant ($3.0x10^8$ copies/ml of rumen fluid) among the three species, followed by *R. flavefaciens* ($2.5x10^8$ copies/ml of rumen fluid) and *F. succinogenes* ($1x10^8$ copies/ml of rumen fluid). Similarly, Ozutsumi *et al*., (2006) reported that the numbers of *R. albus* ($\log_{10}$ 8.3 $\pm$ 6.3 per ml) and *R. flavefaciens* ($\log_{10}$ 8.8 $\pm$ 7.9 per ml) were higher than *F. succinogenes* ($\log_{10}$ 7.7 $\pm$ 6.8 per ml) in the unfaunated cattle rumen fed 66% Sudangrass hay and 34% concentrate mixture. However, Wanapat and Cherdthong (2009) reported that *F. succinogenes* was the major cellulolytic bacteria of rumen digesta (roughage) in swamp buffalo and was present 2.65 x 10^9 to 3.54 x 10^9copies/ml in ruminal fluid. Koike and Kobayashi (2001) also reported that *F. succinogenes* was the major cellulolytic bacteria of rumen digesta in sheep and were present at only 0.1% of total population and that *ruminococci* were relatively minor. The scarcities of the two ruminococci were surprising, considering that they were representative cellulolytics (*i.e*. ruminal densities ranging from 0.1% to 6.6% for *F. succinogenes*, (Stahl *et al*., 1988; Briesacher *et al*., 1992) and from 1.3% to 2.9% for *Ruminococcus spp*. (Krause *et al*., 1999). *Ruminal prevotella* are known to possess oligosaccharolytic and xylanolytic activities and to occupy the ecological niches of the second line degraders. A density of *P. ruminicola* was 0.97 % of total bacteria at 24 hrs after feding (Table 6.2). The quantity of other representative of the genus, *P. bryantii* was decline from 0.000043%, 0.000036% and 0.000017% of total bacteria at 4hrs, 8 hrs and 24 hrs respectively after feeding, demonstrated the opposite kinetics, suggesting its role in starch degradation.

Non-fibrolytic bacteria such as *S. ruminantium* and *T. bryantii* were detected in the fiber-associated community, using comparative 16S rRNA gene analysis (Koike *et al*., 2003). The present study quantitatively confirmed the attachment of nonfibrolytic bacteria to fiber. As expected, they were attached to the fiber at a relatively low level to that of fibrolytic bacteria (Table 6.2). In particular, *S. bovis S. ruminantium* and *Ruminobacter amylophilus* had the highest proportion among the non fibrolytic bacterial species assessed in the present study. This finding suggests the fiber-attachment ability of *S. ruminantium.* Minato and Suto (1978) indicated that

some of the non-fibrolytic bacteria possessed the ability to attach to cellulose at a similar extent to that of fibrolytic bacteria. In addition, non-fibrolytic species such as *Selenomonas spp*. and *Streptococcus spp*. were isolated from plant cell material in rumen digesta (Cheng *et al*., 1984). Attachment of non-fibrolytic bacteria to fiber may be mediated by glycocalyces (Minato *et al*., 1993), which are commonly found in rumen bacteria during their colonization in plant cell wall (Cheng *et al*., 1980). These reports support the significant number of non-fibrolytic bacteria in ruminal fluid of surti buffalo as observed in the present study. The synergism between fibrolytic and non-fibrolytic bacteria during fiber degradation has been noted. In such a relationship, fibrolytic bacteria provide the hydrolyzed product from cellulose to non-fibrolytic bacteria, while non-fibrolytic bacteria indirectly facilitate fiber degradation by preventing the accumulation of bacterial metabolites such as succinate and cellodextrins (Wolin *et al*., 1997). Utilization of cellodextrins from cellulose hydrolysis is crucial in further fiber digestion (Russell, 1985), because cellulases are highly sensitive to feedback inhibition by cellobiose, which is confirmed in *F. succinogenes* (Maglione *et al*., 1997). In the present study, the population sizes of *S. bovis* and *S. ruminantium* in fluid were greater than those of the other non-fibrolytics (Figure 6.3). After 24 hrs ruminal incubation, they were estimated to be 0.11 and 0.025 % of total bacteria, respectively (Table 6.2). This result indicates that these two species were dominant non-fibrolytics and it is known that *S. bovis* and *S. ruminantium* are able to utilize hydrolysis product of polysaccharide such as cellodextrins and maltodextrins for their growth (Cotta, 1992). Therefore, *S. bovis* and *S. ruminantium* in rumen might grow by utilizing the maltodextrins released during cellulose degradation (Nouaille *et al*., 2005). The metabolic interactions mentioned here may explain why non-fibrolytic bacteria are abundant on the ruminally incubated fiber diet. Moreover, this fact strongly highlights the importance of non-fibrolytic bacteria for fiber digestion.

In this study, the population size of hydrogenotrophic methanogens (*Methanomicrobiales* and *Methanobacteriales*) was higher than methanococcales. Because the only carbon source available in plenty for methanogens is H_2/CO_2, thus hydrogenotrophic methanogens (Methanomicrobiales and Methanobacteriales) that are capable of using H_2/CO_2 can multiply easily and are observed in high abundance.

In previous study on diversity of rumen methanogens in Sutri buffalo (reported in chapter-3), hydrogenotrophic methanogens are predominant in surti rumen ecology. Similarly, Denman *et al*. (2007) reported that *Methanobacteriales* order was the major hydrogenotrophic methanogens in rumen of Brahman-crossbred (*Bos indicus*) steers.

We used real- time PCR assay to enumerate, *Dasytricha ruminantium* was to found as the most abundant ciliate protozoa in the Surti rumen and also have the largest number of species. In the present study large numbers of ciliate protozoa were present in rumen, are involved in host metabolism and digestion of plant material (Williams and Coleman, 1992.) and play an important role in the rumen microbial ecosystem by producing hydrogen as a by-product of plant digestion. However, Skillman *et al*., (2006b) reported that *Entodinium sp*. was the major ciliate protozoa in sheep rumen fed hay diet and detected 2.45 x 10^5 per ml of ruminal fluid. Approximately one-quarter to one-third of the fiber degradation in the rumen is protozoal (Demeyer, 1981). Takenaka *et al*., (1999) employed molecular techniques to demonstrate that ruminal protozoa possess fibrolytic enzymes. When the protozoa were removed from the rumen, the total viable counts and cellulolytic bacteria increased (Arakaki *et al*., 1999), and the digestion of fiber in the rumen was reduced (Williams and Coleman, 1997).

Tichopad *et al*., (2002) demonstrated that primer selection is crucial to the accuracy of real-time PCR and that reactions with higher amplification efficiency proceed with lower variability and are better suited to measurement purposes. The primer Ento-472R was sequence specific to *Entodinium* and had >5 base mismatches with all other rumen protozoa. The forward primer (Oph-151F) matched the 18S gene sequences of *Entodinium spp*., and *Ophyroscolex caudatus* and would be expected to detect these three protozoal genera. However, Oph-151F had three nucleotide mismatches between the 3' end of the primer and *E. maggii*, four mismatches with *Diplodinium* and *Polyplastron*, and five mismatches with *Dasytricha* as the predicted specificities of primers may differ from the practical outcome, it is important to test primer specificity against target and nontarget groups. In this study the real-time PCR primers were amplified the 18S rRNA genes of specific genera of *Entodinium* species (Oph-151F and Ento-472R) and *D. ruminantium* (Iso-Das-151F and Das-472R) of rumen protozoa only.

Culture-independent molecular studies clearly indicate an abundance of uncultured bacteria, protozoa and archaea in the rumen (Shin *et al*., 2004b). In the present study, the sum of proportions of 10 representative bacterial species was calculated to be 12.73% of total bacteria, protozoa species to be 0.05 % of ciliate protozoa and methanogens to be 6.7% of total archaea after 24 hrs feeding. This result strongly suggests the significance of uncultured bacteria and protozoa in plant fiber degradation and removal of hydrogen by uncultured archaea Therefore, it is necessary to perform quantitative determination of the community structure of rumen microbes including the uncultured microbes and also to cultivate such microbe.

CONCLUSIONS

In conclusion, four major representative groups of ruminal microbes covering fibrolytic and non-fibrolytic bacterial communities, methanogens and protozoans were detected. Result revealed that *R. albus* was the most dominant fibrolytic among the all detected fibrolytic species. Non-fibrolytic *S. bovis* and *S. ruminantium* were also detected with abundance in rumen fluid. The high magnitude of non-fibrolytic group on the plant fiber suggests the development of mutual relationships between fibrolytic and non-fibrolytic bacterial communities. The population size of *Methanomicrobiales* and *Methanobacteriales* was recorded higher compare to the *Methanococcales.* Among ciliate protozoa, *D. ruminantium* was found to be the predominant over *Entodinium sp.* and propbably help in fiber digestion. Present study indicates that rumen system of Surti buffalo (*B. bubalis*) is harbours the diverse group of microbial communities with high population densities.

Table 6.1: Primers and conditions for Real time PCR Assay

Target	Sequences (5'-3')	Amplicon (bp)	Annealing Temp.	Reference
Total Bacteria	P1 CCTACGGGAGGCAGCAG P 2 ATTACCGCGGCTGCTGG	194	60^0C-30 Sec	Muyzer *et al*., (1993)
Bovine Bacteroides	367f GAAG(G/A)CTGAACCAGCCAAGTA 467r GCTTATTCATACGGTACATACAAG	100	60^0C -30 Sec	Layton *et al*., (2006)
Fibrolytic bacteria				
Ruminococcus albus	P1 CCCTAAAAGCAGTCTTAGTTCG P2 CCT CCTTGCGGTTAGAACA	175	62^0C -15 Sec	Koike and Kobayshi, (2001)
Fibrobacter succinogenes	P1 GGTATGGGATGAGCTTGC P2 GCCTGCCCCTGAACTATC	445	62^0C -15 Sec	Tajima *et al.,* (2001a)
Ruminococcus flavefaciens	P1 GGACGATAATGACGGTACTT P2 GCAATC(CT)GAACTGGGACAAT	835	62^0C -30 Sec	Tajima *et al.,* (2001a)

Prevotella ruminicola	P1 GGTTATCTTGAGTGAGTT P2 CTGATGGCAACTAAAGAA	485	63^0C -35 Sec	Tajima *et al*., (2001a)
Prevotella bryantii	P1 ACTGCAGCGCGAACTGTCAGA P2 ACCTTACGGTGGCAGTGTCTC	540	68^0C -30 Sec	Tajima *et al.,* (2001a)
Non fibrolytic bacteria				
Ruminobacter amylophilus	P1 CAACCAGTCGCATTCAGA P2 CACTACTCATGGCAACAT	642	57^0C -30 Sec	Tajima *et al*., (2001a)
Streptococcus bovis	P1 CTAATACCGCATAACAGCAT P2 AGAAACTTCCTATCTCTAGG	869	57^0C -30 Sec	Tajima *et al*., (2001a)
Treponema bryantii	P1 AGTCGAGCGGTAAGATTG P2 CAAAGCGTTTCTCTCACT	421	57^0C -30 Sec	Tajima *et al*., (2001a)
Anaerovibrio lipolytica	P1 TGGGTGTTAGAAATGGATTC P2 CTCTCCTGCACTCAAGAATT	597	57^0C -30 Sec	Tajima *et al*., (2001a)
Selenomonas ruminantium	P1 TGCTAATACCGAATGTTG P2 TCCTGCACTCAAGAAAGA	513	57^0C -30 Sec	Tajima *et al*., (2001a)

Archaea	P1 ATTAGATACCCSBGAGTCC P2 GCCATGCACCWCCTCT	273	60^0C -30 Sec	Yu *et al*., (2004)
Methanobacteriales	MBT857F CGWAGGGAAGCTGTTAAGT	343	60^0C -30 Sec	Yu *et al*., (2004)
	MBT1196R TACCGTCGTCCACTCCTT			
Methanomicrobiales	MMB282F ATCGRTACGGGTTGTGGG	506	60^0C -15 Sec	Yu *et al*., (2004)
	MMB832R CACCTAACGCRCATHGTTTAC			
Methanococcales	P1 CGWAGGGAAGCTGTTAAGT	337	60^0C -35 Sec	Yu *et al*., (2004)
	P2 TACCG TCGTC CACTC CTT			
Ciliate protozoa	P1 GCTTTCGWTGGTAGTGTATT	223	54^0C -30 Sec	Sylvester
	P2 CTTGCCCTCYAATCGTWCT			*et al*., (2004)
Dasytricha	IsoDas151F CTAGAGCTA ATA CATGCC	300	55^0C -30 Sec	Skillman
ruminantium	Das472RCTA CAATCACAATTA AATTGC			*et al*., (2006b)
Entodinium sp.	Oph151FGAGCTAATACAT GCTAAGGC	300	55^0C -30 Sec	Skillman
	Ento472R CCCTCACTACAATCGAGA			*et al*., (2006b)
	TTTAAGG			

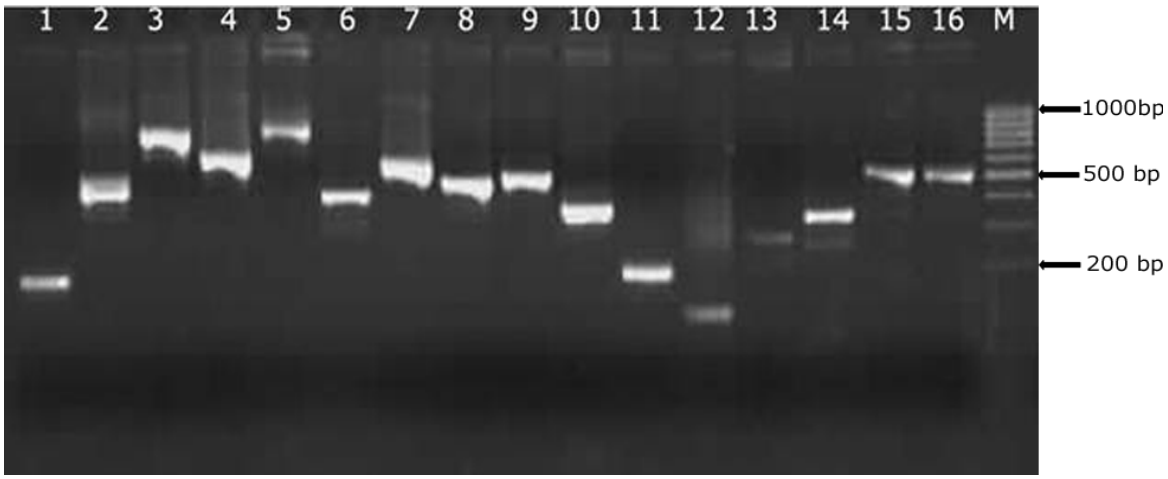


Figure 6.1. Qualitative PCR detection of fibrolytic bacteria, Non fibrolytic bacteria and methanogens. Lane: 1, *Ruminococcus albus*; 2, *Fibrobacter succinogenes*; 3, *Ruminococcus flavefaciens*; 4, *Ruminobacter amylophilus*; 5, *Streptococcus bovis*; 6, *Treponema bryantii*; 7, *Anaerovibrio lipolytica*; 8, *Prevotella ruminicola*; 9, *Selenomonas ruminantium*; 10, *Treponema bryantii*; 11, Total bacteria; 12, bacteroides; 13, Total archaea; 14, Methanobacteriels; 15, Methanococcales; 16, Methanomicrobials. Lane M, DNA size marker.

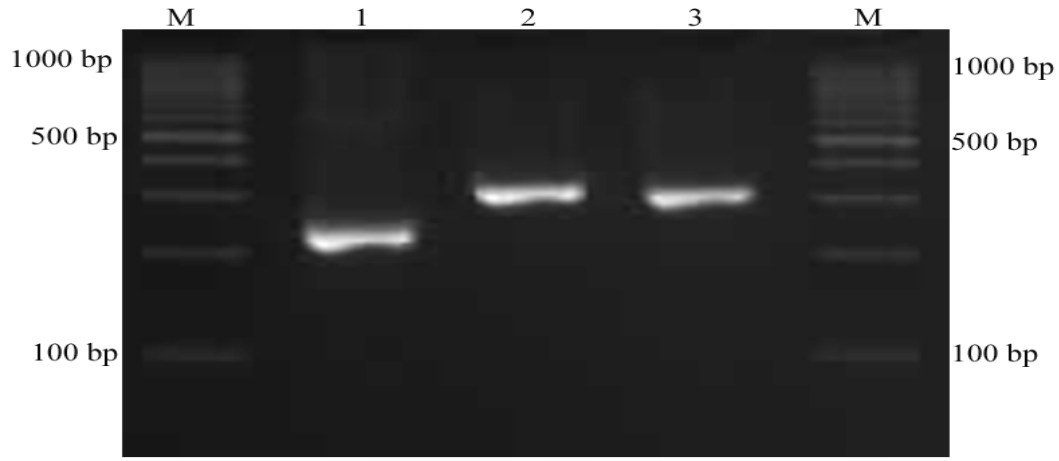
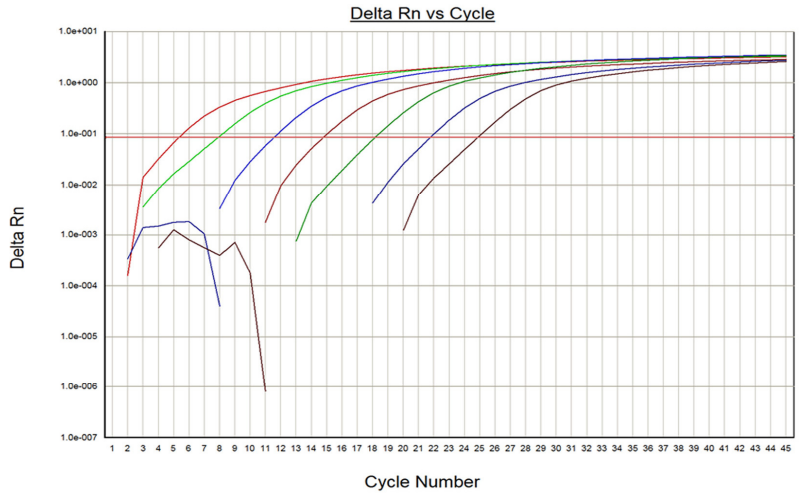
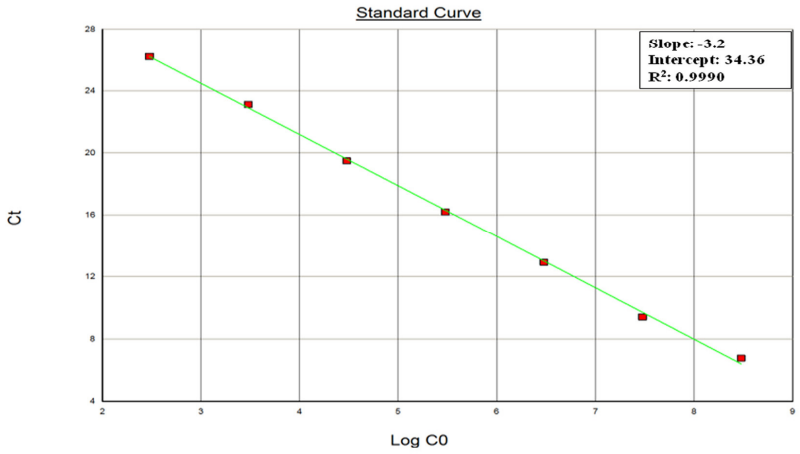


Figure 6.2. Qualitative PCR detection of ciliate protozoa (lane 1), *Entodinium sp.* (lane 2), *Dasytricha ruminantium* (lane 3) and lane M, DNA size marker

Plate 6.1 Amplification curve, standared curve and dissociation curve Total Bacteria





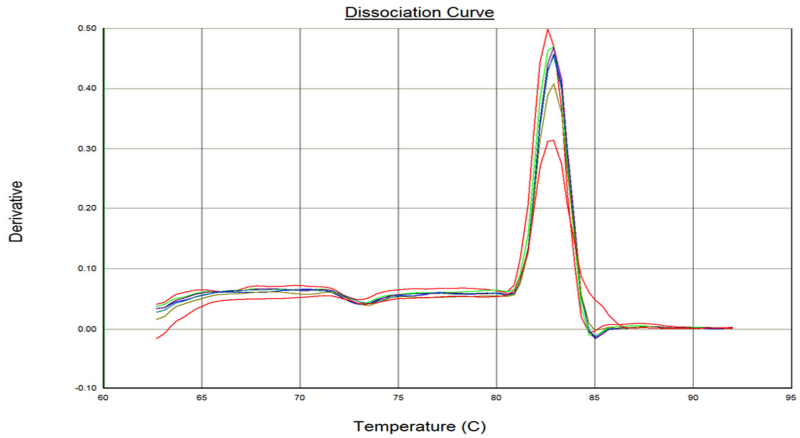
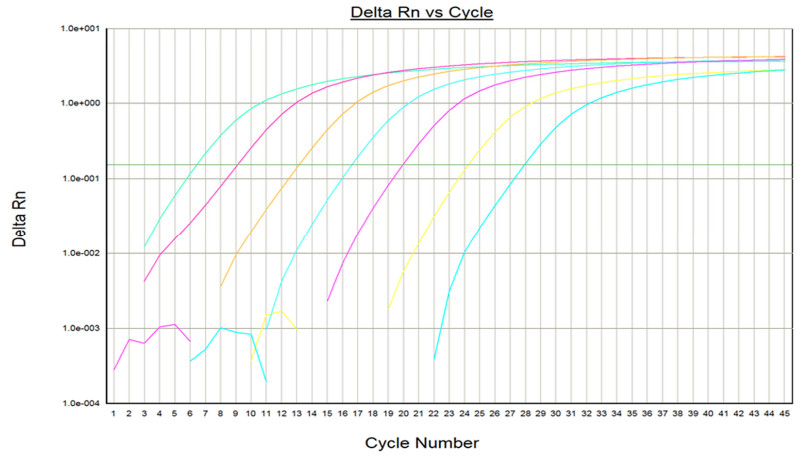
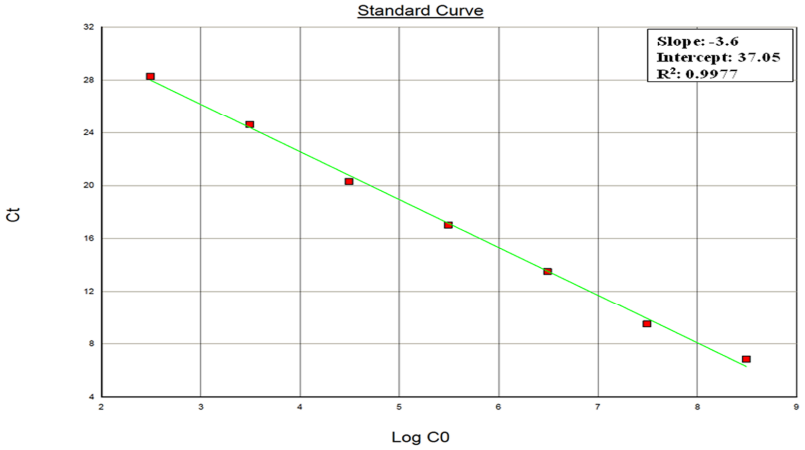


Plate 6.2 Amplification curve, standared curve and dissociation curve of Bacteroides





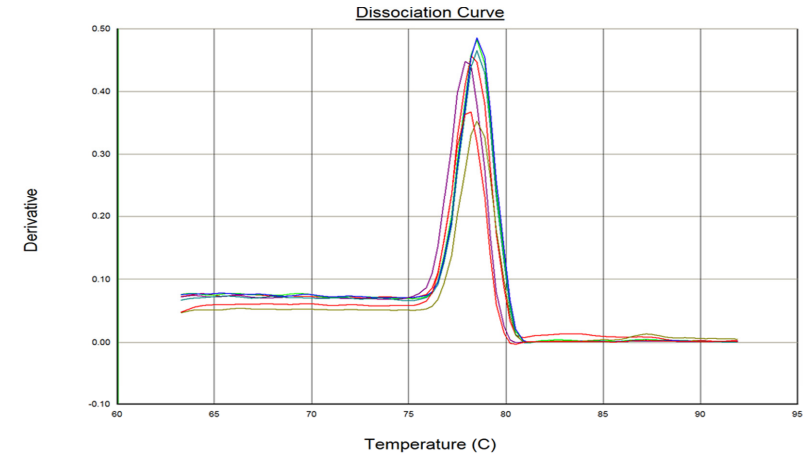


Plate 6.3 Amplification curve, standared curve and dissociation curve of *Ruminicoccus albus*

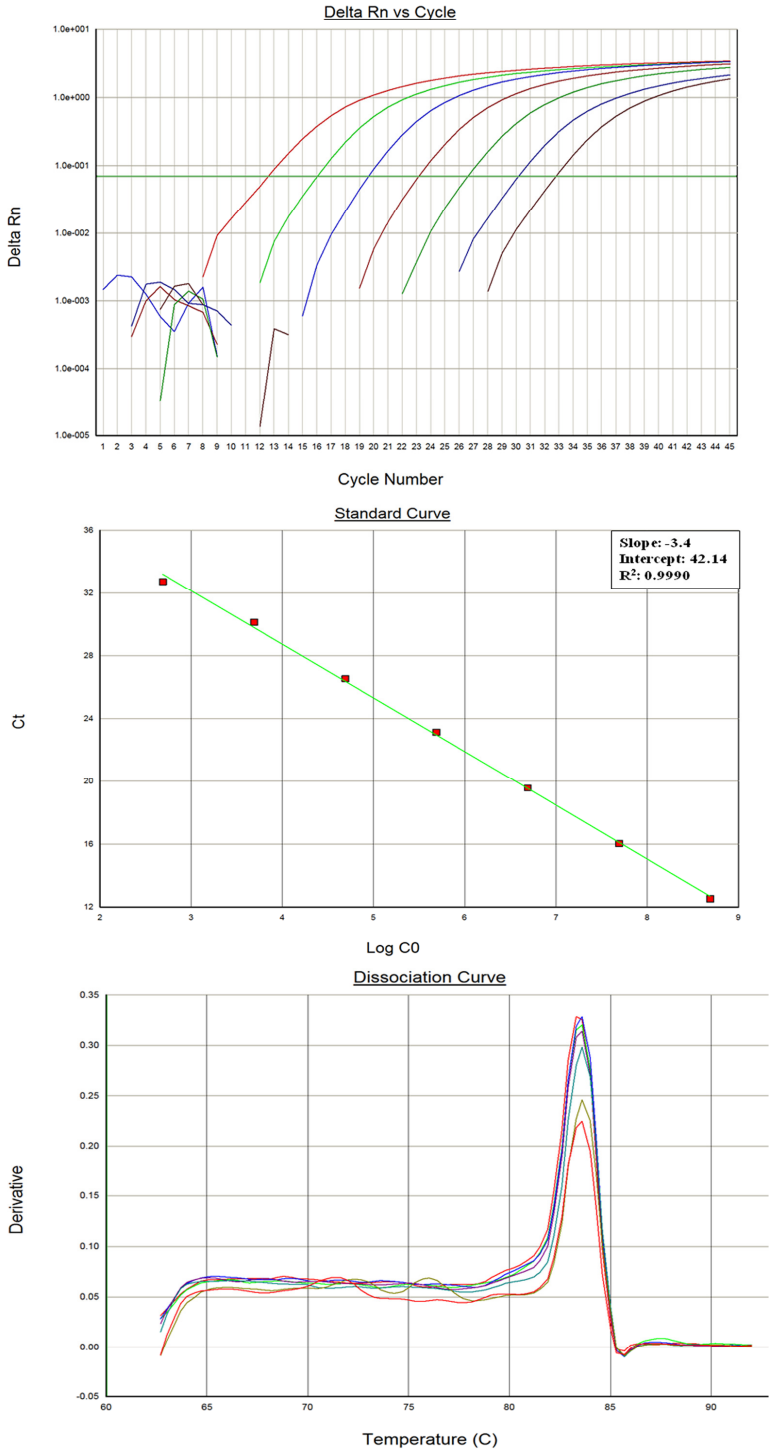


Plate 6.4 Amplification curve, standared curve and dissociation curve of *Ruminicoccus flavefaciens*

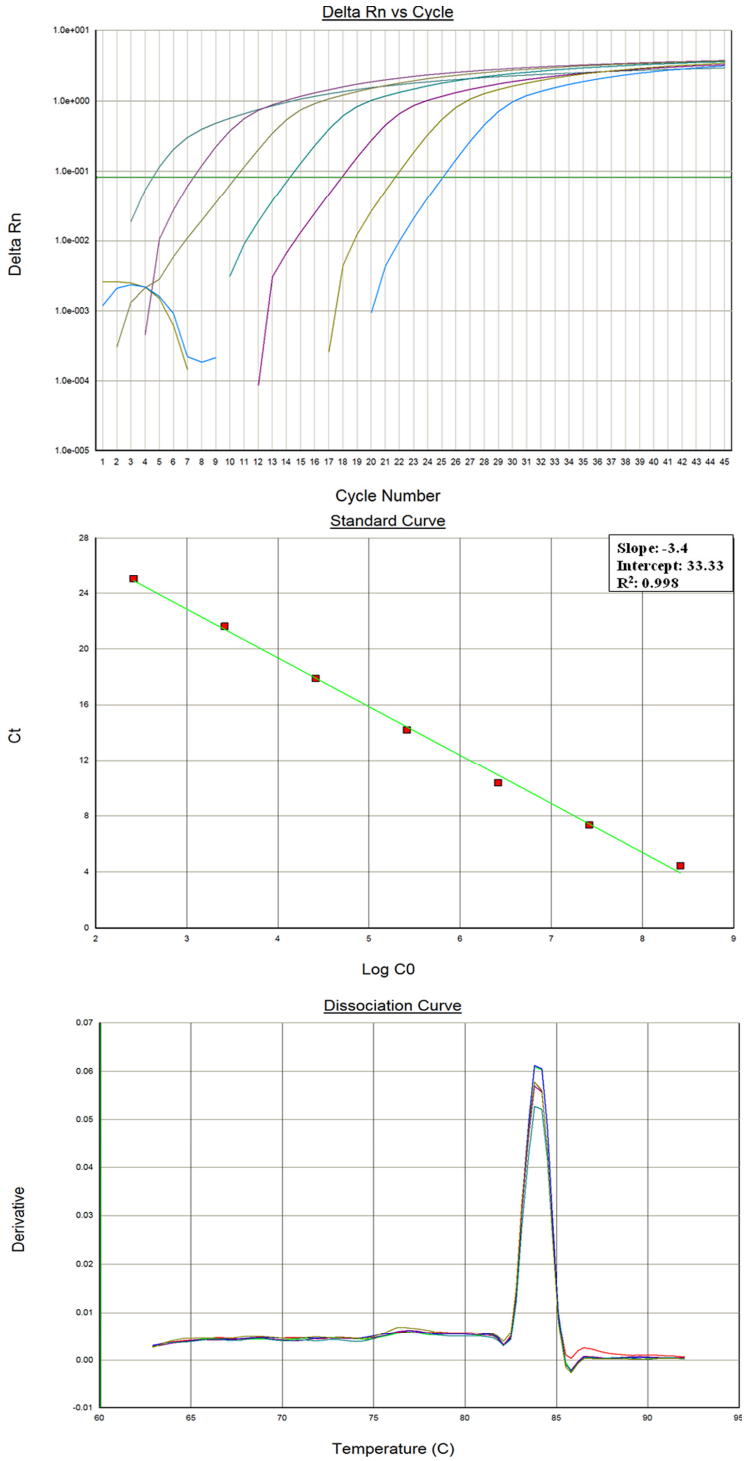


Plate 6.5 Amplification curve, standared curve and dissociation curve of *Fibrobactor succinogenes*

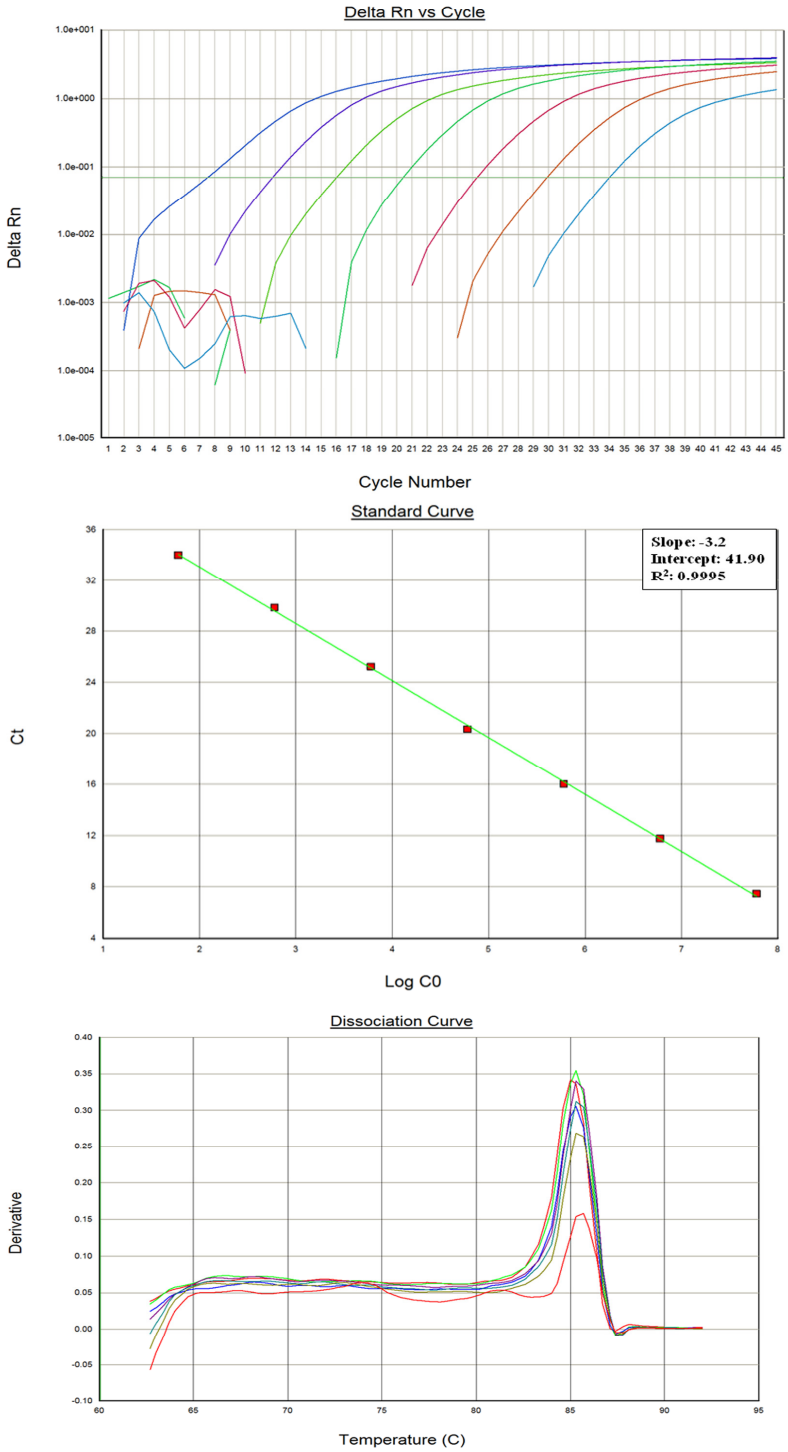
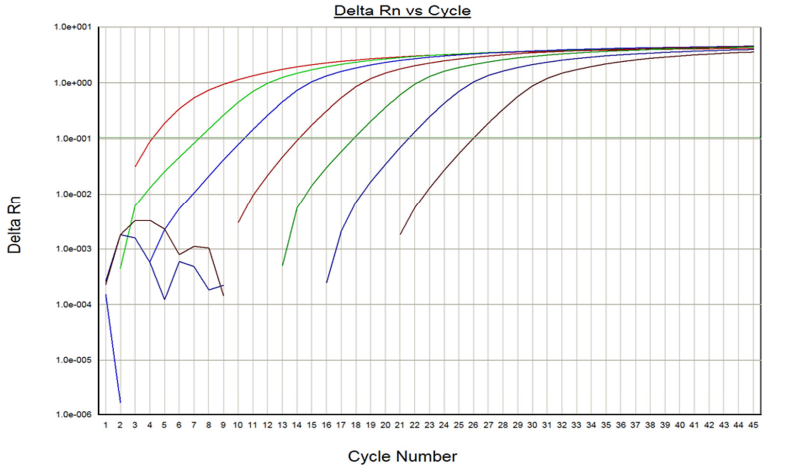
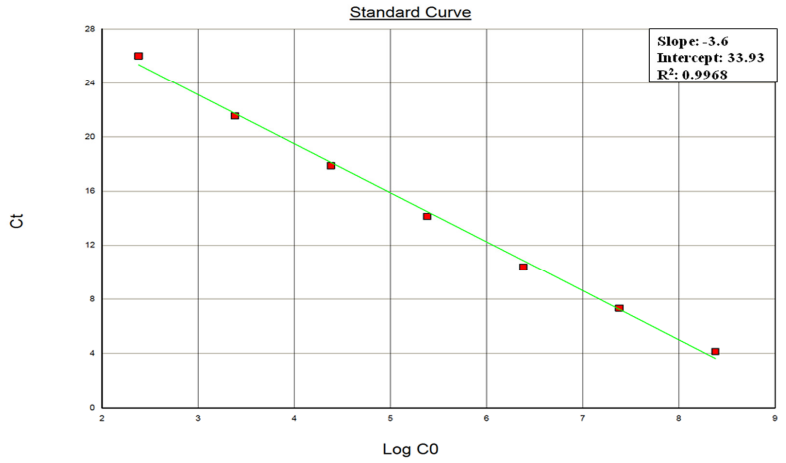


Plate 6.6 Amplification curve, standared curve and dissociation curve of *Prevotella ruminicola*





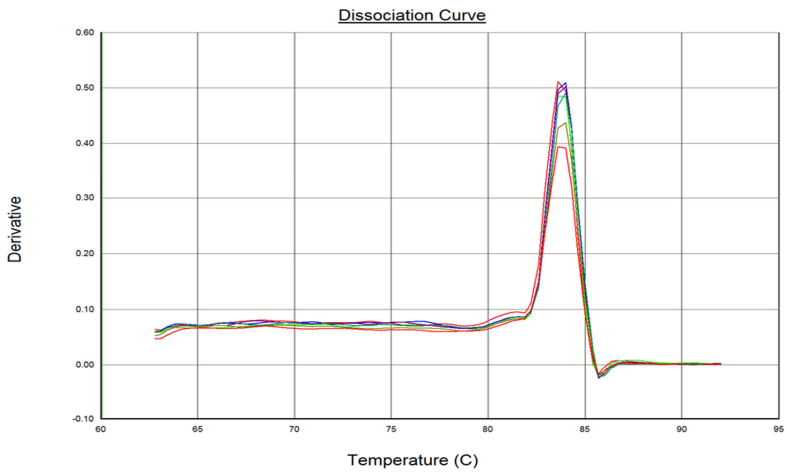
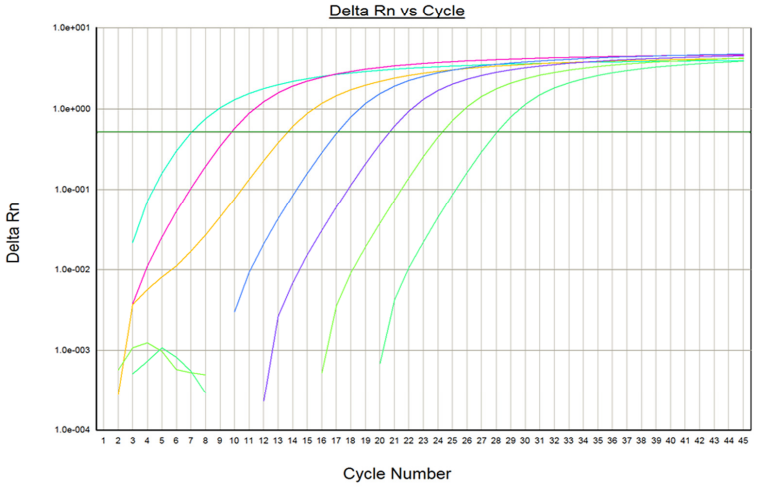
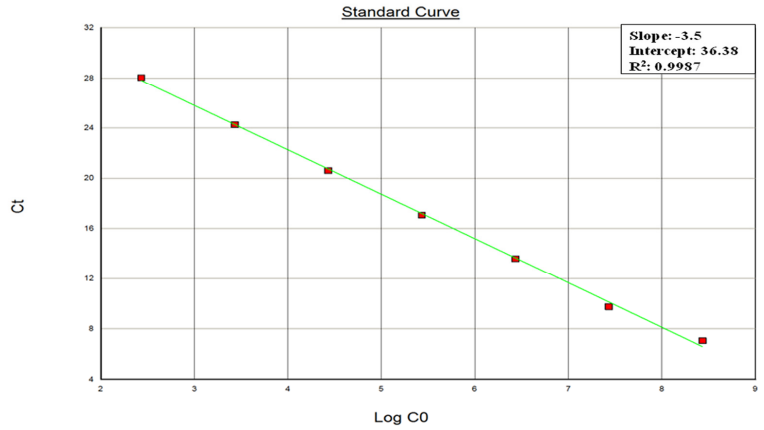


Plate 6.7 Amplification curve, standared curve and dissociation curve of *Prevotella bryantii*





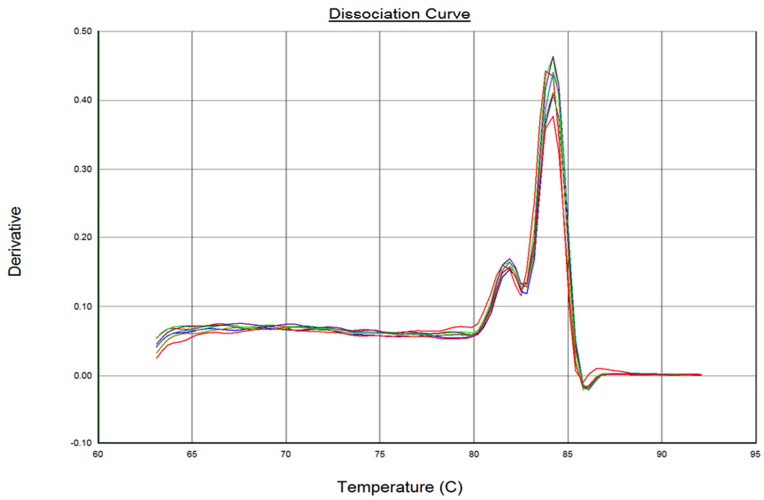


Plate 6.8 Amplification curve, standared curve and dissociation curve of *Streptococcus bovis*

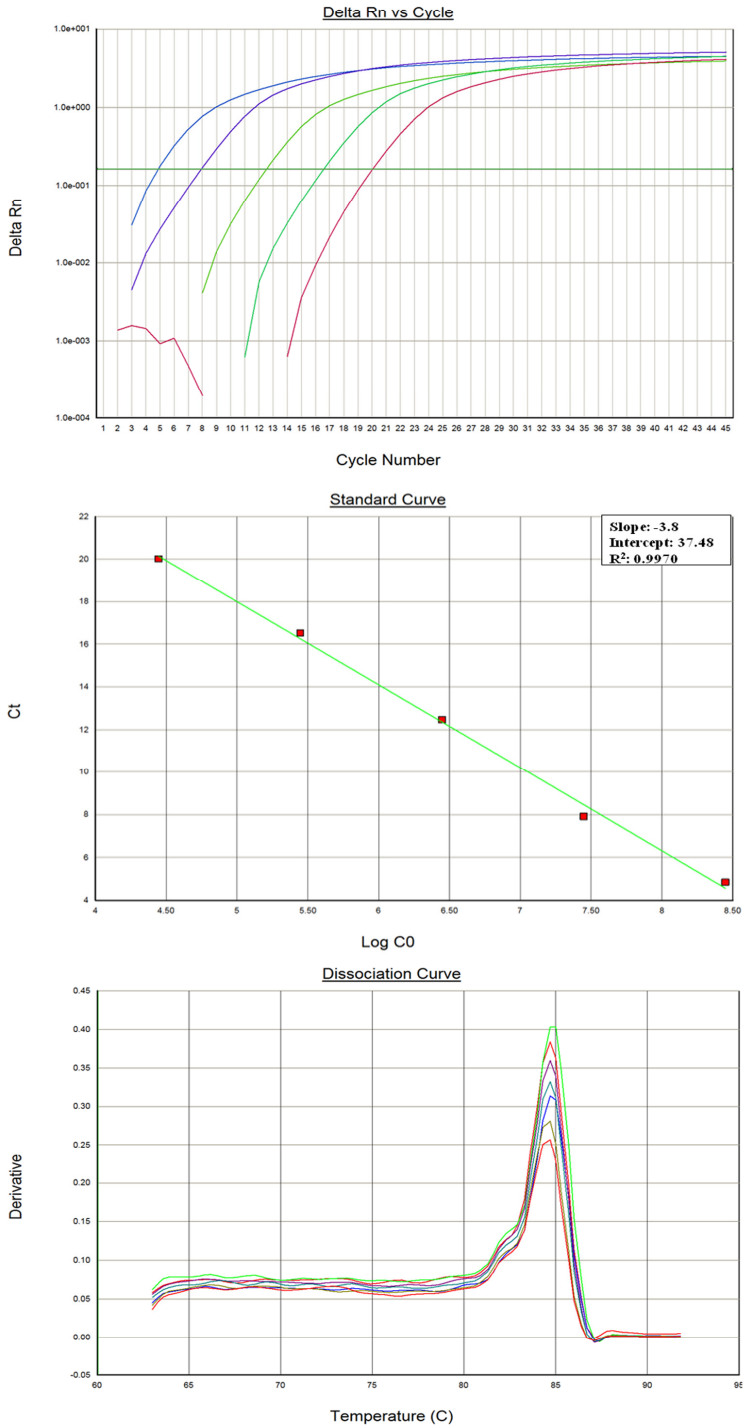


Plate 6.9 Amplification curve, standared curve and dissociation curve of *Selenomonus ruminantium*

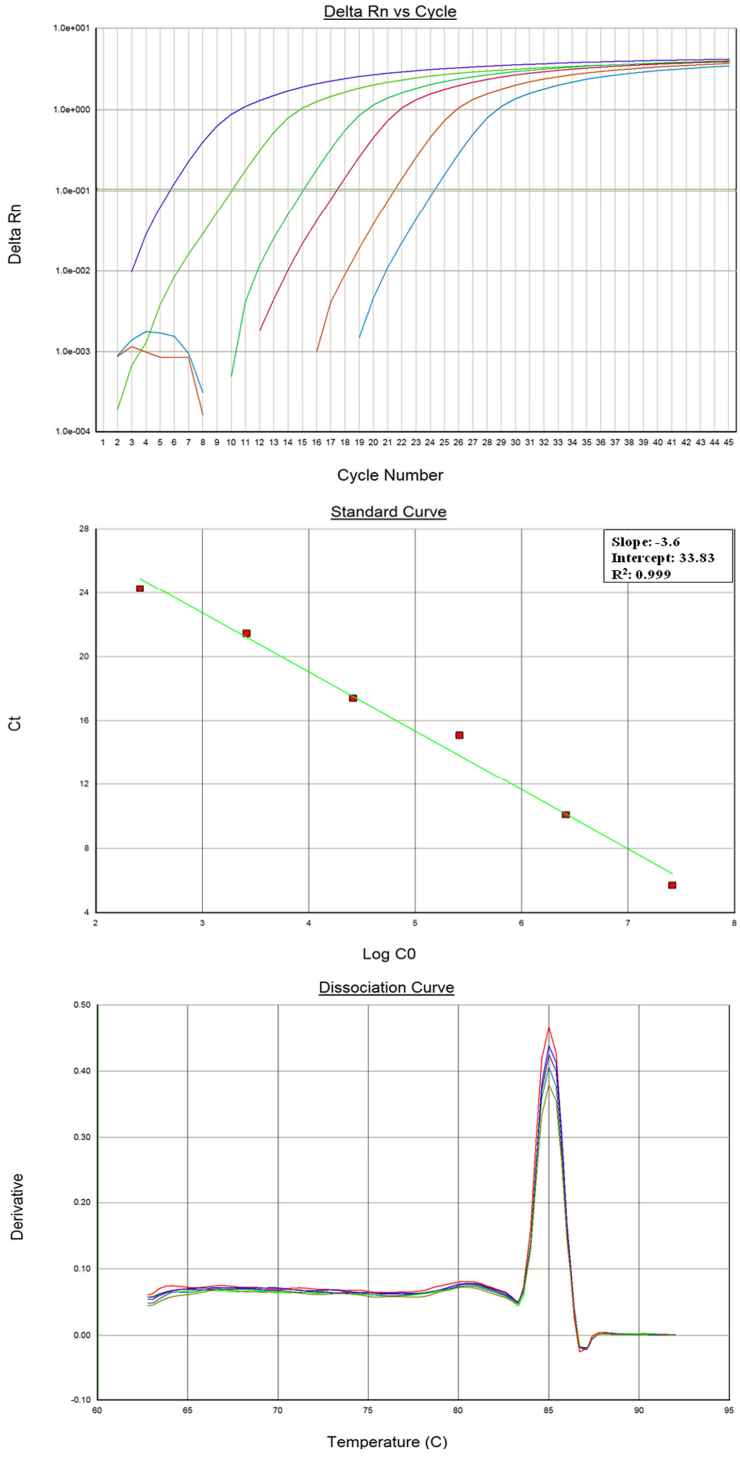


Plate 6.10 Amplification curve, standared curve and dissociation curve of *Ruminobacter amylophilus*

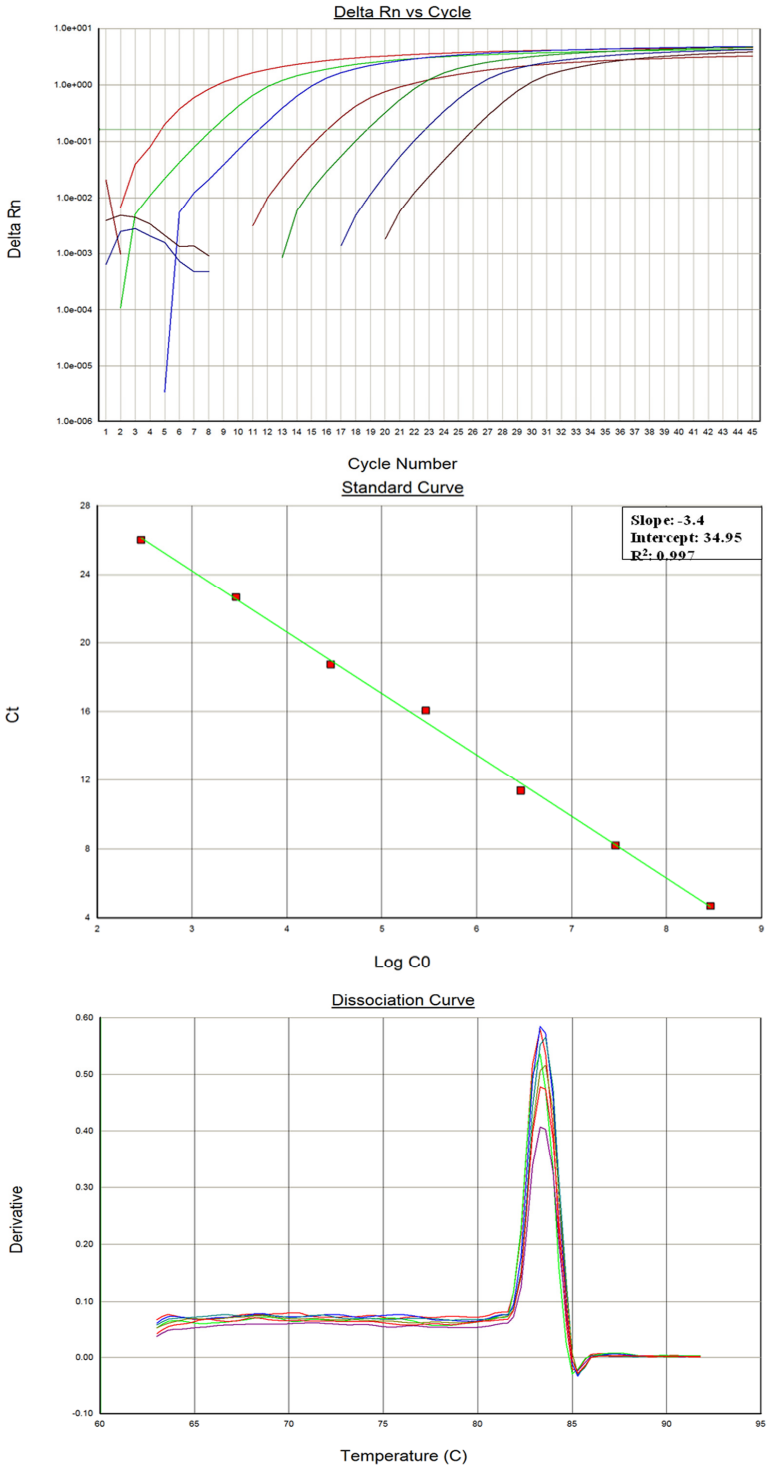


Plate 6.11 Amplification curve, standared curve and dissociation curve of *Treponema bryanti*

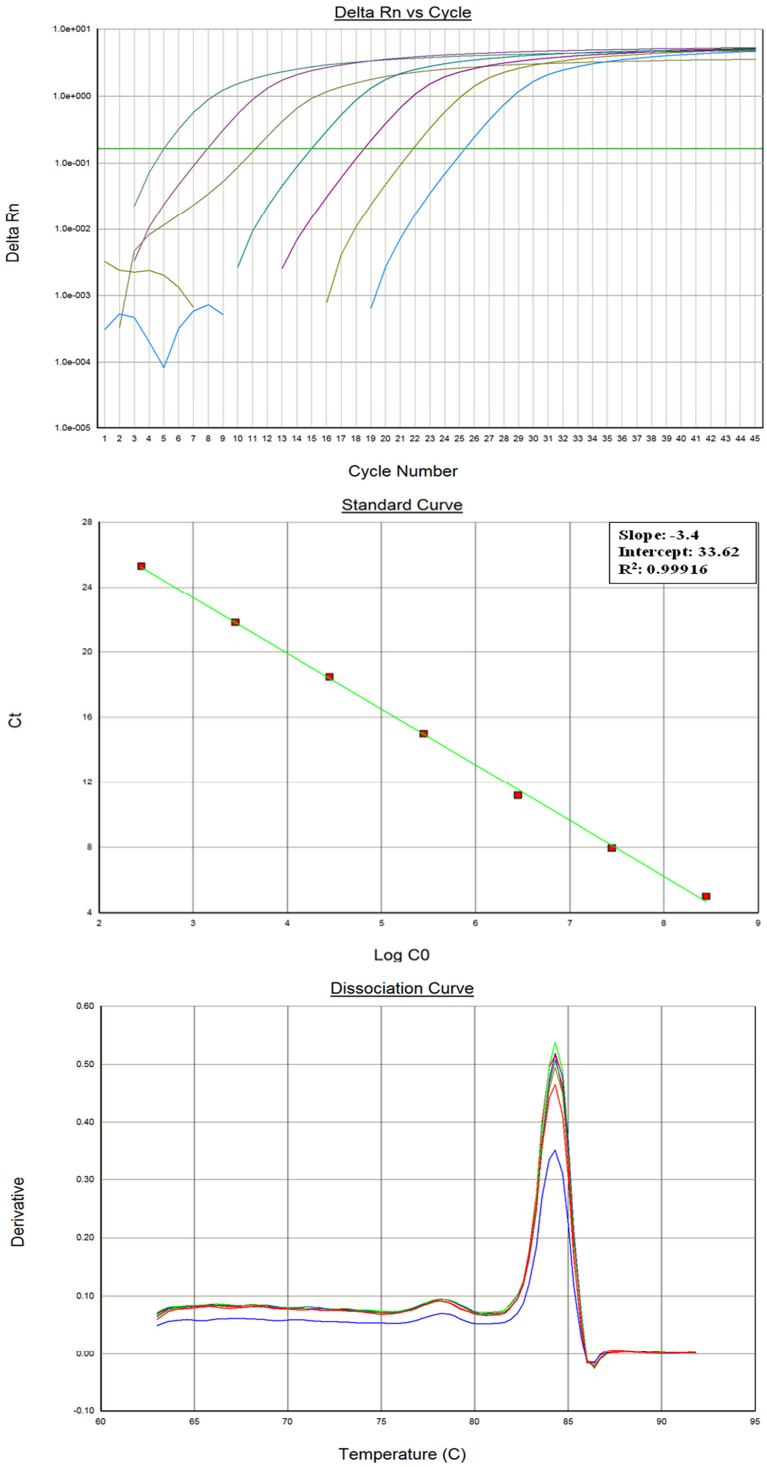
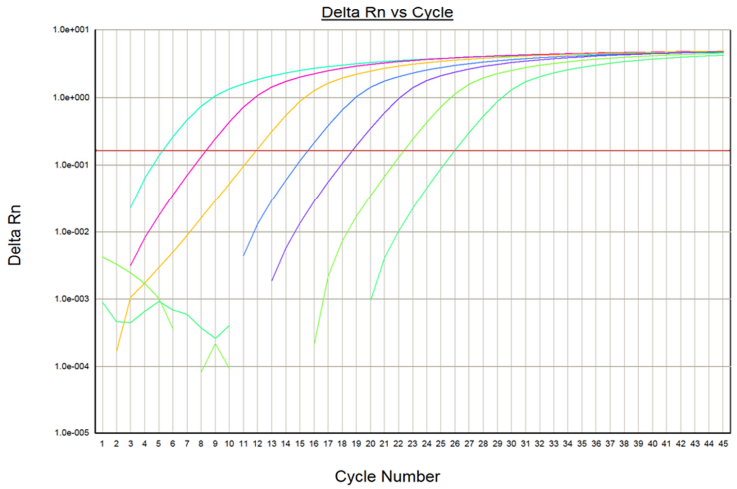
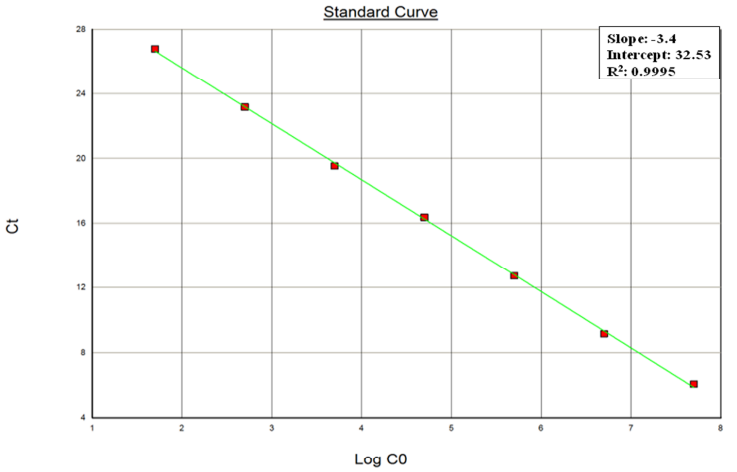


Plate 6.12 Amplification curve, standared curve and dissociation curve of *Anaerovibrio lipolytica*





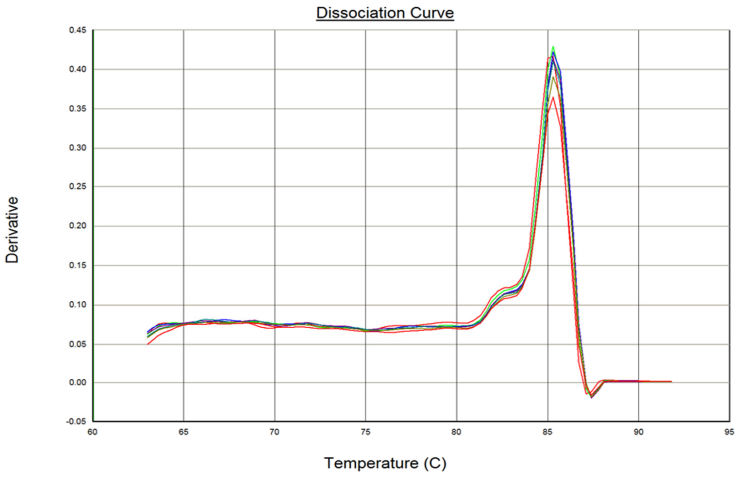


Plate 6.13 Amplification curve, standared curve and dissociation curve of Archaea

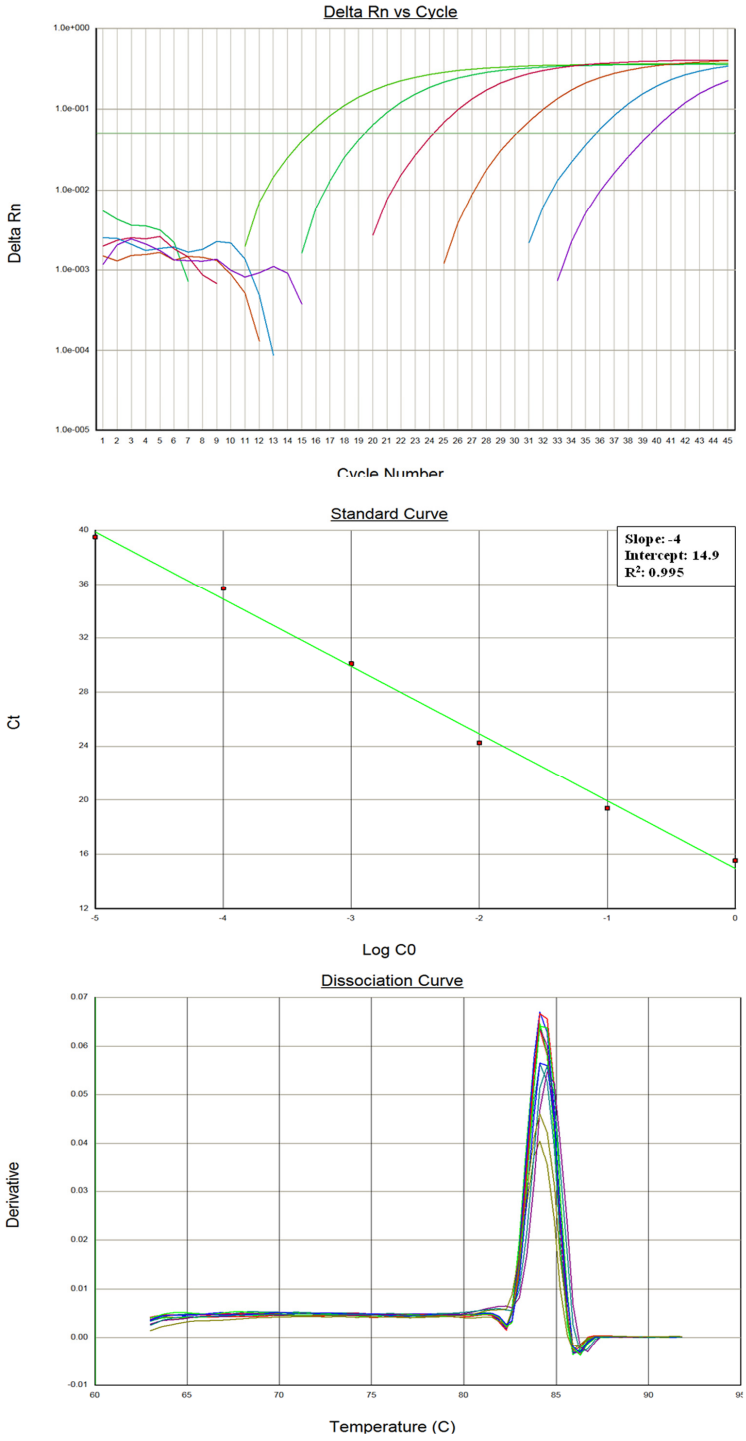


Plate 6.14 Amplification curve, standared curve and dissociation curve of Methanomicrobiales

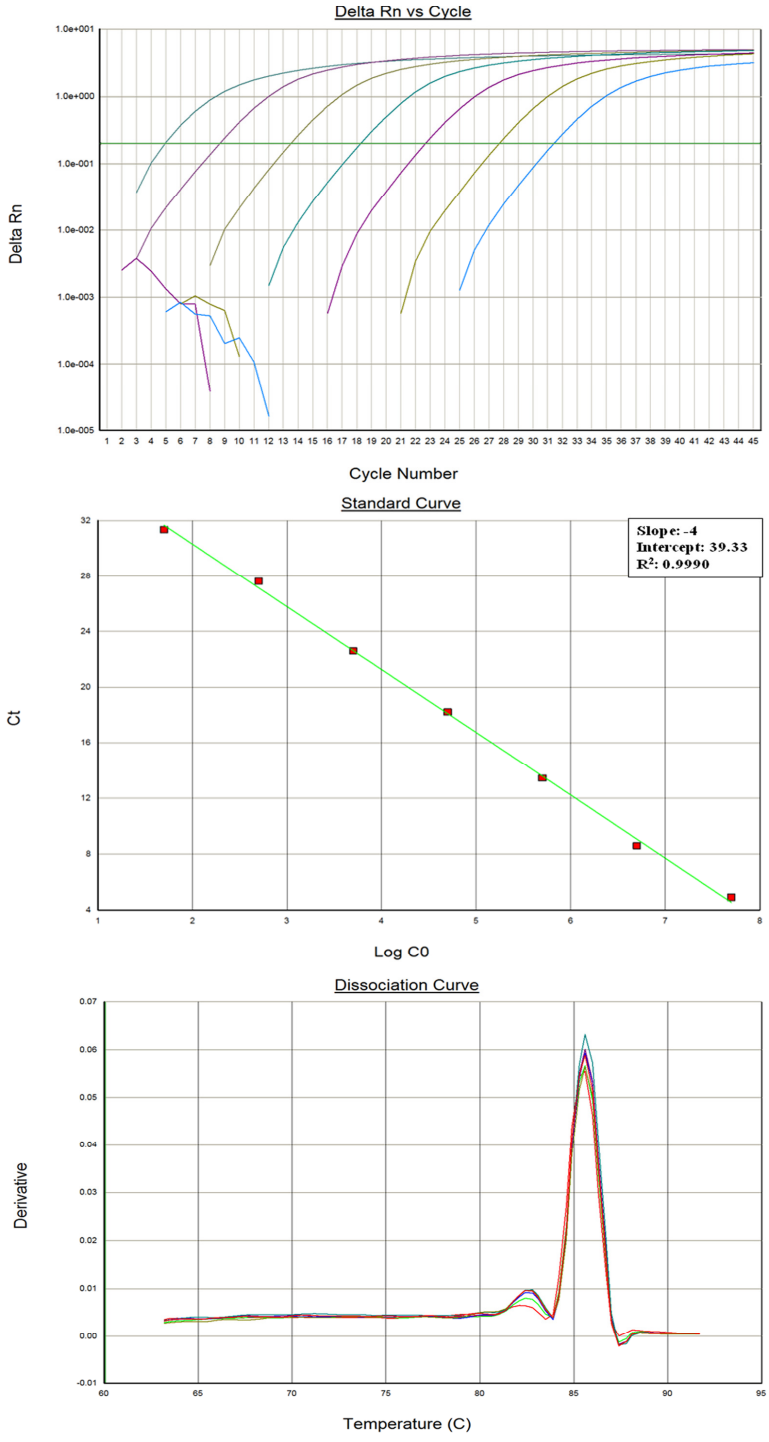


Plate 6.15 Amplification curve, standared curve and dissociation curve of Methanobacterials

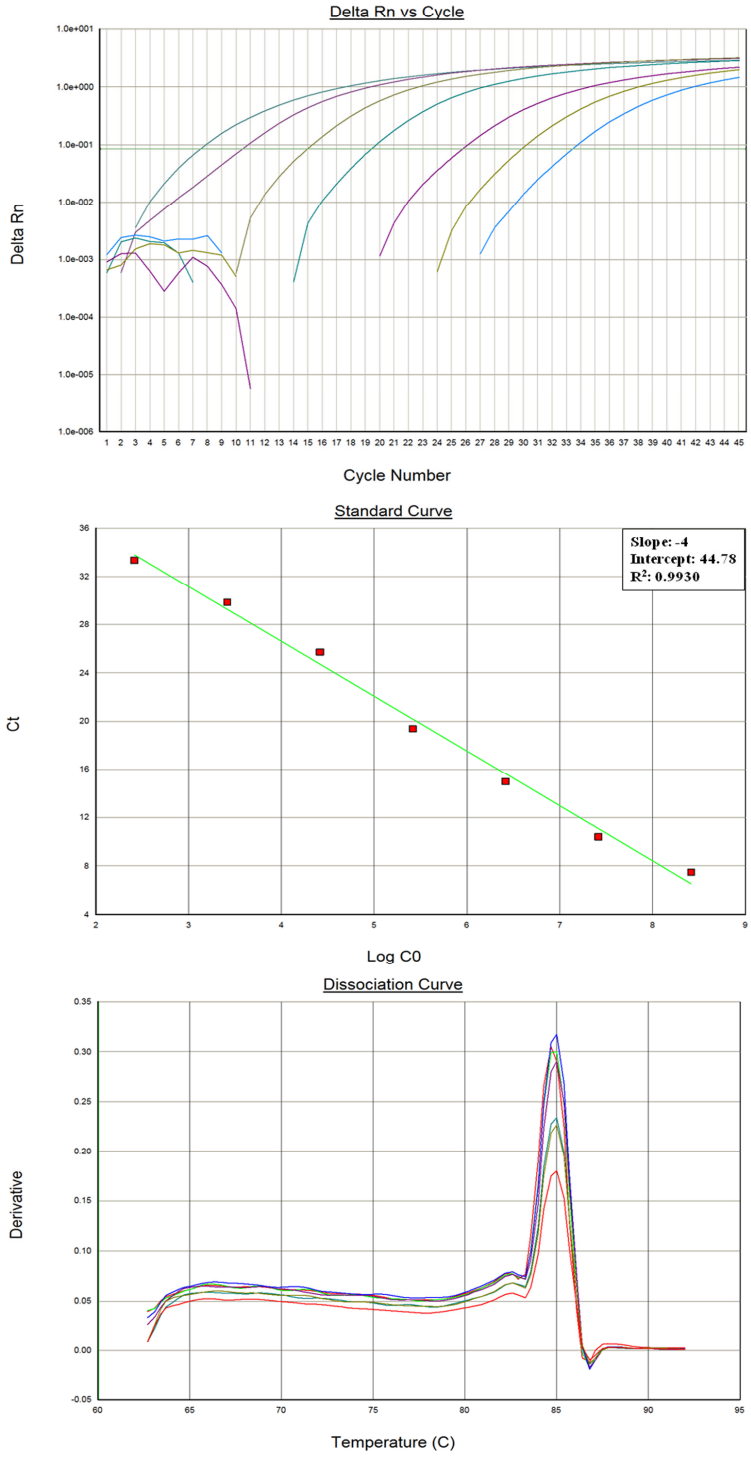


Plate.6.16 Amplification curve, standard curve and dissociation curve of Methanococcales

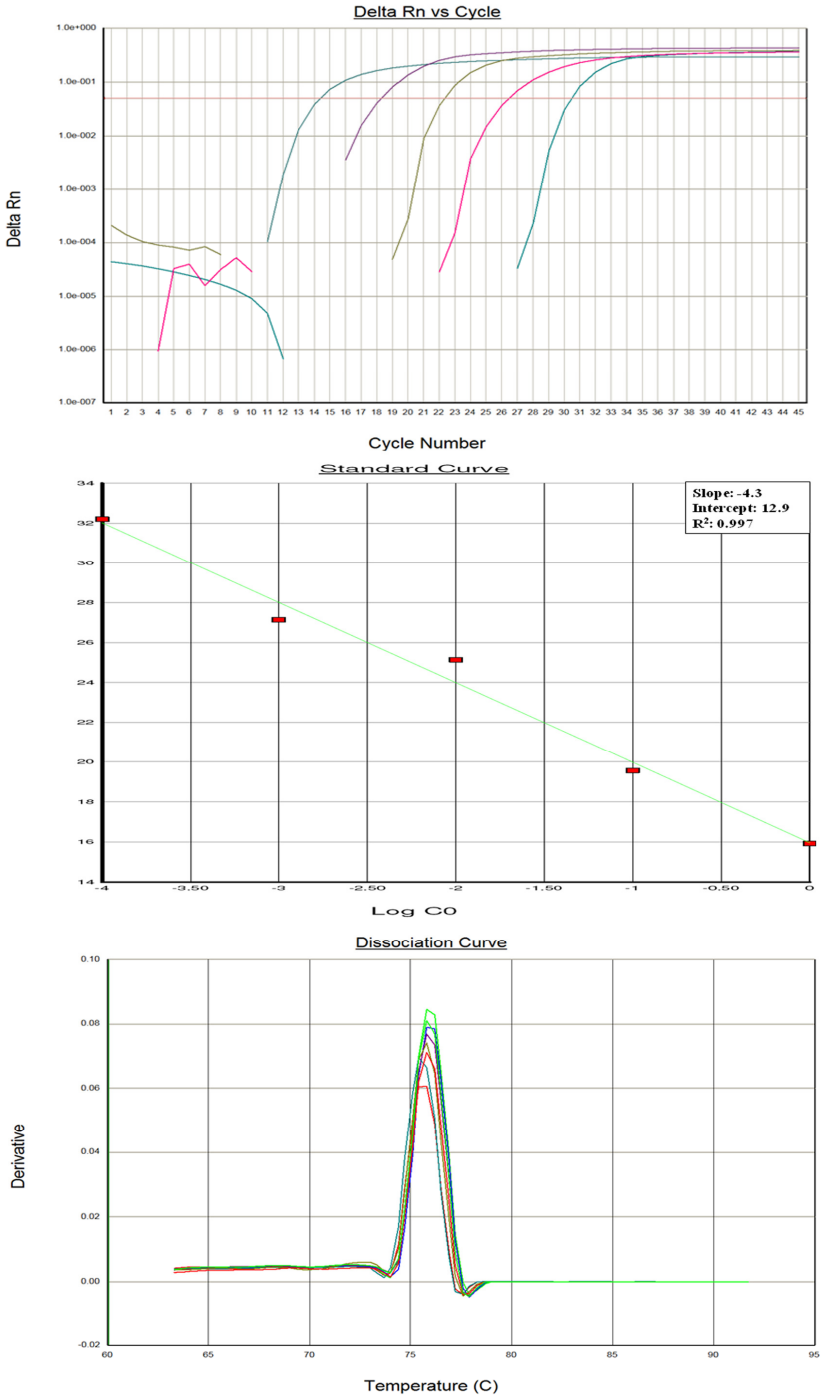


Plate 6.17 Amplification curve, standared curve and dissociation curve of Ciliate protozoa

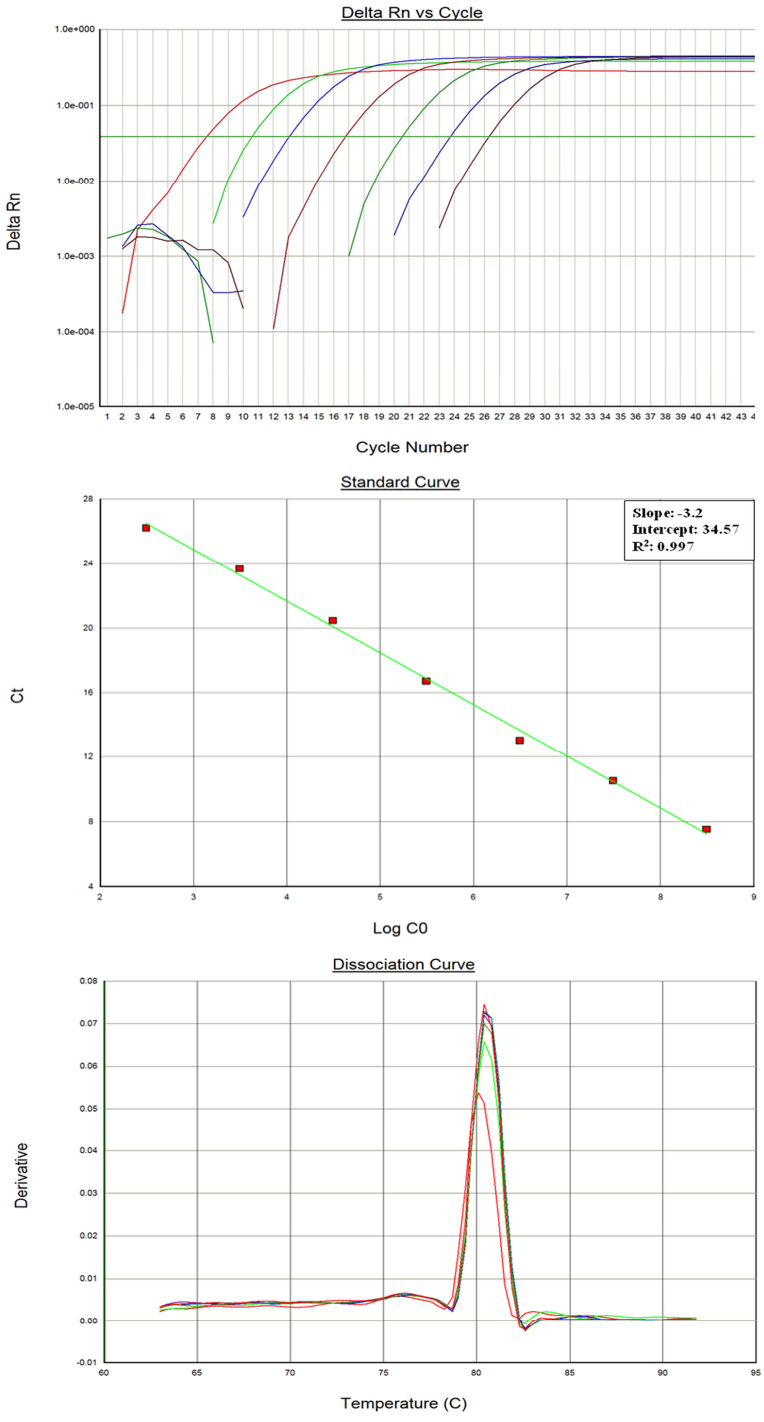


Plate 6.18 Amplification curve, standared curve and dissociation curve of *Entodinium sp.*

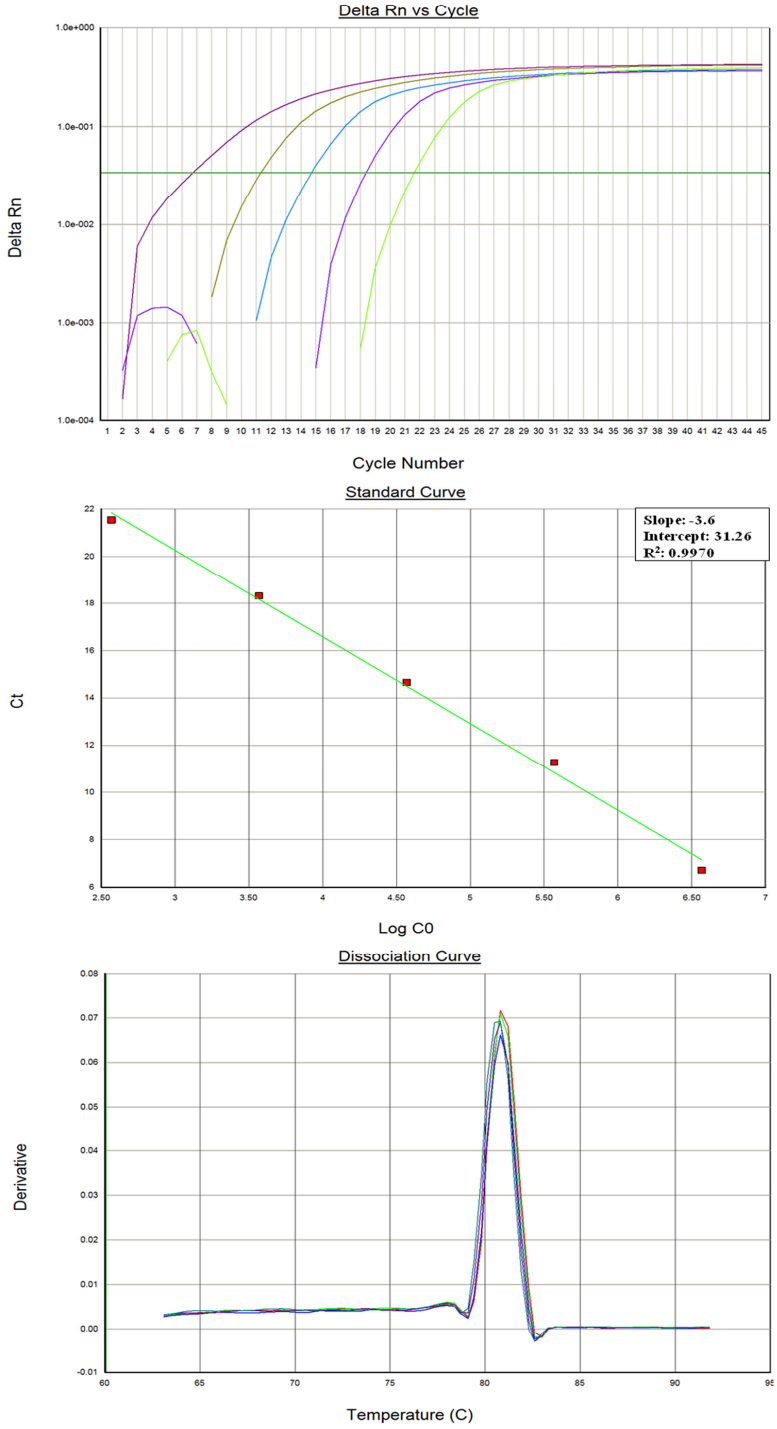
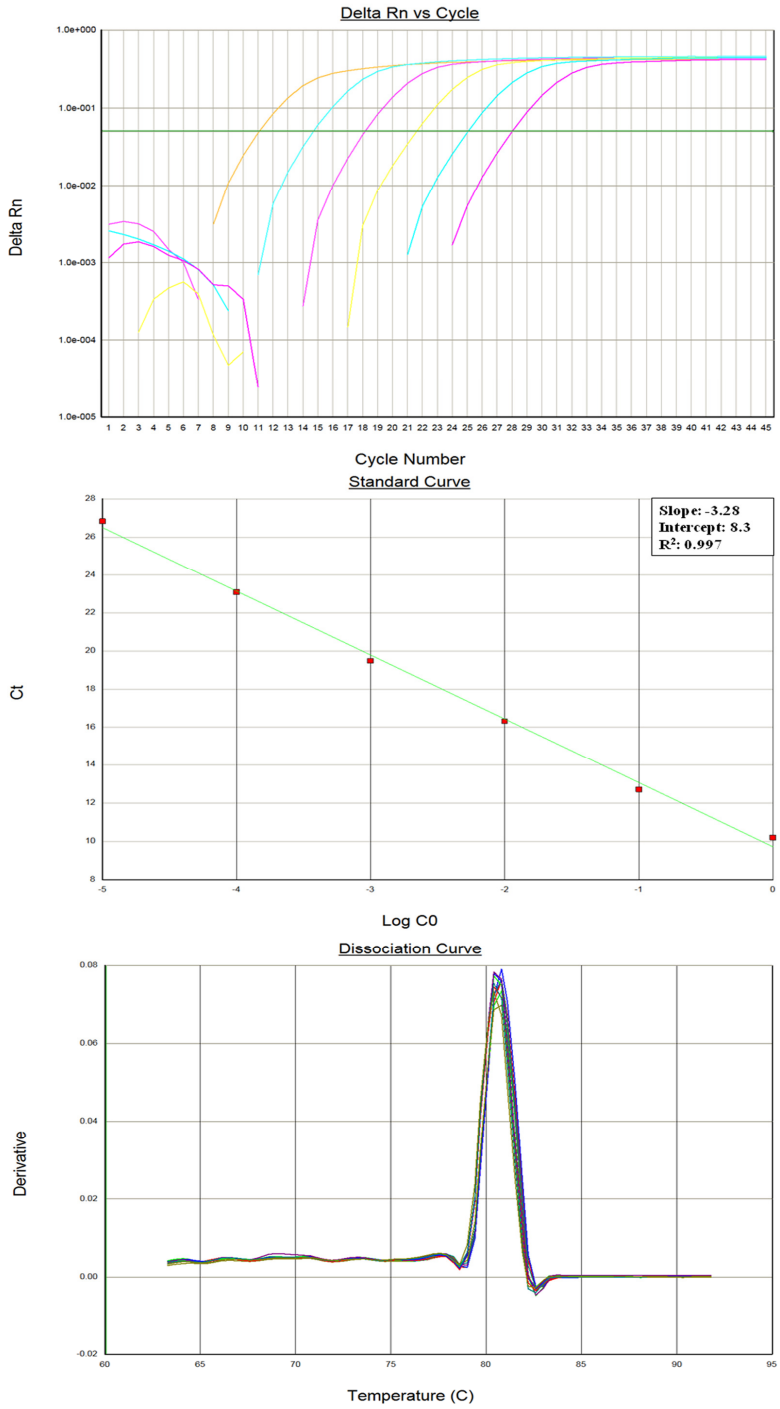


Plate 6.19 Amplification curve, standard curve and dissociation curve of *Dasytricha ruminantium*



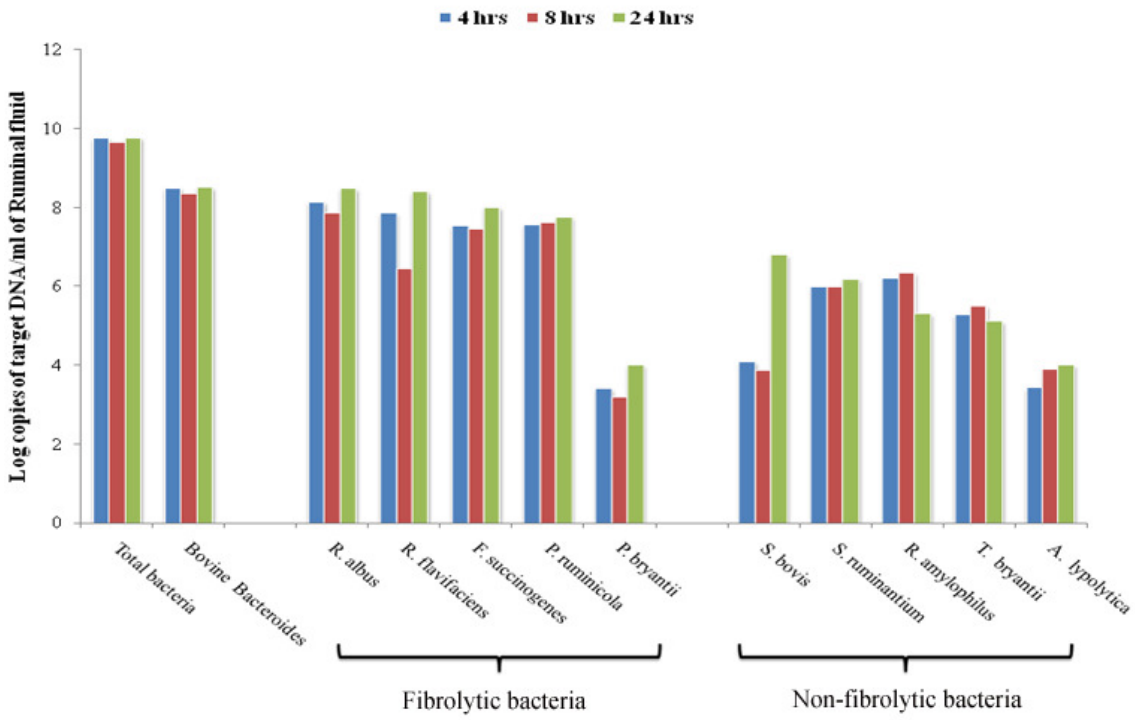


Figure 6.3. Distribution of total bacteria, bacteroides (Bovine) and each representative species in rumen of Surti buffalo, determined by real-time polymerase chain reaction assay at 4 hrs, 8 hrs and 24 hrs after feeding.

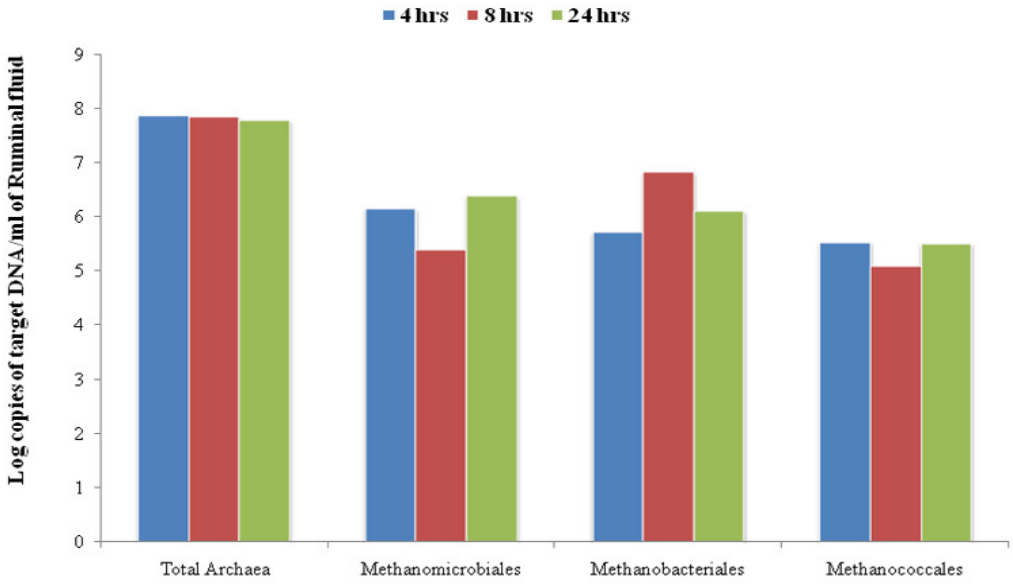


Figure 6.4. Distribution of total archaea, methanomicrobiales, methanobacteriales and methanococcales in rumen of Surti buffalo, determined by real-time polymerase chain reaction assay at 4 hrs, 8 hrs and 24 hrs after feeding.

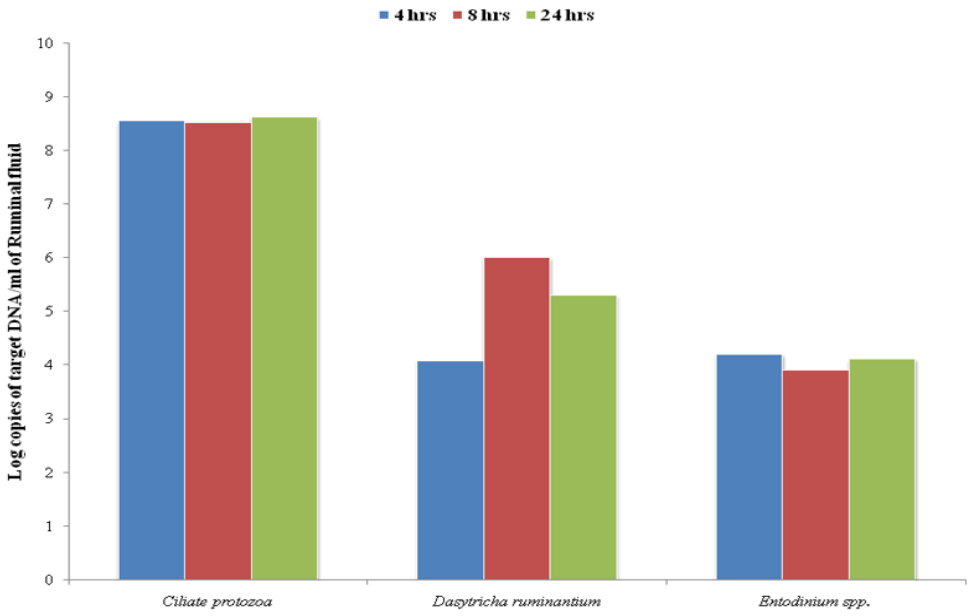


Figure 6.5. Distribution of ciliate protozoa, *Dasytricha ruminantium* and *Entodinium* sp. in rumen of Surti buffalo, determined by real-time polymerase chain reaction assay at 4 hrs, 8 hrs and 24 hrs after feeding.

Table 6.2: Proportions of total fibrolytic and non-fibrolytic bacteria from ruminal fluid of Surti buffalo (*B. bubalis*)

	% Total bacteria		
	4 hrs	**8 hrs**	**24 hrs**
Fibrolytic bacteria			
R. albus	2.36	1.62	5.66
R. flavifaciens	1.24	0.06	4.24
F. succinogenes	0.6	0.62	1.72
P. ruminicola	0.62	0.93	0.97
P. bryantii	0.000043	0.000036	0.000017
Non-fibrolytic bacteria			
S. bovis	0.00021	0.00016	0.11
S. ruminantium	0.016	0.016	0.025
R. amylophilus	0.028	0.049	0.0034
T. Bryantii	0.0033	0.0071	0.0022
A. lypolytica	0.000047	0.00018	0.00017

Table 6.3: Proportions of methanogens of total archaea to ruminal fluid of Surti buffalo (*B. bubalis*)

	% Total archaea		
	4 hrs	**8 hrs**	**24 hrs**
Methanomicrobiales	1.94	0.36	4.0
Methanobacteriales	0.72	9.71	2.17
Methanococcales	0.47	0.17	0.53

Table 6.4: Proportions of protozoa of total ciliate protozoa to ruminal fluid of Surti buffalo (*B. bubalis*)

	% Total ciliate protozoa		
	4 hrs	**8 hrs**	**24 hrs**
D. ruminantium	0.0034	0.03	0.049
Entodinium sp.	0.0046	0.0024	0.0031

Chapter 7. Studying bacterial population size as affected by level of roughage in rumen of Surti buffalo (*Bubalus bubalis*), using real time PCR assay

ABSTRACT

Absolute quantification real-time PCR approach was used to quantify the population change in major ruminal fibrolytic bacterial species (*Ruminococcus albus, Fibrobacter succinogenes, Ruminococcus flavefaciens, Prevotella ruminicola* and *Prevotella bryantii*) and non fibrolytic (*Ruminobactor amyllophilus, Selenomonus ruminantium, Treponema bryantii* and *Anaerovibrio lipolytica*) in rumen fluid of Surti buffalo (*Bubalus bubalis*). Eight female Surti buffalo were randomly assigned in four groups (2 animals each) to evaluate the effect of the wheat straw (roughage source) to-concentrate ratio on bacterial distribution. Animals were fed roughage-to-concentrate (R:C) ratios of 100:0 (T1), 75:25 (T2), 50:50 (T3), and 25:75 (T4), respectively. At the end of 30 days period, rumen fluid was collected at 0 h and 2 h after feeding. It was found that among fibrolytic bacteria *R. flavefaciens* (2.22×10^8 copies/ml) were highest in T2 group, 1.11×10^8 copies /ml for *F. succinogenes* (T2), 2.56×10^7 copies/ ml for *Prevotella ruminicola* (T1) and 1.25×10^7 copies/ml per for *Ruminococcus albus* (T4). In non fibrolytic bacteria, the *Selenomonus ruminantium* (2.62×10^7 copies/ ml) was predominant in group T3 and followed by *Treponema bryantii* (2.52×10^7copies/ ml) in group T1, *Ruminobactor amyllophilus* (1.31×10^7copies/ ml) in group T1 and *Anaerovibrio lipolytica* (2.58×10^6 copies/ml) in group T4. The distributions of the cellulolytic bacteria were higher at 0 h found than at 2 h after feeding. It is most notable that *R. flavefaciens* were the highest in population in the rumen of Surti buffalo and cellulolytic bacteria mostly present in ruminal fluid fed wheat straw as roughage source.

Key words: Surti buffalo; fibrolytic and non-fibrolytic bacteria; roughage: concentrate, 16S RNA gene.

INTRODUCTION

The rumen is a complex habitat in which feedstuffs are fermented primarily to a mixture of volatile fatty acids (VFA, predominantly acetic, propionic, and butyric acids) that serve as the major nutrient source for the ruminant animal. Much of our knowledge of the ruminal metabolism of these feedstuffs was gained through *in vitro* study of bacterial species, most of which were isolated in the early decades of rumen microbiology (Hungate, 1966; Krause and Russell, 1966). According to Wanapat (2000), buffalo had different rumen microorganisms than those in beef cattle, particularly the rumen bacteria, which belong to more than 500 different species (Collado and Sanz, 2006) and have the ability to recycle nitrogen to the rumen. Thus, any variations between cattle and buffalo in the proportions and number of rumen bacteria, protozoa, and fungal zoospores might attribute to the explanation of the differences in digestive capability due to fermentation end products available for the absorption and utilization by ruminants (Wanapat and Rowlinson, 2007). The complex symbiotic microbiota of the rumen is responsible for the breakdown of plant fiber which commonly occurs. This microbiota is highly responsive to changes in diet, age, antibiotic use, and the health of the host animal, which varies according to geographical location, season, and feeding regimen (Hunagte, 1966). Anaerobic rumen fibrolytic bacteria, protozoa, and fungi degrade fibrous material, allowing ruminants to utilize plant fiber for nutrition. Bacteria are the most numerous of these microorganisms and play a major role in the biological degradation of dietary fiber. *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* are presently recognized as the major cellulolytic bacterial and non fibrolytic bacterial (*S. ruminantium*, *S. dextrinosolvens* and *T. bryantii*) species found in the rumen (Forster *et al*., 1997; Shinkai and Kobayashi, 2007; Koike *et al*., 2007; Wanapat and Cherdthong, 2009). Techniques of molecular microbial ecology provide an opportunity to quantify these ruminal species with great sensitivity and precision, and several recent reports have provided a sense of which species are most abundant in the rumen under particular feeding conditions (Tajima *et al.,* 2001a; Klieve *et al*, 2003; Kobayashi, 2006). Moreover, DNA-based methods offer the option of storing samples

until their analysis, which could be an important advantage in field conditions (Castillo *et al*., 2006). The recent development of real-time polymerase chain reaction (PCR) has been successfully used for quantifying protozoa (Skillman *et al*., 2006b; Sylvester *et al*., 2004), cellulolytic fungi (Denman and McSweeney, 2006), and cellulolytic bacterial species (McSweeney and Denman, 2007; Tajima *et al*., 2001a). Real-time PCR is an approach that allows continuous monitoring of PCR product formation, and techniques vary according to the method of fluorescence generation. Real-time PCR has the ability to enumerate targeted bacteria with high sensitivity (Zimmermann and Mannhalter, 1966) and has been used to analyze various environmental samples, such as water (Lyaton *et al*., 2006) and rumen digesta (Reilly and Attwood, 1998). This technique is both reliable and simple to perform. Increased knowledge concerning the fibrolytic and non fibrolytic bacterial population will allow insight into the fiber-digestion capabilities of ruminant animals. However, very limited research has been conducted on Indian buffalo (*Bubalus bubalis*) with regard to the ruminal bacterial population using molecular techniques. Therefore, this study was conducted to determine by real-time PCR techniques the ruminal bacterial (fibrolytic and non fibrolytic) population of rumen fluid in Surti buffalo fed roughage: concentrate diet.

MATERIALS AND METHODS

Animal's diet and samples collection

The experiment was carried out on eight 2-3 year old female Surti buffaloes randomly assigned to four groups (2 in each group) reared at the Department of Animal Nutrition, College of Veterinary Science and A.H., Anand. The permission of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) was obtained prior to initiation of the study. The animals were received four different total mixed ration (TMR) of roughage to concentrate (R: C) of 100:0 (T1), 75:25(T2), 50:50 (T3), and 25:75 (T4) in individual feeding stall. All animals received feed according to respective R:C ratios *ad lib* daily, and Wheat straw was used as roughage. Concentrates are high-quality, low-fiber feeds that contain a high concentration of digestible energy per unit weight and volume. Under this study,

the concentrate diet consisted of 20.11% crude protein, 10.28 % crude fibre, 3.8 % ether extract, 52.43 % nitrogen free extract, 13.38 % ash, 3.38 % silica, 1% phosphorus and 1.22% calcium. All Animals were let loose daily for 2 hrs morning and evening, during which they had free access to drinking water. The feeding experiment was conducted for a period of 30 days. Samples of rumen content were collected from all the animals on 30th day of experiment to study the dynamics of rumen microbes. Samples of rumen content (about 500 ml) were collected at 0 and 2 hrs after feeding by a suction pump using a flexible stomach tube (Khampa *et al*., 2006). Finally the ruminal fluids collected from eight animals were pooled among 0 hrs and 2 hrs treatments wise and were immediately used for DNA extraction.

DNA extraction and PCR amplification of 16S RNA genes

Total genomic DNA was isolated from 1.0-mL aliquot of rumen fluid from four groups separately by using Qiagen stool kit as per manufacturer instruction with little modification. The sample was taken using a wide-bore pipette, so as to ensure a homogeneous sample containing plant particles and liquid. The DNA quality and quantity were checked by 0.8% (W/v) agarose gel electrophoresis and NanoDrop spectrophotometer (ND 1000, NanoDrop Technologies, Inc, Wilmington, DE, USA) at 260 nm.

Species specific PCR primers used for the amplification of target region of the 16S rRNA (target DNA) were chosen from the literatures (Table 7.1). The primers sequences of all targets are given in Table 7.1. The target DNA of all fibrolytic and non fibrolytic bacteria were amplified from the metagenomic DNA with respective primer sets, as described previously (Muyzer *et al.*, 1993; Tajima *et al*., 2001a; Koike and Kobayashi, 2001). All quantification Real-time PCR amplification and detection were performed using ABI 7500 system software (ABI 7500, USA). The reaction was conducted in a final volume of 25µl containing the following: 12.5µl Qiagen DNA Master SYBR Green I, 10 pM as a forward primer, 10 pM as a reverse primer, 7.5µl distilled water, and 2.0µl of DNA solution of unknown concentration. Amplicon specificity was judged based on dissociation curve of PCR end products by increasing the temperature at a rate of 1^0C every 30s from 60 to 95 ^{0}C. All PCRs were performed in duplicate. Before starting the real time PCR assay, conventional PCRs for the

validation of the specificity of the primers against target genes were performed in 25µl and reactions were performed using a minicycler under the respective PCR conditions. The PCR products were analysed by running on 1.5% agarose gels containing ethidium bromide and visualizing for a single specific band and the absence of primer dimmer products.

Preparation of standard plasmid for real-time PCR assays

Plasmid DNA containing the respective target gene sequence, used as the standard DNA in real-time PCR, was obtained by PCR cloning using the species-specific primer sets. After the confirmation of a specific amplification of the correct size (Table 7.1) on an agarose gel, the PCR products were excised from the gel. The PCR products were purified using the Qiagen gel Purification Kit (Qiagen CA), and ligated to the PTZ57T/R vector (Fermetas, UK). The ligation products transformed competent *Escherichia coli DH5α* cells by heat shock. Plasmids were purified from transformed *E. coli* using a QIAprep spin miniprep kit (Qiagen, Valencia, CA, USA), and the plasmids containing the correct insert were screened out by PCR amplification with respective primer sets. Prior to preparation of the standard, DNA sequences of each cloned target DNA were confirmed by sequencing and BLAST. The concentration of the plasmid was determined with a Nanodrop spectrometer. Copy number of each standard plasmid was calculated using formula; Copy No/ µl= Concentration of plasmids (gm/µl) x 6.022 x 10^{23} / length of recombinant plasmid (bp) x 660, (660 = Molecular weight of nucleotide base, 6.022 x 10^{23} = Avogadro's number). Ten-fold dilution series ranging from 10 to 10^{9} copies were prepared for each target. Real-time PCR was performed with ABI system (ABI7500), using the Qiagen DNA Master SYBR Green I. The optimal amplification conditions for each primer set were obtained with 10 pM each primer with the combination of annealing temperature and extension time are shown in Table 7.1. The 10-fold dilution series of the standard plasmid for the respective target was run along with the samples. Amplification of each sample was performed in duplicate. Quantification was made using standard curves obtained from the amplification profile of known concentrations of the standard plasmid for the respective target.

Statistical analysis

Statistical analysis of data was performed by using software of the Sigma Stat 32 (SPSS Inc., Chicago, Illinois). The significant differences at the 5% level ($P<0.05$) for treatment (dietary ratio) were statistically compared using Tukey test available in Sigma Stat32. Paired t-tests were also carried out to determine the significance of differences in data of rumen fluid at 0 h and 2 h after feeding.

RESULTS AND DISCUSSIONS

Real-time PCR-based quantification of representative rumen bacteria was reported in a few studies (Tajima *et al*., 2001a; Ozutsumi *et al*., 2006). In the present study, we assessed the rumen bacterial species (fibrolytic and non fibrolytic) using real-time PCR (Figure 7.1 and Figure 7.2). In the reaction for all standard, nearly perfect linear regressions (r^2 = 0.9930 to 0.9995) and slope (-3.3 to -4) were obtained between threshold cycle and quantities of standard. The graphs of standard curve, amplification curve and dissociation curve are given in Plate 7.1 to 7.10.

Figure 7.2 & 7.3 shows the population sizes of the fibrolytic and non fibrolytic bacteria in the rumen fluid of Surti buffalo while in response to ratio of dietary change, as enumerated by the real-time PCR assays. Total bacteria (averaged over four diets) were detected to be 2.16×10^{10} copies per ml ruminal fliud (Figure 7.2). Among the fibrolytic bacteria, *R. flavefaciens* was the most abundantly detected (2.22×10^8 copies/ml of ruminal fluid (in T2) followed by, 1.11×10^8 copies / ml for *F. succinogenes* (in T2), 2.56×10^7 copies/ ml for *Prevotella ruminicola* (in T1), 1.25×10^7 copies/ml per for *Ruminococcus albus* (in T4) and 2.56×10^4 copies/ml per for *Prevotella bryantii* (in T4). In non fibrolytic bacteria, the maximum population of *Selenomonas ruminantium* was observed 2.62×10^7 copies/ml (in T3), followed by 2.53×10^7copies/ml for *Treponema bryantii* (in T1),1.31×10^7copies/ml per for *Ruminobactor amyllophilus* (in T1) and 2.58×10^6 copies/ml per for *Anaerovibrio lipolytica* (in T4). The population of *P. ruminicola* in T1, *P. bryantii* in T4, *F. succinigenes* in T2, *R. amyllophilus* in T1, *S. ruminantium* in T3 and *A. lipolytica* in T4 group were significantly higher among treatment groups ($P < 0.05$). However, the *R. albus* and *F. succinogenes* was not significantly different ($P > 0.05$) between all treatment groups.

To the best of our knowledge, no previous study has reported the diet dependent changes of population size of rumen microbes of Indian buffalo. Our observations indicate that a large number of bacteria were present in the ruminal fluid. Because each bacterial species has a different copy number of 16S rRNA gene (Ozutsumi *et al*., 2006). This is comparable to the level of rumen bacteria counted by microscopy in the literature (Hungate, 1966).

The results showed that the rumen of Surti buffalo harboured *R. flavefaciens* and *F. succinogenes* at high levels (*R. flavefaciens* > *F. succinogenes*), with both species out numbering *R. albus*. Previous studies performed with other molecular approaches targeting either RNA (16S rRNA-targeting oligonucleotide probes) or DNA (competitive PCR) also showing that *R. albus* is the least abundant species among the three fibrolytic species in the rumen of sheep (Klappenbach *et al*., 2001; Chaucheyras *et al*., 1997; Koike and Kobayashi, 2004; Michalet-Doreau *et al*., 2002; Koike *et al*., 2003). However, the data presented here differ from those reported by (Chaucheyras *et al*., 1997), who found *F. succinogenes* to be the most abundant species in the rumen of sheep fed with alfalfa hay and concentrate. Wanapat and Cherdthong, (2009), also observed the *F. succinogenes* to be the most dominant in digesta and rumen fluid of swamp buffalo fed roughage and concentrate as estimated by real time PCR assay. This difference may be attributed to various host and environmental factors (diet, age and health of animal, geographical location and season) (Mosoni *et al*., 2007) and to the technique used (Primers, real time PCR versus competitive PCR). For instance, the estimation of the number of the three cellulolytic species is much higher than that reported by competitive PCR (Chaucheyras *et al*., 1997) whereas it is of the same order of magnitude with reported data obtained from cattle by real-time PCR (Ozutsumi *et al*., 2006).

The dynamics of fibrolytic bacteria were in good correlation with the response to diet shift, particularly the changes of concentrate (Koike *et al*., 2003). In this study, changes the feeding of wheat straw from 75 % to 25 % remarkably decreased the population of these three cellulolytic bacteria numbers from 2.22×10^8 to 1.70×10^8 copies/ml for *R. flavefaciens*, 1.11×10^8 to 1.33×10^6 copies/ml for *F. succinogenes* and 2.56×10^8 to 2.76×10^6 copies/ml for *Prevotella ruminicola* in rumen fluid

(Figure 7.2) respectively. On the other hand, the population size of *R. albus* and *Prevotella bryantii* increased from 9.42 x 10^6 to 1.25 x 10^7 copies/ml and 3.5 x 10^3 to 2.5 x 10^5 copies/ml respectively in response to this diet change. The proportion of roughage in the diet might influence the population size or the proportion of fibrolytic bacterial numbers in the rumen. In addition, all the fibrolytic bacterial numbers examined in the present study were different, responding to a change in proportion of wheat straw and concentrate. As the results show, the lowest numbers of the two fibrolytic bacteria (*R. albus* and *Prevotella bryantii*) were found when increasing the level of concentrate. It is possible that dietary conditions might have influenced on reduced numbers of cellulolytic bacteria. Moreover, rumen pH together with microbial population, nature of substrates, environmental factors such as temperature, and the existence of cations and soluble carbohydrates have been suggested as factors governing bacterial attachment (Stewart and Bryant, 1988). Ruminal pH is one of most important of these factors, because the fibrolytic bacteria are very sensitive to the pH change (Miron *et al*., 2001). When ruminants are fed fiber- deficient rations, ruminal pH declines, microbial ecology is altered, and the animals become more susceptible to metabolic disorders (Sung *et al*., 2007). As Koike *et al*., (2003), quantified the numbers of *F. succinogenes*, *R. flavefaciens*, and *R. albus*, attached to straw and they were analyzed by competitive PCR showing that the numbers of all the three species increased gradually with increased neutral-detergent fiber. On the other hand, Wora-anu *et al*., (2000) reported that roughage- to-concentrate ratios of 100:0, 60:40, and 40:60 could decrease the cellulolytic bacterial population in swamp buffalo (5.62 x 10^{10}, 4.06 x 10^{10}, and 4.57 x 10^{10} CFU/ ml), respectively. In addition, Tajima *et al*., (2001a) reported that the quantity of *F. succinogenes* DNA predominant in animals on the hay diet fell 20-fold on the third day of the switch to a grain diet and further declined on day 28, with a 57-fold reduction in DNA. The *R. flavefaciens* DNA concentration on day 3 declined to 10% of its initial value in animals on the hay diet and remained at this level on day 28.

Non-fibrolytic bacteria such as *S. ruminantium* and *T. bryantii* were detected in the fiber-associated community, using comparative 16S rRNA gene analysis (Michalet-Doreau *et al*., 2002). The present study quantitatively confirmed the

attachment of nonfibrolytic bacteria to roughage. As per current understanding, they attached to the fibre at a relatively low level to that of fibrolytic bacteria (Figure 7.3). *S. ruminantium* (2.62 x 10^7 copies/ml in T3) *Treponema byrantii* (2.53 x 10^7 copies/ml in T1) and *Ruminobacter amylophilus* (1.31 x 10^7 copies/ml in T1) had the highest proportion among the non fibrolytic bacterial species assessed in the present study. While the proportion of *A. lipolytica* was 2.56 x 10^6copies/ml in T4 groups. This observations suggests that the fiber-attachment ability of *S. ruminantium.* Minato and Suto, (1978) indicated that some of the non-fibrolytic bacteria possessed the ability to attach to cellulose at a similar extent to that of fibrolytic bacteria. Attachment of non-fibrolytic bacteria to fiber may be mediated by glycocalyces (Minato and Suto, 1978), which are commonly found in rumen bacteria during their colonization in plant cell wall (Minato *et al*., 1993). These reports support the significant number of non-fibrolytic bacteria in ruminal fluid of Surti buffalo as observed in the present study. The synergism between fibrolytic and non-fibrolytic bacteria during fiber degradation has been noted. In such a relationship, fibrolytic bacteria provide the hydrolyzed product from cellulose to non-fibrolytic bacteria, while non-fibrolytic bacteria indirectly facilitate fiber degradation by preventing the accumulation of bacterial metabolites such as succinate and cellodextrins (Cheng *et al*., 1980).Utilization of cellodextrins from cellulose hydrolysis is crucial in further fiber digestion (Wollin *et al*., 1997), because cellulases are highly sensitive to feedback inhibition by cellobiose, which is confirmed in *F. succinogenes* (Russel, 1985). This result indicates that these one species were dominant non-fibrolytics and it is known that *S. ruminantium* are able to utilize hydrolysis product of polysaccharide such as cellodextrins and maltodextrins for their growth (Wollin *et al*., 1997; Maglione *et al*., 1997). Therefore, *S. ruminantium* in rumen might grow by utilizing the maltodextrins released during cellulose degradation (Cotta, 1992). The metabolic interactions mentioned here may explain why non-fibrolytic bacteria are abundant on the ruminally incubated fiber diet. Moreover, this fact strongly highlights the importance of non-fibrolytic bacteria for fiber digestion.

The distribution of the fibrolytic and non fibrolytic bacterial species in rumen fluid of the Surti buffalo are shown in Table7.2 and Table 7.3. As found among

fibrolytic in the rumen, specially two cellulolytic bacterial populations, *R. flavefaciens F. succinogenes* were higher to be 1.78 x 10^8 and 4.53 x 10^7 copies/ml at 0 h than 2 h after feeding (Table 7.2). Similarly, in non fibrolytic bacteria, the distribution of population size of *Ruminobactor amyllophilus* was higher to be 2.03 x 10^7copies/ml at 0 h than 2 h after feeding, while the population of *S. ruminantium* (1.47 x 10^7copies/ml) was higher at 2 h than 0 h (Table 7.3). Indeed, the distribution of bacterial population were not significant different (P >0.05) between sampling hours. Although, the high distribution of the fibrolytic bacteria in rumen fluid is reasonably explained by the fact that the fluid mainly consisted of plant fiber particles that were likely to have been colonized by the fibrolytic bacteria. Similarly, Hungate, (1966) reported that fibrolytic bacteria were more abundant in the whole digesta, of rumen, presumably because many attached to the solids particles. In the study by Koike *et al*., (2003), the authors suggested that the increase in attached cell numbers observed could be mostly attributed to cell proliferation on the straw, at 6 h, the numbers of attached cells of the three species gradually increased and peaked at 24 h (10^9/gram dry matter (DM) for *F. succinogenes* and 10^7/ gram DM for *R. flavefaciens*) or 48 h (10^6 per gram DM for *R. albus*). There are two possible explanations of the increased cell populations on the 6-h post feeding, cell proliferation after feeding and the additional attachment of new bacteria from the liquid phase or other particles.

Although real-time PCR quantifies both viable and non-viable cells, this technique has the advantage of targeting true cellulolytic species and being a very sensitive, accurate and reproducible method, enables underlining of slight changes and allows the differentiation between the population sizes of the key cellulolytic bacteria. However, quantification of cellulolytic species numbers does not reflect the real activity of these species in the rumen. Indeed, linking community structure to activity and functionality is a central but still poorly studied issue in microbial ecology. Quantifying 16S transcripts would provide additional information as it would better reflect total bacterial activity. However, fibrolytic activity can only be measured by the quantification of glycoside hydrolase transcripts. This is particularly difficult with fibrolytic species (cellulolytic), they each carry numerous genes involved in the fibrolytic function, whose relative importance is not known (Nouille *et*

al., 2005; Krause *et al*., 2003). But recently Brulc *et al*., (2009) observed that the glycoside hydrolase is the key enzyme in fiber-adherent bovine microbiome through pyrosequencing (Gene-centric Metagenomics).

CONCLUSIONS

In conclusion, real-time PCR is a relevant and useful tool to study the dynamics of microbial populations in Surti buffalo (*Bubalus bubalis*) rumen as long as several precautions in sampling, DNA extraction and real-time PCR (primer specificity, standard curves for each target species, PCR efficiency, etc.) are taken. Its sensitivity and accuracy allows the differentiation of slight changes in bacterial numbers that cannot be seen using microbial techniques or other molecular approaches (16S probing and competitive PCR) (Qi *et al*., 2005). In this study, two major representative groups of ruminal microbes covering fibrolytic and non-fibrolytic bacterial communities were demonstrated. Result revealed that *Ruminococcus flavefaciens* and *Fibrobactor succinogens* were the most dominant fibrolytic among the all detected fibrolytic species, which may forms a multi-enzyme cellulosome complex that could play an integral role in the ability of this bacterium to degrade plant cell wall polysaccharides. Non-fibrolytic, *S. ruminantium* and *Treponema bryantii* were also detected with abundance in rumen fluid. The high magnitude of non-fibrolytic group on the roughage suggests the development of mutual relationships between fibrolytic and non-fibrolytic bacterial communities. Present study may be useful in manipulating feeding regimes for Indian buffaloes.

Table 7.1: Primers and PCR Conditions for Real time PCR Assay

Target	Sequences (5'-3')	Product size (bp)	Annealing Temp.	Reference
Total Bacteria	P1 CCTACGGGAGGCAGCAG P 2 ATTACCGCGGCTGCTGG	194	60^0C-30 sec	Muyzer *et al*., (1993)
Fibrolytic bacteria				
Ruminococcus albus	P1 CCCTAAAAGCAGTCT TAGTTCG P2 CCTCCTTGCGGTTAGAAC A	175	62^0C-15 sec	Koike and Kobayshi (2001)
Fibrobacter succinogenes	P1 GGTATGGGATGAGCT TGC P2 GCC TGCCCC TGAACTATC	445	62^0C-15 sec	Tajima *et al.*, (2001a)
Ruminococcus flavefaciens	P1 GGACGA TAA TGACGGTACTT P2 GCAATC(CT)GAACTGGGACAAT	295	55^0C-20 sec	Tajima *et al.*, (2001a)
Prevotella ruminicola	P1 GGTTATCTTGAGTGAGTT P2 CTGATGGCAACTAAAGAA	485	53^0C-35 sec	Tajima *et al.*, (2001a)
Prevotella bryantii	P1 ACTGCAGCGCGAACTGTCAGA P2 ACCTTACGGTGGCAGTGTCTC	540	68^0C- sec	Tajima *et al.*, (2001a)

Non fibrolytic bacteria

Ruminobacter amylophilus	P1 CAACCAGTCGCATTCAGA P2 CACTACTCATGGCAACAT	642	57^0C-30 sec	Tajima *et al.*, (2001a)
Treponema bryantii	P1AGTCGAGCGGTAAGATTG P2 CAAAGCGTTTCTCTCACT	412	57^0C-30 sec	Tajima *et al.*, (2001a)
Anaerovibrio lipolytica	P1 TGGGTGTTAGAAATGGATTC P2 CTCTCCTGCACTCAAGAATT	597	57^0C-30 sec	Tajima *et al.*, (2001a)
Selenomonas ruminantium	P1 TGCTAATACCGAATGTTG P2 TCCTGCACTCAAGAAAGA	513	57^0C-30 sec	Tajima *et al.*, (2001a)

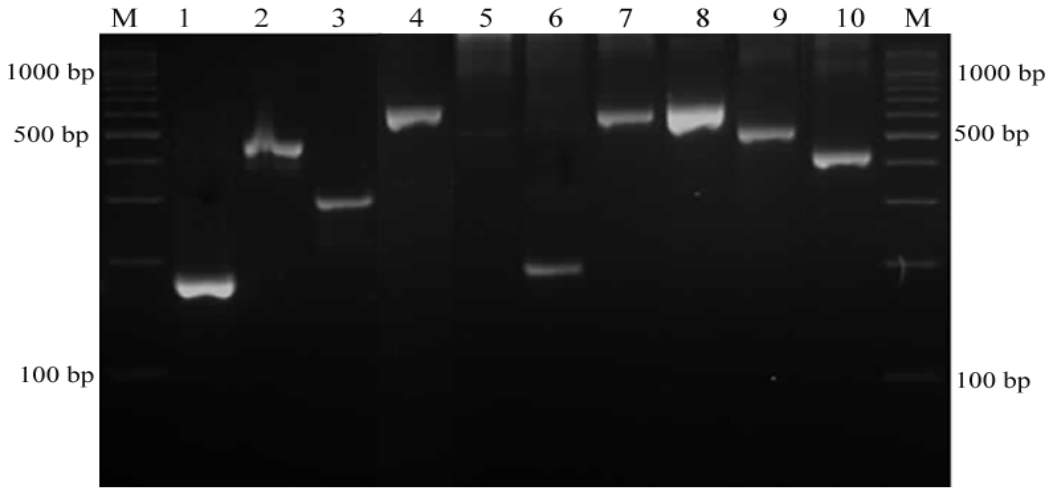


Figure 7.1. Qualitative PCR detection of fibrolytic bacteria and Non fibrolytic bacteria. Lane: 1, *Ruminococcus albus*; 2, *Fibrobacter succinogenes*; 3, *Ruminococcus flavefaciens*; 4, *Prevotella bryantii*; 5, *Prevotella ruminicola*; 6, Total bacteria; 7, *Ruminobacter amylophilus*; 8, *Anaerovibrio lipolytica;* 9, *Selenomonas ruminantium*; 10, *Treponema bryantii*; Lane M, DNA size marker.

Plate 7.1 Amplification curve, standard curve and dissociation curve of Total Bacteria

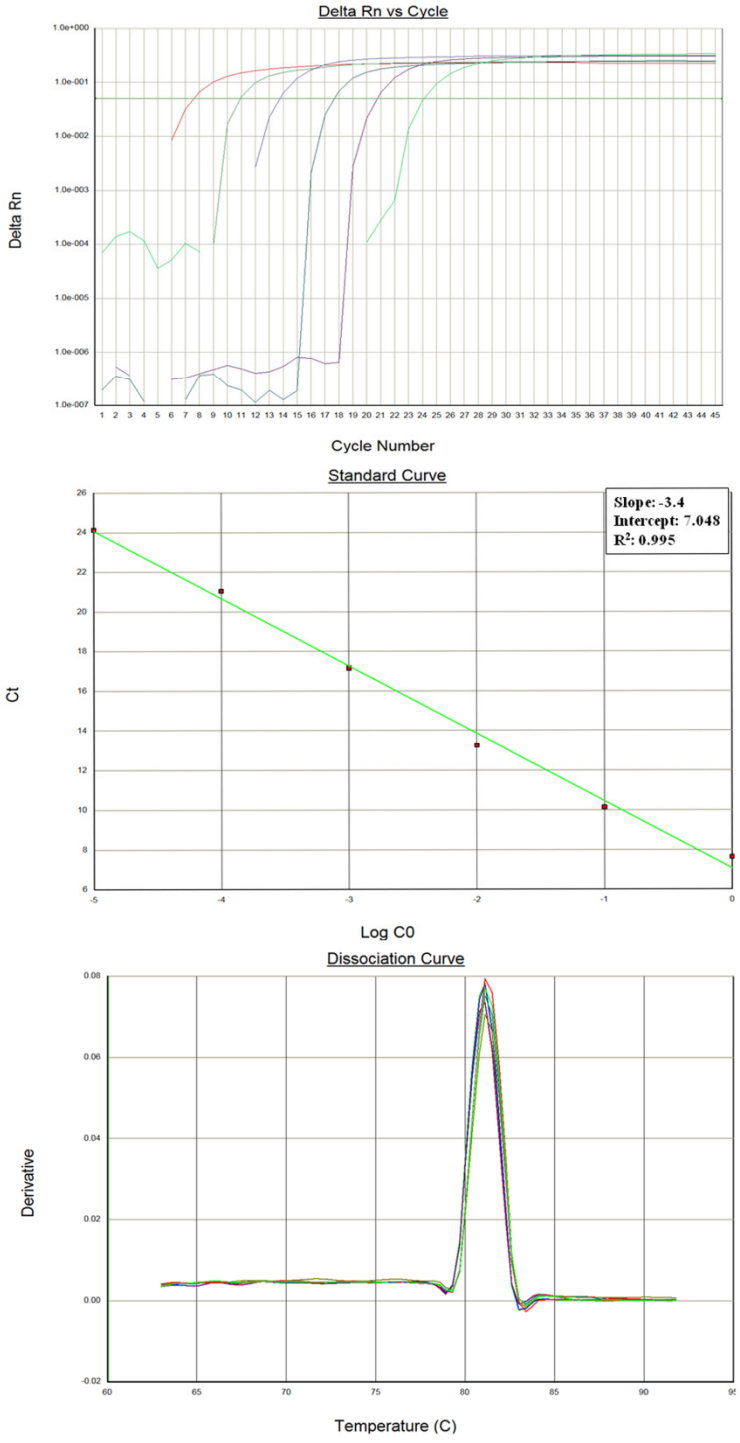


Plate 7.2 Amplification curve, standard curve and dissociation curve of *Ruminococcus albus*

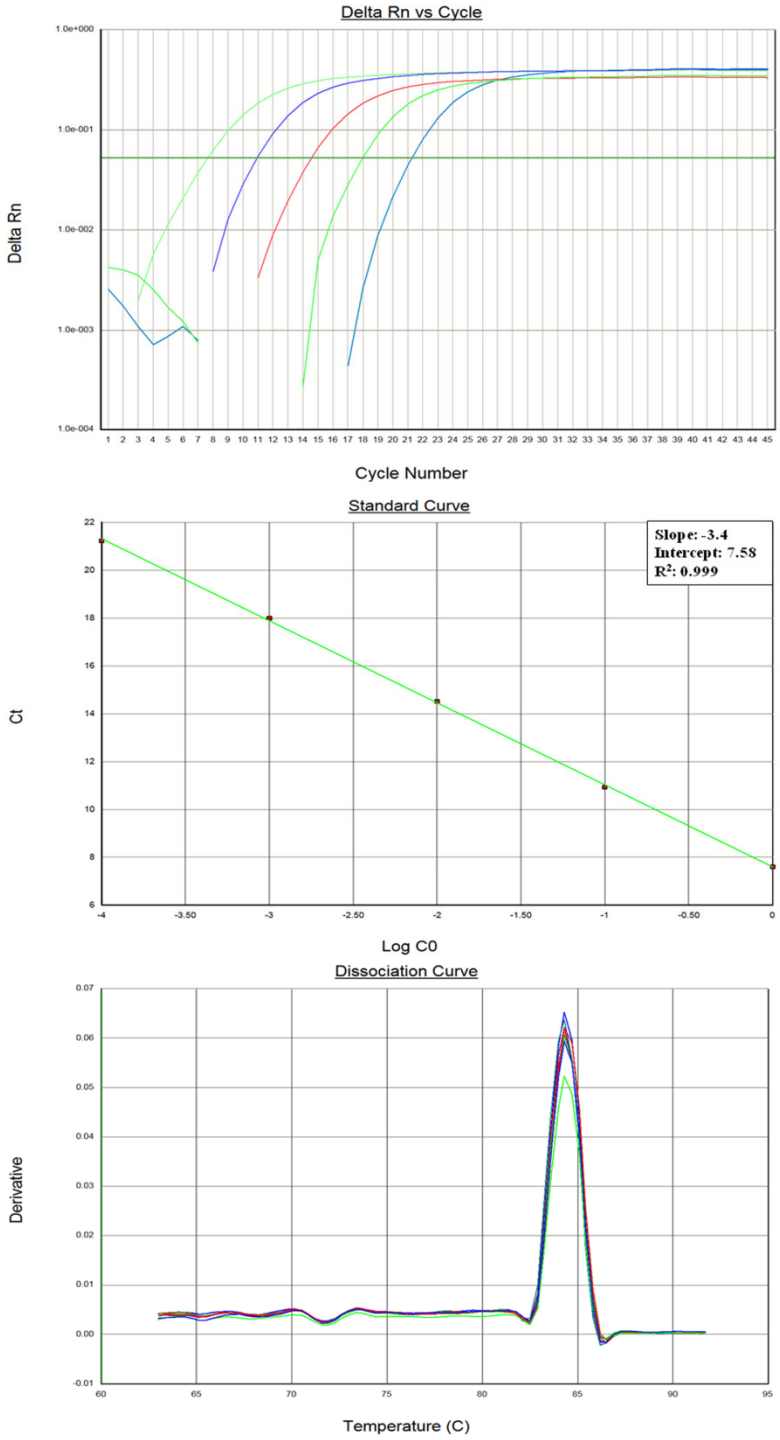


Plate 7.3 Amplification curve, standard curve and dissociation curve of *Fibrobactor succinogenes*

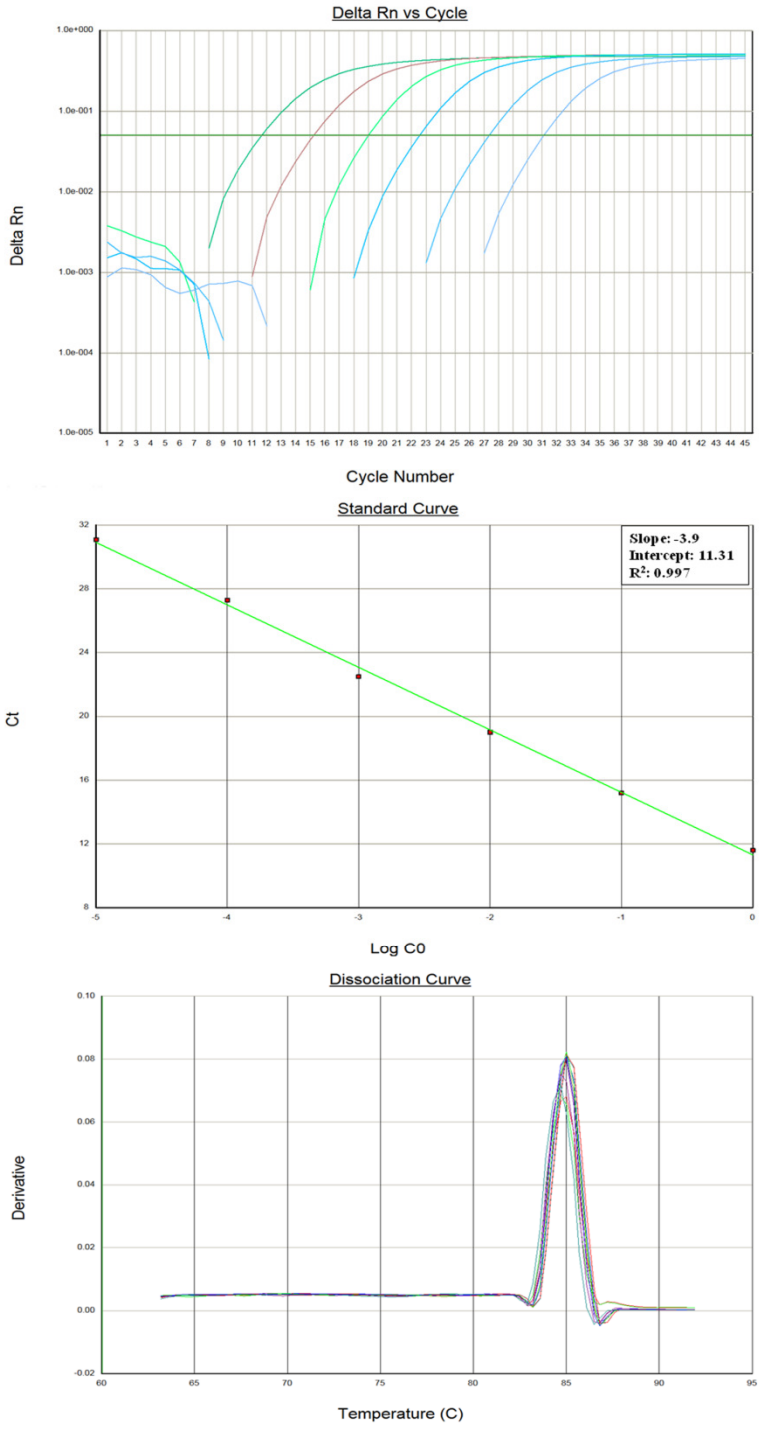


Plate 7.4 Amplification curve, standard curve and dissociation curve of *Ruminococcus flavefaciens*

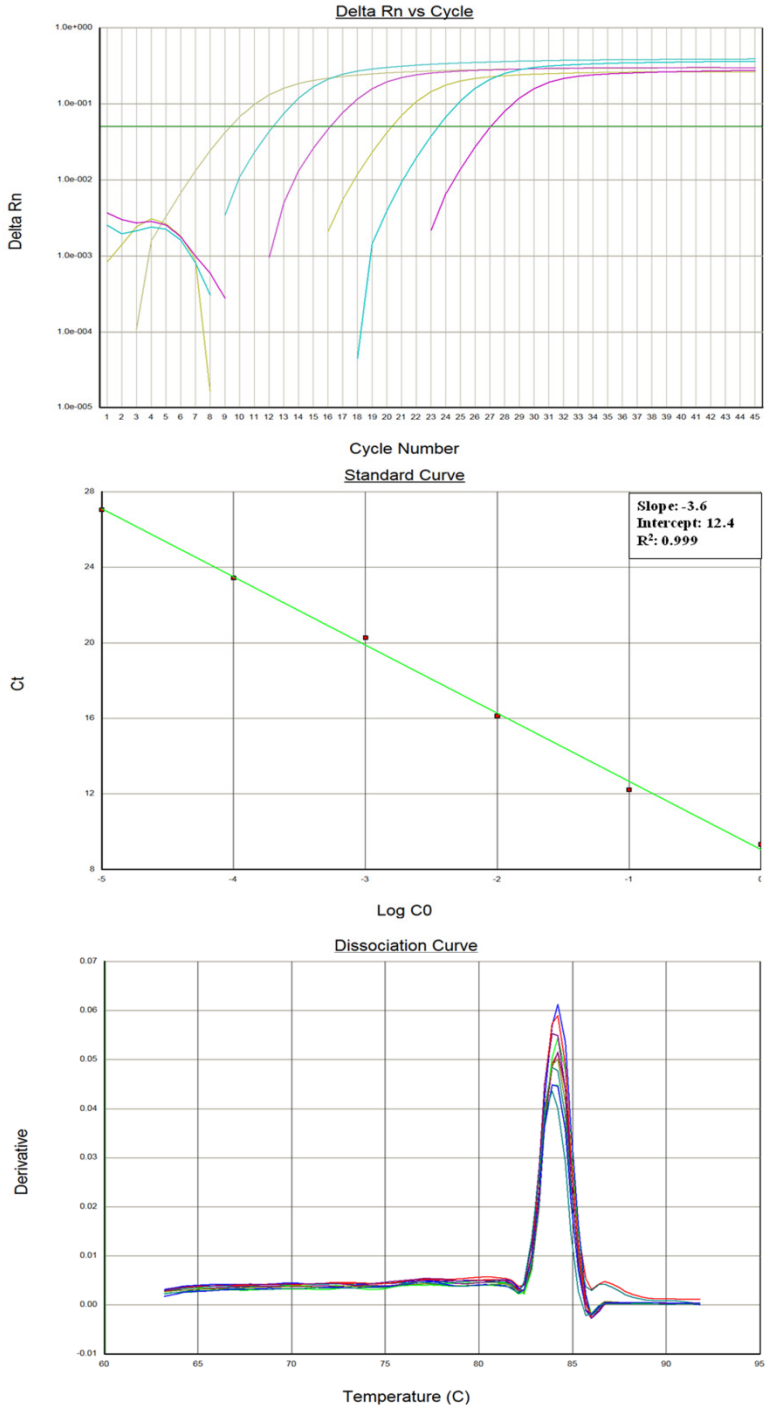
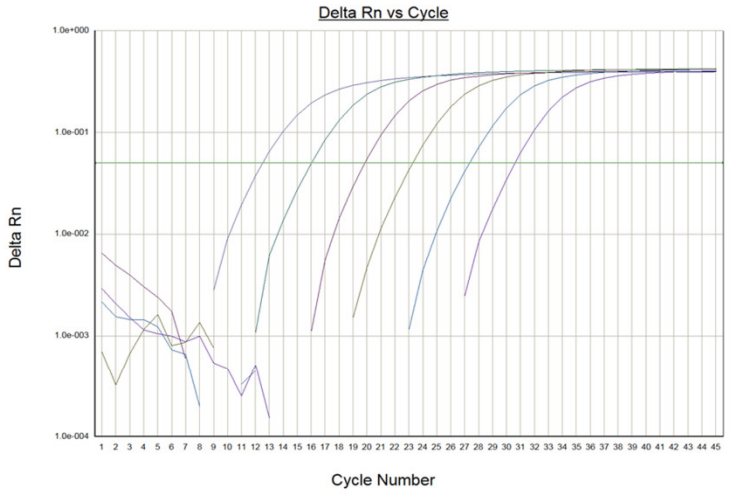
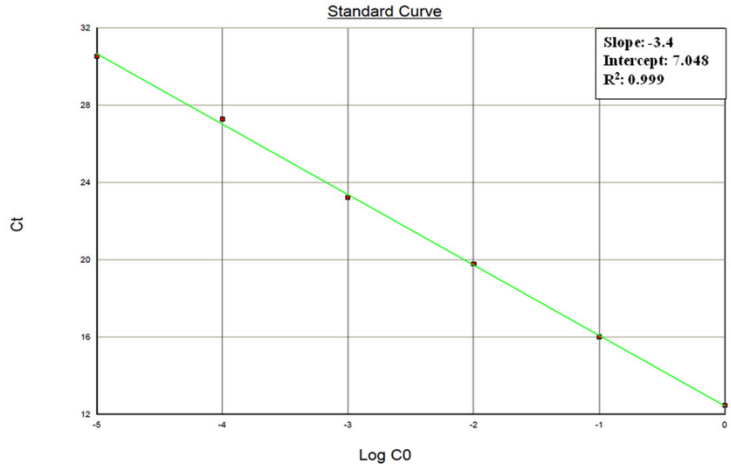


Plate 7.5 Amplification curve, standard curve and dissociation curve of *Prevotela ruminicola*





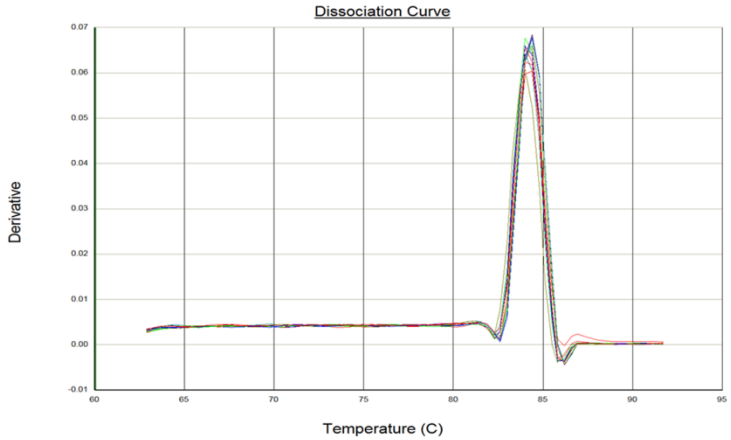


Plate 7.6 Amplification curve, standard curve and dissociation curve of *Prevotella bryantii*

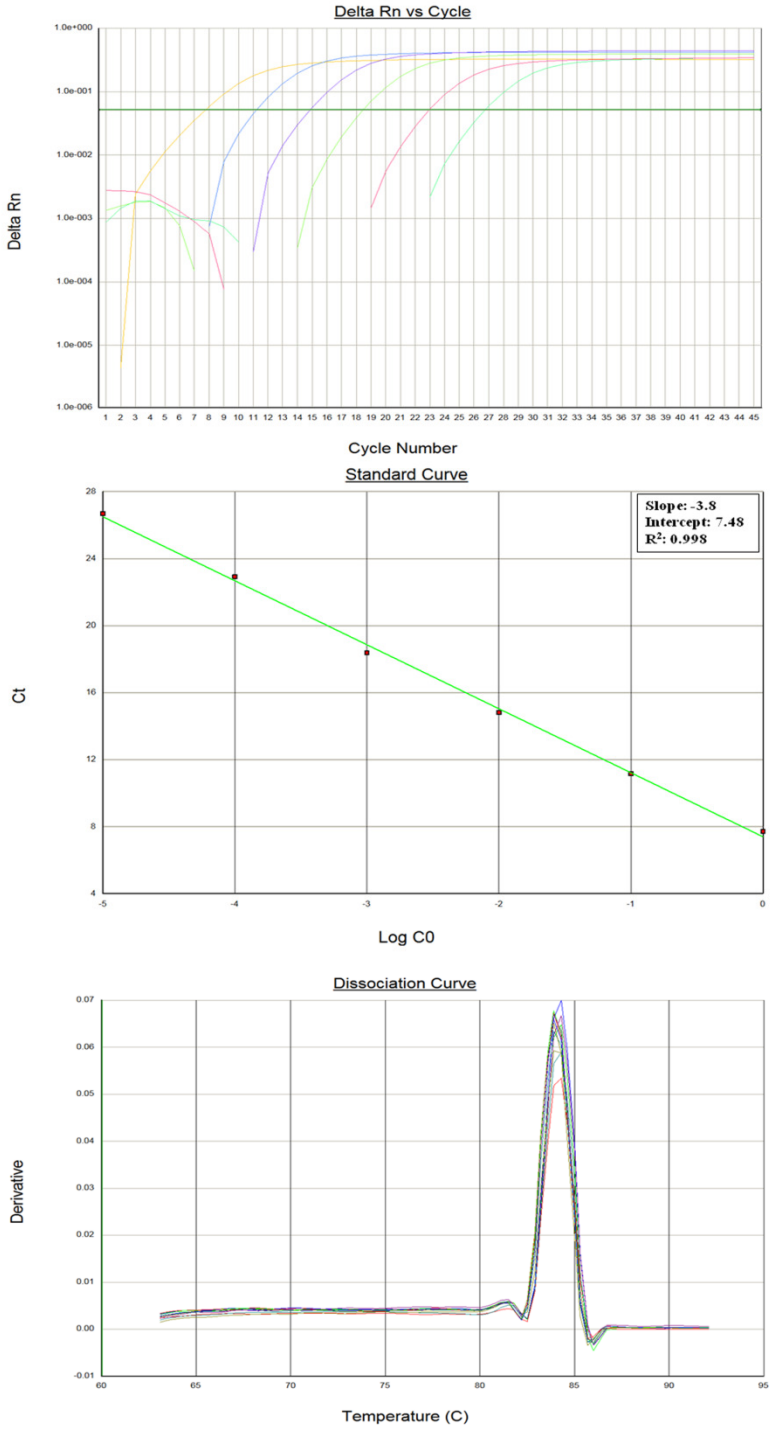


Plate 7.7 Amplification curve, standard curve and dissociation curve of *Ruminobator amylophilus*

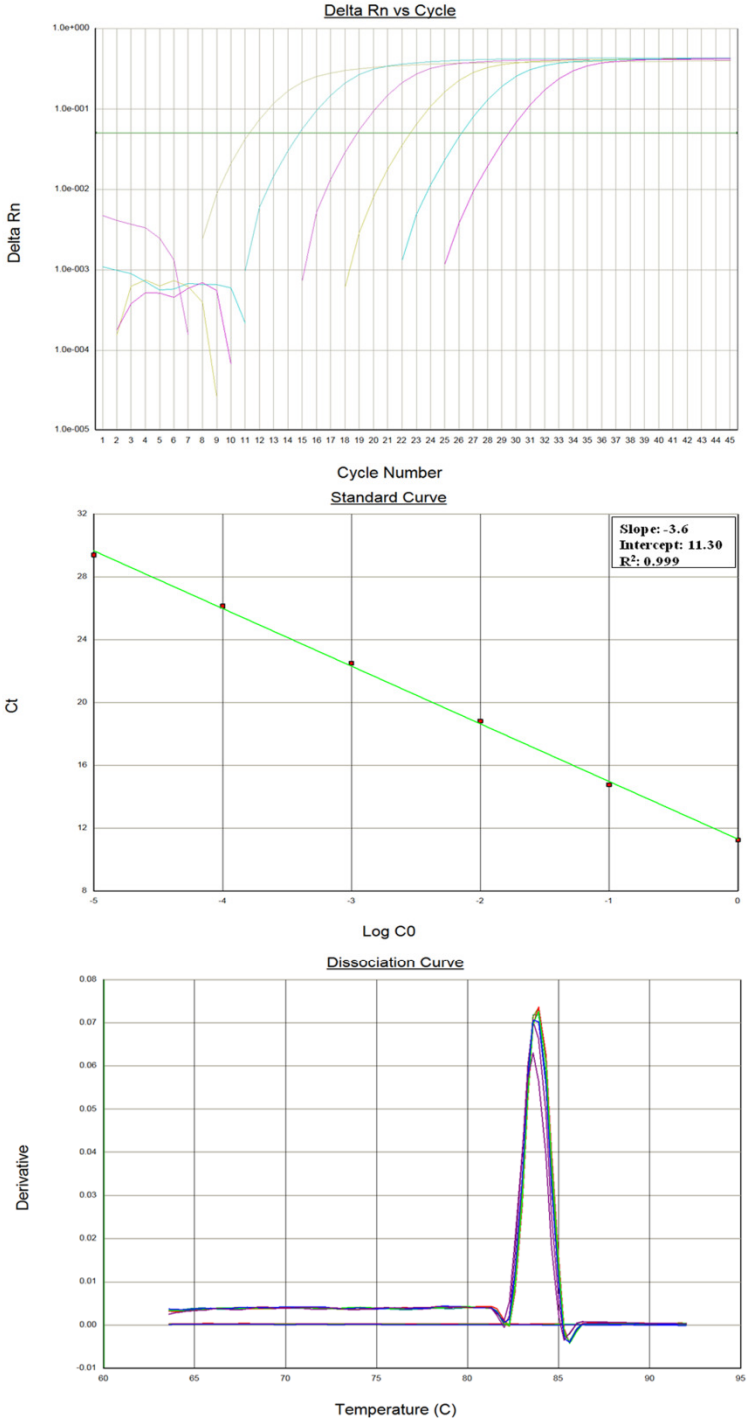


Plate 7.8 Amplification curve, standard curve and dissociation curve of *Selenomonas ruminantium*

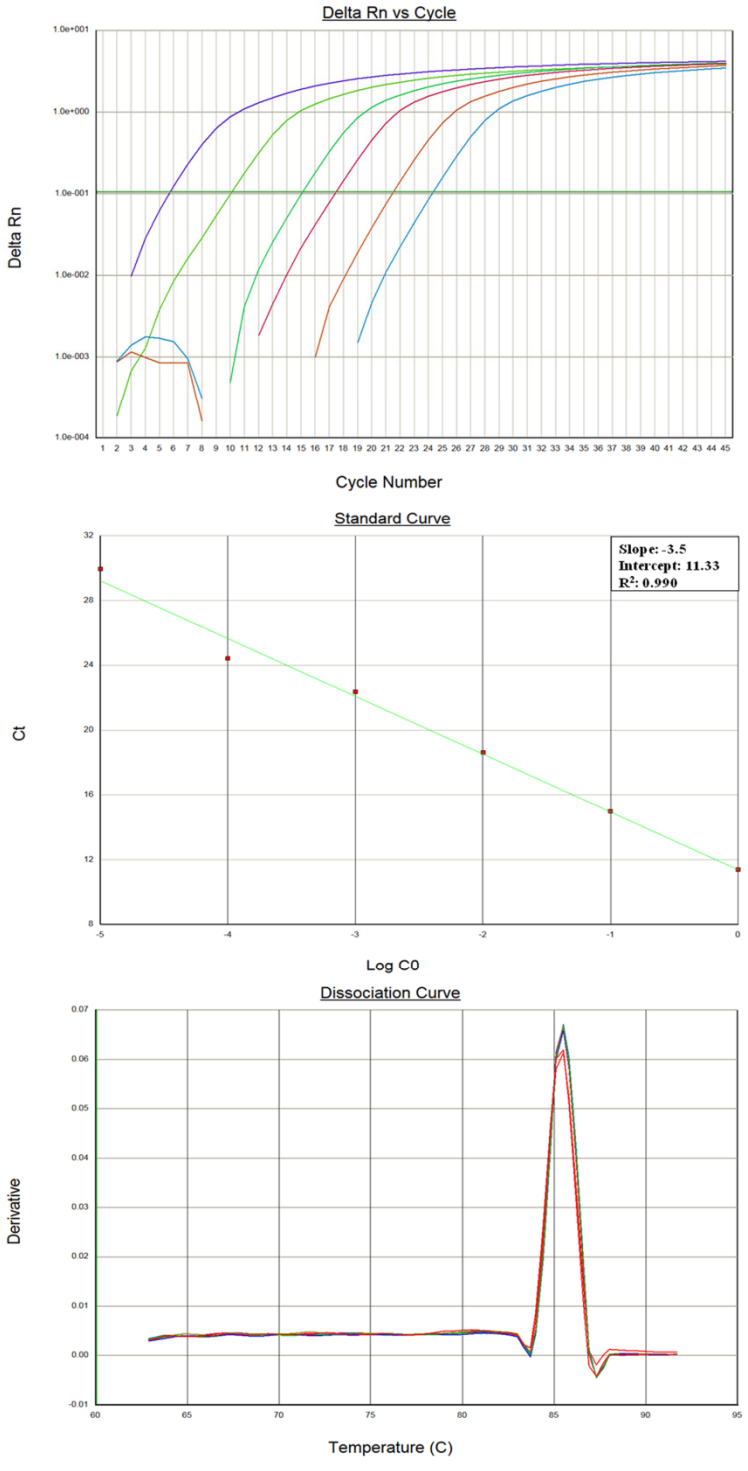


Plate 7.9 Amplification curve, standard curve and dissociation curve of *Treponema bryantii*

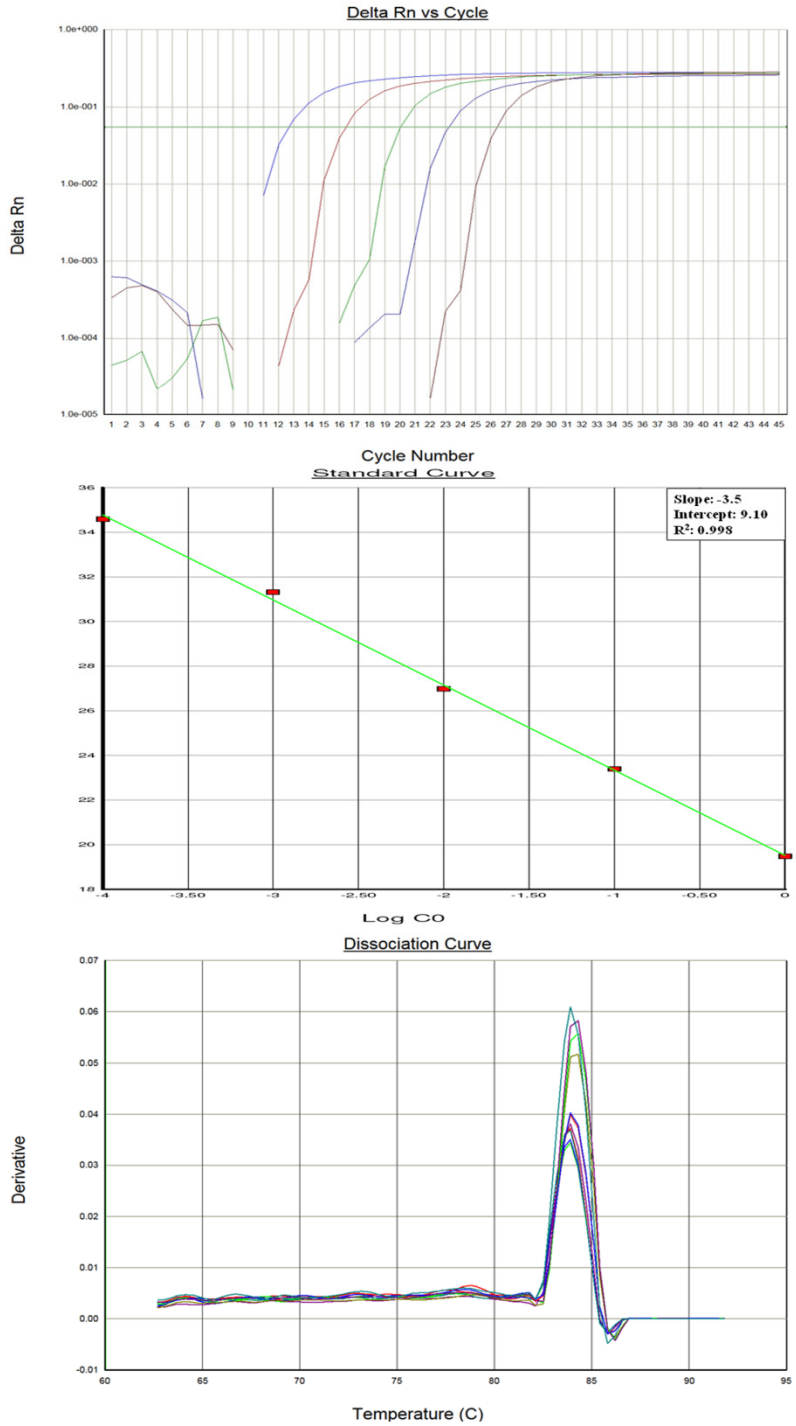
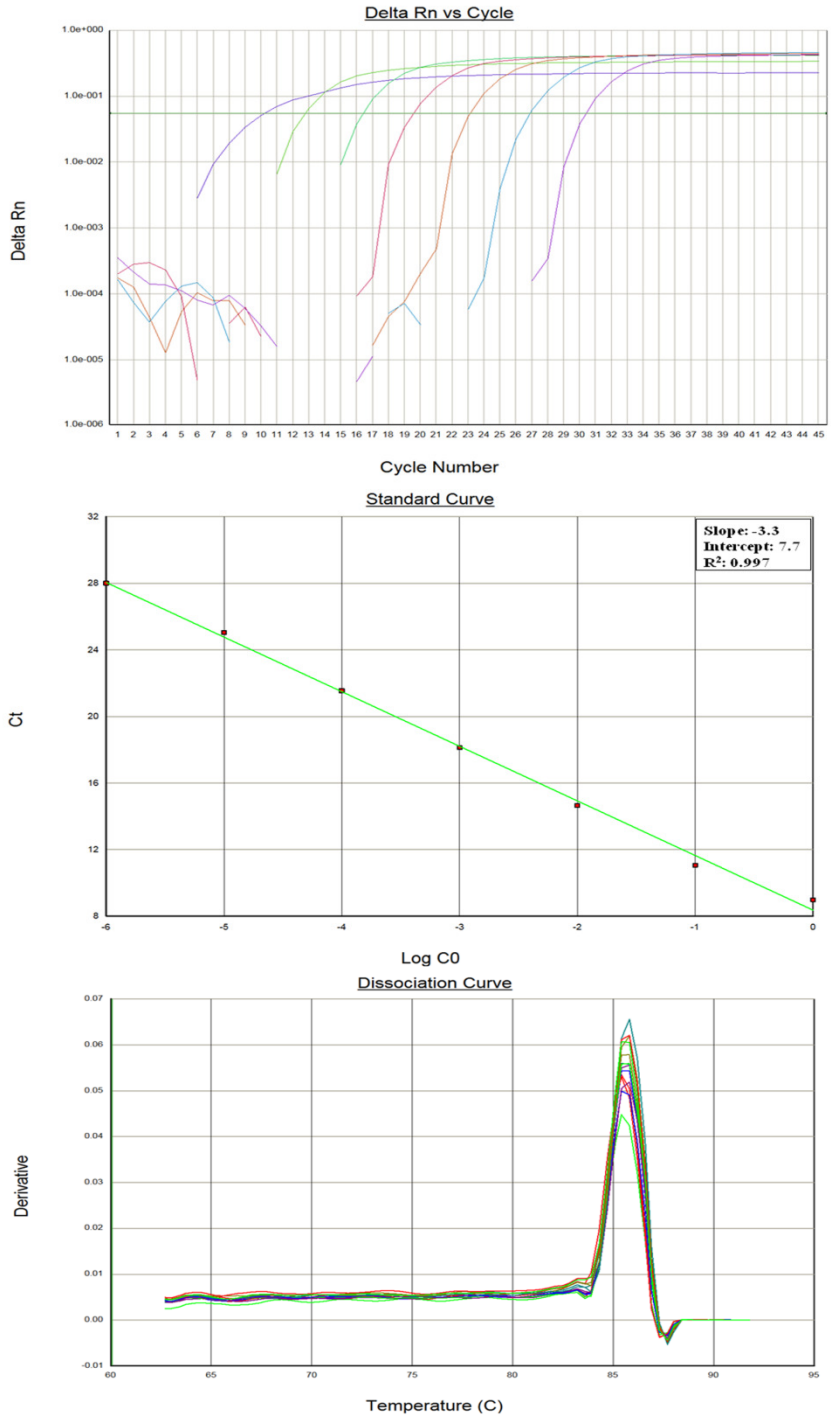


Plate 7.10 Amplification curve, standard curve and dissociation curve of *Anaerovibrio lypolytica*



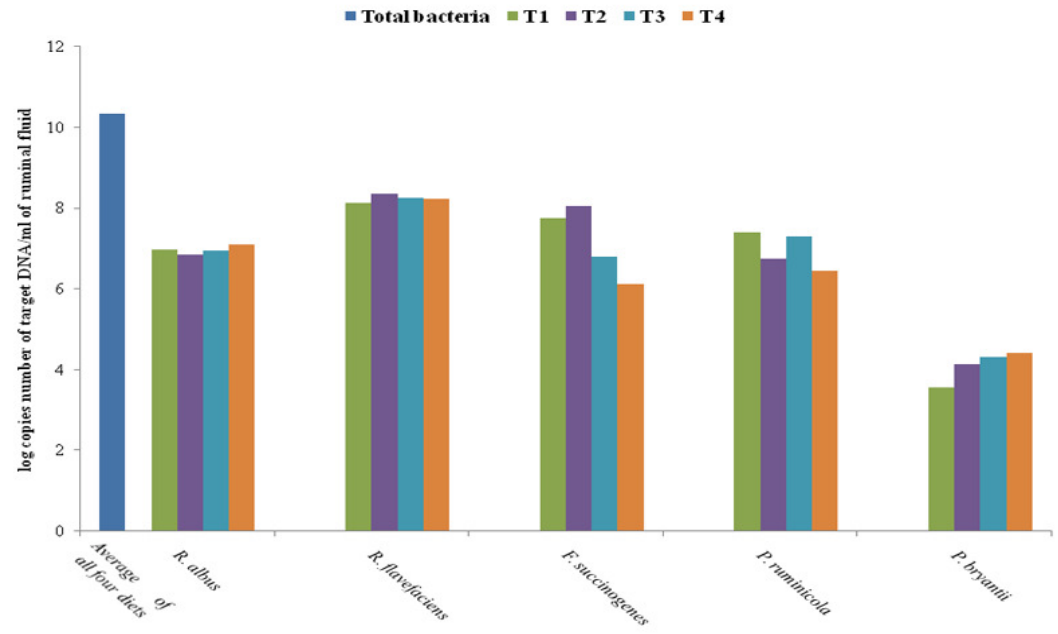


Figure 7.2. Population of the fibrolytic bacterial species of Surti buffalo fed different roughage (wheat straw)-to-concentrate ratios while values were averaged from samples taken at 0 and 2 h after feeding.

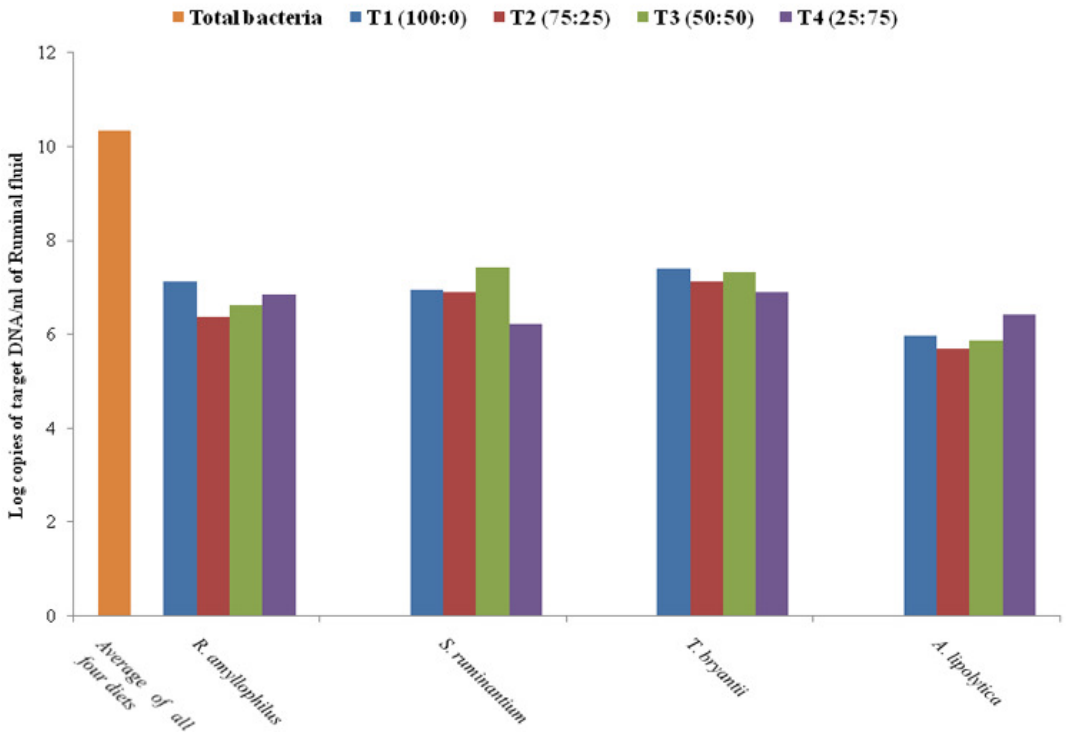


Figure 7.3. Population of the Non fibrolytic bacterial species of Surti buffalo fed different roughage (wheat straw)-to-concentrate ratios while values were averaged from samples taken at 0 and 2 h after feeding.

Table 7.2: Distribution of fibrolytic bacterial DNA from sampling hours using real-time PCR techniques.

	Species (Copies/ml[a])				
Sampling hours	*Ruminococcus albus*	*Ruminococcus flavefaciens*	*Fibrobactor Succinogenes*	*Prevotella ruminicola*	*Prevotella bryantii*
0 h	1.00×10^7	1.78×10^8	4.53×10^7	1.34×10^7	1.52×10^4
2 h	9.30×10^6	1.76×10^8	4.18×10^7	1.34×10^7	1.67×10^4
p- value	0.91	0.94	0.89	1.00	0.79

[a] The values were averaged on all the four diets

Table 7.3: Distribution of non fibrolytic bacterial DNA from sampling hours using real-time PCR techniques.

	Species (Copies/ml[a])			
Sampling hours	*Ruminobactor amyllophilus*	*Selenomonus ruminantium*	*Treponema bryantii*	*Anaerovibrio lipolytica*
0 h	2.03×10^7	7.54×10^6	1.67×10^7	1.19×10^6
2 h	9.05×10^6	1.47×10^7	1.67×10^7	1.19×10^6
p-value	0.21	0.14	1.00	1.00

[a] The values were averaged on all the four diets

Chapter 8. Microbiome of Surti buffalo (*Bubalus bubalis*) rumen ecology as affected by level of roughage, using high throughput sequencing

ABSTARCT

The complex microbiome of the rumen functions as an effective system for the conversion of plant cell wall biomass to microbial proteins, short chain fatty acids and gases. As such, it provides a unique genetic resource for plant cell wall degrading microbial enzymes that could be used in the production of biofuels. The rumen and gastrointestinal tract harbor a dense and complex microbiome. To gain a greater understanding of the ecology and metabolic potential of this microbiome, we used comparative metagenomics (phylotype analysis and SEED subsystems-based annotations) to examine randomly sampled pyrosequence data from four different feeding regime microbiomes liquid sample. Considering phylogenetic composition and metabolic potentials in the rumen may remarkably be different with respect to nutrient utilization. The distribution of phylotypes and environmental gene tags (EGTs) detected within each rumen sample were primarily the bacteroidetes/chlorobi, firmicutes and proteobacteria, consistent with previous SSU rDNA libraries of the Surti buffalo rumen microbiome.

Key words: MG-RAST, subsystem, pyrosequencing, Surti buffalo, SEED.

INTRODUCTION

Livestock production in India is subsidiary to plant agriculture. In tropical countries, the ruminant are fed on lignocellulosic agriculture by-products like cereal straws, tree foliages etc, and cakes of oil seeds, and digest such plant materials by virtue of the efficient microbial process. The microbiome inhabiting the rumen is characterized by its high population density, wide diversity, and complexity of interactions. Bacteria predominate the rumen, with a variety of anaerobic protozoa and fungi (Hespell *et al*., 1997), and the associated occurrence of bacteriophage is

well documented (Klieve and Bauchop, 1988). The use of small subunit (SSU) rRNA sequence analysis has allowed a more complete description of rumen microbiome and these inventories have demonstrated that a large microbial component remains uncultured (Tajima *et al*., 2001a) and that a high proportion of the fibrolytic population has not been thoroughly described (Larue *et al*., 2005). The rumen habitat contains a consortium of microbes that harbor the complex lignocellulosic degradation system for the microbial attachment and digestion of plant biomass. However, the complex chemical processes required to break down the plant cell wall are rarely carried out by a single species. Evidence also suggests that the most important organisms and gene sets involved in the most efficient hydrolysis of plant cell wall are associated with the fiber portion of the rumen digesta (Forsberg *et al*., 1997).

Bovine rumen provides a unique genetic resource for the discovery of plant cell wall-degrading microbial enzymes for use in biofuel production, presumably because of coevolution of microbes and plant cell wall types. There are, however, limitations to metagenome mining (Henne *et al*., 1999), and the number of clones needed to represent the entire metagenome is staggering (Handelsman *et al*., 1998). Nonetheless, this approach does allow one to begin to harvest the remarkable and vast diversity present in a given metagenome (Gill *et al*., 2006).

The sequencing of the genomes from several hundred microbial and numerous eukaryotic species has laid the foundation for generating genomic sequence data from whole environments avoiding a culturing step. This approach, also known as ‘‘metagenomics’’, is defined as the genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms (Handelsman, 2004). Pyrosequencing is the base for a promising new generation sequence technology developed by 454 Life Sciences Technologies (http://www.454.com/) (Margulies *et al*., 2005) and is now being applied to metagenomics. One approach has been the use of the pyrosequence technology to increase the depth of SSU rDNA surveys by sequencing amplicons from the variable region of the SSU molecule. This has been applied to ocean microbial samples (Sogin *et al*., 2006), soils (Roesch *et al*., 2007) and human (Larsen *et al*., 2010). The second approach uses random sample

pyrosequencing to generate environmental gene tags (EGTs) and protein families (Tringe *et al*., 2005) from microbiomes. This approach, applied to environmental biomes (Edwards *et al*., 2007), allows one to highlight significant differences in metabolic potential in each environment.

Massive depth metagenomic sequencing is an invaluable complement to what has already been learned about lignocellulose degradation in the rumen. With help of this technology, buffalo rumen (Surti) can be fistulated, this allows for simple and rapid sampling strategies, and changes in diet and management can be easily implemented for metagenomic investigations on the microbial community and metabolic potential. Presented in this study is a comparative metagenome analysis of Surti buffalo microbiome by using the inexpensive, massively parallel, and rapid method of pyrosequencing.

MATERIALS AND METHODS

Rumen Sampling and DNA extraction

Experimental material consisted of Surti buffaloes of 2-3 years of age divided in to four groups with two animals in each group. Samples of whole rumen contents were obtained from four groups, each group was given different treatment namely T1, T2, T3 and T4 averaging 200 kg of weight that were housed at the Department of Animal Nutrition, College of Veterinary Science and A. H. Anand. The permission of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) was obtained prior to initiation of the study. The all animals were received four different total mixed ration (TMR) of roughage to concentrate (R: C) of 100:0, 75:25, 50:50, and 25: 75 respectively in individual feeding stall. All animals received feed according to respective R: C ratios *ad lib* daily and roughage was in form of wheat straw. Concentrates were high-quality, low-fiber feeds that contain a high concentration of digestible energy per unit weight and volume. Under this study, the concentrate diet consisted of 20.11% crude protein, 10.28 % crude fibre, 3.8 % ether extract, 52.43 % nitrogen free extract, 13.38 % ash, 3.38 % silica, 1% phosphorus and 1.22% calcium. All Animals were let loose daily for 2hrs in the morning and evening during which they had free access to drinking water under

control condition. The experiment was conducted for all groups for a period of 30 days. Sample of the rumen liquor (500 ml) was collected separately from all group of animals on 30th day of experiment after 2 hrs of morning feeding by a suction pump using a flexible stomach tube as described earlier by (Khampa *et al*., 2006). About 100 ml rumen fluid was passed through four layers of cheese cloth to remove particulate matter. Remaining rumen fluid was stored at -80°C for further study. Total genomic DNA was extracted from four groups by using a protocol similar to the extraction of high molecular weight DNA for rumen separately with little modification (enzyme-chemical lysis method). DNA purity and concentration was analyzed by spectrophotometric quantification and gel electrophoresis.

Pyrosequencing and Sequence Analysis

Four samples were subjected to a single pyrosequence run by 454 Life Sciences by using a 454 Life Sciences technology based high throughput sequencer (GS FLX 454 Life Sciences). In brief, the about 10-15µg each samples were nebulised to generate smaller fragments of size 500-800 bp and the fragments were processed as described by manufacturer (Roche GS FLX Titanium Sequencing Method Manual) to apply adaptors on both the end, emulsion PCR and pyrosequencing. The detail protocol is given in appendix B. Sequencing was carried out for 200 cycles with the flow of A, T, G and C nucleotides sequentially and image capture. Capture images were processed by image processing software. The data were analyzed using the SEED annotation engine (http://seed.sdsu.edu/FIG/index.cgi) (Overbeek *et al*., 2005) under metagenome rapid annotation using subsystem technology (MG-RAST). The sequences were compared using the BLASTX algorithm with an expected cut off of $1x10^{-5}$ (Edwards *et al*., 2006). The BLASTN algorithm ($E<1\ X10^{-5}$ and a sequence length hit >50 nt) was used to identify small sub unit rRNA (SSU rRNA) genes from the Ribosomal database project (RDP database) (Cole *et al*., 2005); (http://rdp.cme.msu.edu/), and the European Ribosomal RNA database (http:// www.psb.ugent.be/rRNA/index.html).RDP was used for robust Bacterial classification and the European Ribosomal RNA database was used to classify eukaryal and archaeal sequences. The metagenomes used in this article are freely

available from the SEED platform (MG-RAST). The IDs used in this study are: 4445089.3, 4445091.3, 4445093.3 and 4446901.3.

RESULTS AND DISCUSSIONS

In order to better understand the functional gene content and metabolic potential of the microbial community in rumen of Surti buffaloes, we undertook comparative metagenomic strategy of four groups using 454 pyrosequencing. The overall goal of this study was to obtain a detailed characterization of the rumen microbiome, with respect to both phylotype (ribosomal DNA gene tags) and functional content (environmental gene tags; EGTs) of Surti buffalo in four different feeding regimes. Dissimilar relationships (Table 8.1) were seen for SSU rDNA hits against the Ribosomal Database Project (Bacterial SSU rDNA) and against European Ribosomal RNA databases (Archaeal and Eukaryotic SSU rDNA) in all four groups may be due to unequal distribution of contig numbers (experimental bias). The number of SSU rDNA hits in the Surti buffalo metagenomic libraries were very rare (0.02% in T1, 0.21% in T2, 0.04% in T3 and 0.13% in T4 for bacteria) (Table 8.1). Although few SSU rDNA hits has been also reported by Brulc *et al*., (2008) in cattle rumen microbiome. Unfortunately, no SSU rDNA hits were observed in Archaea (European Ribosomal Database) and Eukarya (European Ribosomal Database) in our microbiomes. Although 3 sequences of T2 group (0.0295%) were hit with SSU rDNA database of other than bacteria, archaea and eukarya (Table 8.1).

Further insights into the diversity within the four different treatment groups of buffalo rumen metagenomic samples were obtained by comparing the number of EGTs (E value $<1X10^{-5}$) of different bacterial phyla. (Figure 8.1). Sequence length is one of the primary factors in assessing similarity between sequences, and BLAST E values are dependent on both the query sequence lenght and the size of the database to which they are being compared (Altschul *et al*., 1990). However, this will affect the number of significant sequences found in the searches by a factor of two or more (Wommack *et al*., 2008), pyrosequencing yielded more sequence than comparable Sanger sequencing, more than compensating for these missing sequences. The sequences missed in our searches are expected to be randomly distributed, and

therefore, we are not expected to skew the comparative analysis. Indeed, while classifying EGTs from pyrosequencing reads has been challenging, a recent report demonstrates that EGTs as short as 27 amino acids can accurately be classified with an average specificity ranging from 97% for Superkingdom to 93% for Order (Krause *et al*, 2008).

Bacterial specific EGTs represented approximately 70.73% in T1, 83.68% in T2, 79.24% in T3 and 64.88% in T4 of the total EGTs (Table 8.1) and the distribution of phylotypes fell predominantly into the bacteroidetes/Chlorobi, firmicutes and proteobacteria groups, regardless of the microbiome analyzed (Figure 8.1). The distribution of EGTs from the bacteria is congruent with the distribution of SSU rDNA phylotypes, as was found with the Soudan Mine, cattle rumen and chicken microbiome studies (Edwards *et al*., 2006; Brulc *et al*., 2008; Qu *et al*., 2008). Similar observations were also found in our previous study in chicken gut microbiome and goat rumen microbiome (data unpublished) by high throughput sequencing. Archaeal EGTs constituted approximately 0.5% of EGTs in T1, 1.69 % of EGTs in T2, 1.34% of EGTs in T3 and 1.48% of EGTs in T4 of the total EGTs, respectively (Table 8.1), matching well with previous estimates of archaea numbers in the adult chicken cecum microbiome and cattle rumen (Saengkerdsub *et al*., 2007a; Brulc *et al*., 2009). The majority of archaeal EGTs correspond to methanogenic classes with the largest proportion corresponding to the Euryarchaeota. Eukaryotic EGTs were 17.02 %, 4.93%, 10.25% and 17.19 % of total EGTs in T1, T2, T3 and T4 respectively (Table 8.1). The majority of eukarya ETGs were fungi/ metazoan group. These EGT proportions were expected from our current knowledge of the rumen microbiome community structure. Interestingly, few EGTs in the category "other" were also recovered in present microbiome with similar passion in all four groups (Table 8.1).

Virus EGTs were very rare and primarily composed of dsDNA viruses in Surti rumen microbiome. The lack of viral sequences may be because of their extensive diversity and limited representation in public databases. Additionally, viral sequences may have been overlooked because they were not enriched in fluid during sampling procedures.

The subsystems-based annotations (SEED) database (MG-RAST) was utilized to gain a better understanding of these phylogenetic trends and to predict the metabolic potential (content of EGTs) of these microbiomes (Figure 8.2)**.** The EGT proportions were also expected from our current knowledge of the rumen microbiome community structure. The subsystems are annotated across genomes and are based on biochemical pathways, fragments of pathways, and clusters of genes that function together, or any group of genes considered to be related. Much of this analysis is dependent on sequence databases, and while we tried to avoid database bias by using multiple databases and alternative querying algorithms for analysis, we are aware that some sequences have no matched relatives in the databases, or are over-represented in the databases. Further, sequence similarity does not always mean functional similarity and this may influence the interpretation of our results as minor sequence dissimilarities may represent functionaly different or even a completely new functions. Figure 8.2 shows the metabolic profile (subsystems) of all four rumen microbioms (T1, T2, T3 and T4). The distribution of subsystems is strikingly different for the T1 (100 % roughage) microbiome from the rumen of T4 (25 % roughage), which is predominated with metabolisms consistent with a community that has shifted away from a roughage digesta, more easily fermentable carbohydrate-based metabolism. It appears that animal of group T4 had not adapted to the higher-fiber diet. This is consistent with the metabolism represented by the Gammaproteobacteria. Whereas most of the Gammaproteobacteria sequences were most similar to sequences from *Psychrobacter*-like organisms (from arctic samples), they probably are not from this genus, but rather from a close relative (Bozal *et al*., 2003). Our analysis were also similar with other microbiome (Dinsdale *et al*., 2008 ; Qu A *et al*., 2008), the surti rumen microbiomes are dominated by carbohydrate metabolism in high fiber diet (100% roughage), lowest in high concentrate diet (75 % concentrate), and are sparsely populated with genes for respiration, reflecting the more stable anoxic environment in the gastrointestinal tract (Figure 8.3).

When looking solely at the Surti rumen microbiome and the SEED carbohydrate subsystem of all four groups, the central carbohydrate metabolism had higher level (26 % in T1 group) than T4 group (14%) of the EGTs in this subsystem,

while population size of monosaccharides EGTs were less 22 % in T1 and higher 35% in T4. However the carbohydrate EGTs of T2 group and T3 were similar proportion. This may be because of rumen pH together with microbial population, nature of substrates, environmental factors such as temperature, and the existence of cations and soluble carbohydrates have been suggested as factors governing bacterial attachment (Miron *et al*., 2001). Ruminal pH is one of the most important of these factors, because the fibrolytic bacteria numbers are very sensitive to the pH change (Sung *et al*., 2007). When ruminants are fed fiber- deficient rations, ruminal pH declines, microbial ecology is altered, and the animals become more susceptible to metabolic disorders (Russell and Rychlik, 2001).

While a limitation of the random sample pyrosequencing approach is the resulting short read lengths, we were able to assemble all of these reads into 280 contigs of >500 nucleotides (39 from T1, 17 from T2, 187 from T3 and 37 from T4). The summary of blastx results of all assembled contigs from four treatments are given in table 8.2 to 8.5. Translations of these contigs (EGTs) were used for BLASTX analysis. The distributions of these contigs from four groups are shown in Figure 8.4. The majority of these translations showed similarity with genes from the bacteroidetes, firmicutes and actinobacteria, the dominant taxa from this four microbiome. Similar observations have been also reported by Brulc *et al*., (2009) in cattle rumen and Q *et al*., (2008) in chicken ceacum microbiome.

BLASTX analysis indicates all contigs of all four microbiome shared sequence similarities (27 to 100%) with different known organisms from the bacteroidetes, firmicutes, actinobacteria, proteobacteria and spirochetes etc. confirming the results from the non-assembled data and many contigs had sequence similarity with hypothetical proteins found in this four microbiome. However, four contigs (one from T1 and three from T2 group) were not showing any matching with database, due to biasness in assembly process or may be novel.

Metagenomic analysis allows the relative abundances of all genes to be determined and used to generate a dataset for the assessment of the functional potential of each community (Edwards *et al*., 2006; Wegley *et al*., 2007; Fierer *et al*., 2007). Our exercise to assemble genes from primarily the bacteroidetes/chlorobi

group suggests that this is an important phylum in the Surti rumen, similar to that observed in studies of the human faecal microbiome (Gill *et al*., 2006; Kurokawa *et al*., 2007).

CONCLUSIONS

We conclude that the microbiome datasets presented herein represent the first assessment of the metabolic potential of the Surti rumen microbiome at the level functional gene content. As such, they represent a baseline for future studies and will be of great use in understanding the large, complex, and dynamic microbial community of the rumen fluid, the composition of which ultimately reflects the co-evolution/selection of microbes with their host and diet. It is clear that the composition and function the microbiome can be affected by various factors such as dietary ingredients, nutrient levels, environment, probiotic, and antibiotic treatments. Moreover, the gastrointestinal tract microbiome plays an important role in the growth and health of the host through its effects on gastrointestinal tract morphology, nutrition, pathogenesis of intestinal diseases, and immune responses. This comparative microbiome data provides a critical genetic context for understanding animal nutrition, animal health and well-being. Additionally, the combined pyrosequence approach and subsystems-based annotations available in the SEED database allowed us to gain an understanding of the metabolic potential of these microbiomes. Sequence information was recovered in a comparative context based on the ecology of the microbial communities that inhabit the Surti rumen, which in the future will allow us to link metabolic potential to the identity of rumen microbes in their natural habitat. Also, further study of glycobiome of different rumen bacterium to be needed in high polysaccharide- rich environment adaptation.

Table 8.1: Summary of pyrosequencing data obtained from four different Surti buffalo (*Bubalus bubalis*) rumen samples

Parameters	**T1**	**T2**	**T3**	**T4**
Number of sequences	6,220	21,290	18,125	6228
Total length of sequences	1,762,476	8,011,603	5,928,355	1,942,714
Average length of sequences (bp)	283	376	327	311
Total coding sequences (EGTs)	1,922	10,169	7,172	1.623
(% of total sequences)	(32.03%)	(47.76%)	(39.57%)	(26.06%)
Archaea EGTs	10	172	96	24
(% of total EGTs)	(0.50%)	(1.69%)	(1.34%)	(1.48 %)
Bacteria EGTs	1409	8509	5683	1053
(% of total EGTs)	(70.73%)	(83.68%)	(79.24%)	(64.88%
Eukarya EGTs	339	501	735	279
(% of total EGTs)	(17.02%)	(4.93%)	(10.25%)	(17.19%)
Other	234	987	658	267
	(11.75%)	(9.71%)	(9.17%)	(16.45%)
SSU DNA hits (% of total sequences)				
Bacteria (Ribosomal Database Project)	1 (0.02%)	41(0.21%)	7 (0.04 %)	8 (0.13 %)
Archaea (European Ribosomal Database)	0	0	0	0
Eukarya (European Ribosomal Database)	0	0	0	0
Other	0	3 (0.0295%)	0	0

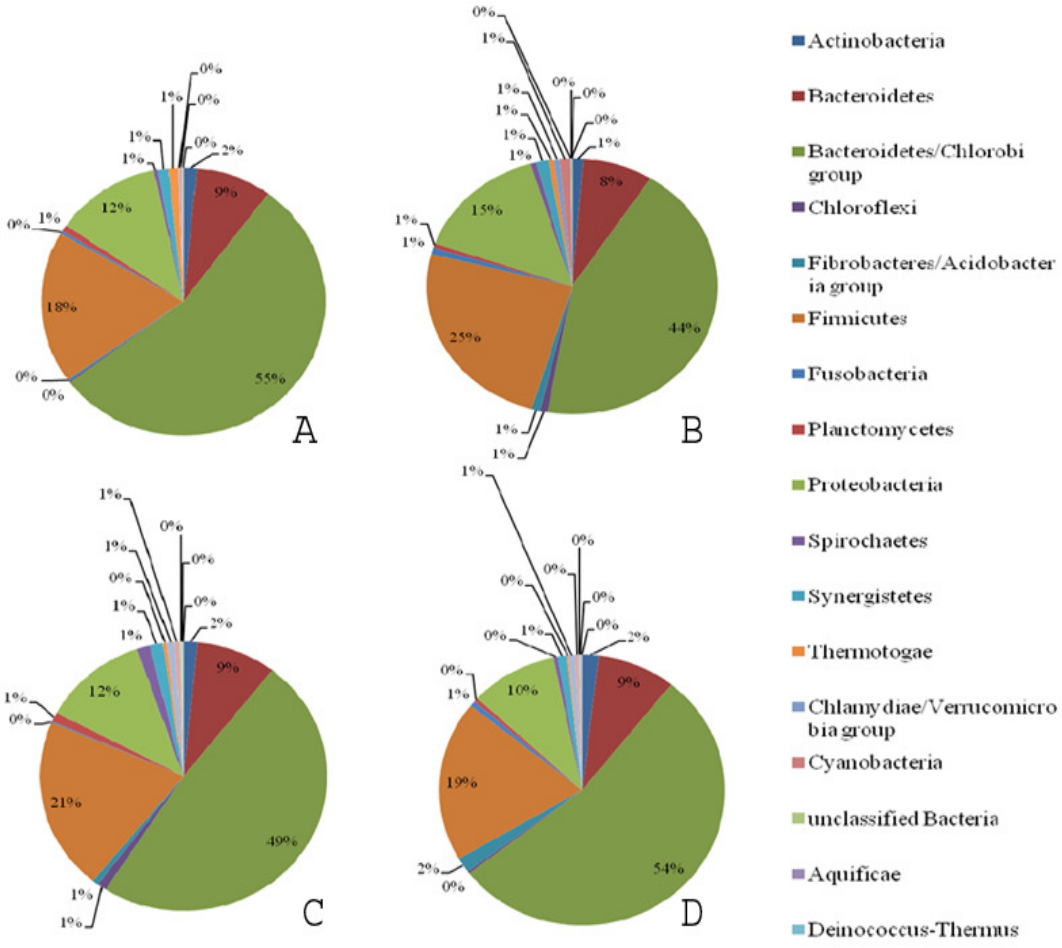


Figure 8.1. Phylogenic composition of bacteria phyla from four pyrosequnce environmental gene tags (EGTs).The percent of sequences in each of the bacterial phyla from A; (T1, 100% roughage), B; (T2, 75% roughage), C; (T3, 50 % roughage) and (T4, 25 % roughage) microbiomes are shown.The BLASTX cutofff of EGTs is 1x 10^{-5} with a minimum length of 50 bp.

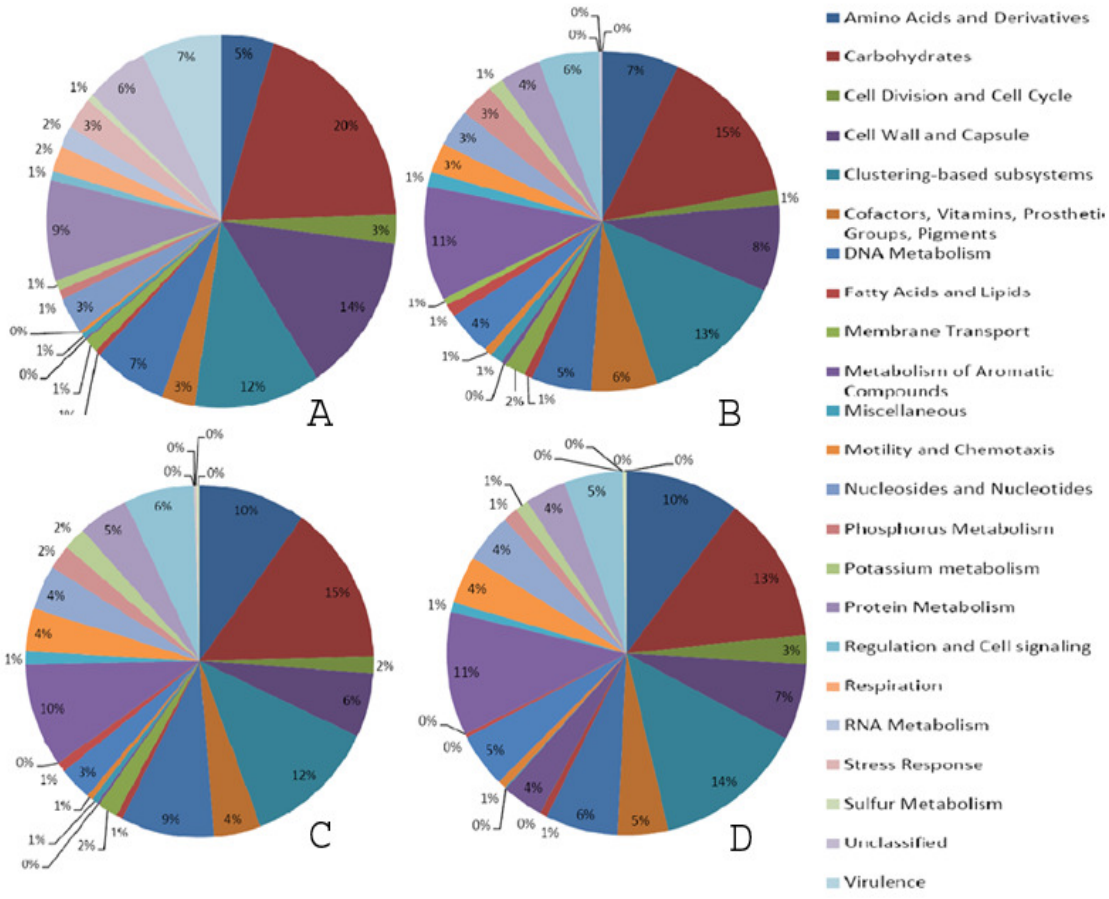


Figure 8.2. SEED subsystem composition of four rumen microbiome A (T1; 100% roughage), B (T2; 75% roughage), C (T3; 50 % roughage) and D (T4; 25% roughage) are shown. The percent of environmental gene tags (EGTs) of the SEED subsystems from the rumen microbiomes (T2) is shown. The BLASTX cut off for EGTs is 1x 10^{-5}.

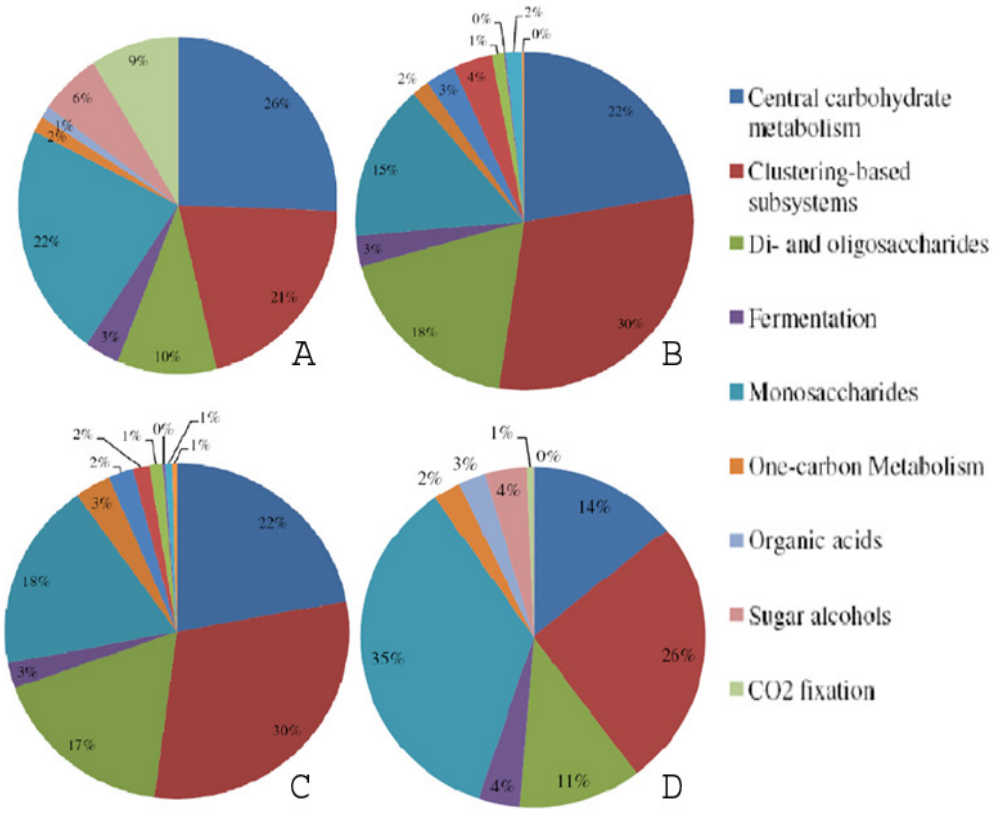


Figure 8.3. Carbohydrate Metabolism subsystem composition of four rumen microbiomes in A (T1; 100 % roughage), B (T2; 75 % roughage), C (T3; 50 % roughage) and D (T4; 25 % roughage) are shown. The percent of environmental gene tags (EGTs) in each of the carbohydrate metabolism subsystems from the rumen microbiomes is shown. The BLASTX cut off for EGTs is 1x 10^{-5}.

Table 8.2: Summary of blastX results of T1 assembled contigs.

Contig	Length (bp)	Gene bank	Annotation	Organism	Score	E value	Identity (%)
00073	503	ZP03990966	Hypothetical protein HMPREF61230905	*Oribacterium sinus* F0268	34.7	2.1	37
00116	648	ZP05544164	conserved hypothetical protein	*Parabacteroides sp.* D13	37.7	0.47	41
00124	533	YP001330061	hypothetical protein MmarC70843	*Methanococcus maripaludis* C7	35.0	1.9	27
00171	505	YP003469362	D-alanine:D-alanine-adding enzyme	*Xenorhabdus bovienii* SS-2004	36.6	0.56	31
00208	527	ZP02733839	hypothetical protein GobsU18692	*Gemmata obscuriglobus* UQM 2246	32.7	7.9	29
00235	513	UQM 2246	abortive infection protein	*Chlorobium phaeobacteroides* DSM 266	60.4	0.021	69
00247	520	ZP01091301	hypothetical protein DSM364505500	*Blastopirellula marina* DSM 3645	60.5	4e-08	29
00291	507	YP002398034	Major tail protein V	*Escherichia coli* ED1a	33.1	6.0	41
00294	550	ZP05394230	amidohydrolase	*Clostridium carboxidivorans* P7	41.6	0.017	55
00304	507	ZP02424760	hypothetical protein ALIPUT00888	*Alistipes putredinis* DSM 17216	102	8e-21	43
00307	528	YP001866379	multi-sensor signal transduction histidine kinase	*Nostoc punctiforme*	34.7	2.1	32
00320	515	YP158770	exonuclease SbcD	*Aromatoleum aromaticum* EbN1	53.1	6e-06	67
00331	501	YP001997233	protein of unknown function DUF323	*Chloroherpeton thalassium* ATCC	78.9	9e-08	71
00333	505	not available	not available	not available	-	-	-

00335	501	ZP03012389	hypothetical protein BACCOP04328	*Bacteroides coprocola* DSM	87.4	3e-16	40
00337	508	YP003574439	hypothetical protein PRU1112	*Prevotella ruminicola* 23	163	3e-39	98
00348	673	ZP04292677	hypothetical protein bcere000955340	*Bacillus cereus* R309803	132	1e-11	76
00358	525	YP001305132	hypothetical protein BDI3826	*Parabacteroides distasonis* ATCC 8503	32.7	7.9	30
00364	513	YP003574722	putative lipoprotein	*Prevotella ruminicola* 23	147	3e-34	48
00365	528	YP001955463	hypothetical protein BLD1520	*Bifidobacterium longum* DJO10A	52.8	7e-06	37
00366	513	YP003048446	protein of unknown function DUF853 NPT hydrolase putative	*Methylotenera mobilis* JLW8	213	3e-54	65
00370	683	ZP03717680	hypothetical protein EUBHAL02765	*Eubacterium hallii* DSM 3353	182	2e-38	63
00386	500	YP001815416	type III restriction protein res subunit	*Exiguobacterium sibiricum*	61.2	2e-08	33
00387	522	ZP02041468	hypothetical protein RUMGNA02237	*Ruminococcus gnavus* ATCC	55.5	1e-06	37
00388	503	ZP03758781	hypothetical protein CLOSTASPAR02802	*Clostridium asparagiforme*	63.9	3e-09	44
00399	506	YP003608269	hypothetical protein BC10024793	*Burkholderia sp*. CCGE1002	38.9	0.11	43
00416	519	ZP04195047	Fibronectin type III domain protein	*Bacillus cereus* AH676	43.1	0.006	27
00419	539	YP001660983	hypothetical protein MAE59690	*Microcystis aeruginosa* NIES-843	34.7	2.1	32
00422	513	ZP05403861	chlorohydrolase family protein	*Mitsuokella multacida* DSM 20544	182	8e-45	67
00435	516	ZP04169881	TPR domain protein	*Bacillus mycoides* DSM 2048	36.2	0.71	32

00440	506	NP519917	putative N-acetylmuramoyl-L-alanine amidase protein	*Ralstonia solanacearum* GMI1000	38.5	0.14	42
00442	518	ZP06604206	conserved hypothetical protein	*Selenomonas noxia* ATCC 43541	42.0	0.013	48
00445	510	ZP05986025	retrovirus-related Pol polyprotein LINE-1	*Neisseria subflava* NJ9703	91.7	1e-17	57
00448	549	YP741225	thiamine-monophosphate kinase	*Alkalilimnicola ehrlichii* MLHE-1	35	1.6	40
00455	510	ZP05623640	RIP metalloprotease RseP	*Treponema vincentii* ATCC 35580	34.7	2.1	27
00458	504	ZP05758666	glycyl-tRNA synthetase	*Bacteroides sp.* D2	169	7e-41	74
00464	530	YP003547704	coagulation factor 5/8 type domain protein	*Coraliomargarita akajimensis*	118	1e-19	47
00470	513	YP003575886	pfkB family kinase	*Prevotella ruminicola* 23	112	6e-24	78
00543	745	ZP03539761	glycine betaine, L-proline ABC transporter	*Borrelia garinii* PBr	39.7	0.064	31

Table 8.3: Summary of blastX results of T2 assembled contigs.

Contig	Length (bp)	Gene bank	Annotation	Organism	Score	E value	Identity (%)
00001	941	YP003575498	PASTA domain-containing protein	*Prevotella ruminicola* 23	112	6e-17	68
00003	532	ZP02211989	hypothetical protein CLOBAR01606	*Clostridium bartlettii* DSM 16795	128	5e-28	72
00007	688	YP002249512	thiamine biosynthesis enzyme	*Thermodesulfovibrio yellowstonii* DSM	191	2e-40	63
00010	502	YP003574244	ABC transporter ATP-binding protein	*Prevotella ruminicola* 23	324	1e-65	100
00011	502	YP003574876	hypothetical protein PRU 786	*Prevotella ruminicola* 23	219	1e-55	64
00012	500	YP003574619	putative 1,4-alpha-glucan branching enzyme	*Prevotella ruminicola*	310	4e-76	94
00031	519	ZP02871253	hypothetical protein cdivTM13371	candidate division TM7 single-cell	118	4e-07	62
00033	604	ZP02478032	possible uncharacterized restriction enzyme	*Haemophilus parasuis*	278	2e-66	82
00034	692	EFH09036	ISBp1 transposase	*Roseomonas cervicalis* ATCC 49957	152	3e-35	43
00040	778	ZP06424012	Low quality protien	*Prevotella sp*	128	4e-28	85
00042	704	ZP04292677	hypothetical protein bcere0009_55340	*Bacillus cereus* R309803	109	3e-22	56
00045	1405	ZP03717680	hypothetical protein EUBHAL02765	*Eubacterium hallii* DSM 3353	156	4e-30	62
00046	680	YP003574071	peptide chain release	*Prevotella*			

			factor 1	*ruminicola* 23	343	8e-93	95
00048	918	ZP06864841	hypothetical protein NpolA408531	*Neisseria polysaccharea* ATCC	244	5e-63	64
00049	526	ZP03717135	hypothetical protein EUBHAL02203	*Eubacterium hallii*	122	7e-20	100
00055	524	YP003575881	polyribonucleotide nucleotidyltransferase	*Prevotella ruminicola* 23	312	8e-77	96
00063	873	ZP06865003	hypothetical protein NpolA409365	*Neisseria polysaccharea* ATCC	41.6	0.065	27

Table 8.4: Summary of blastX results of T3 assembled contigs

Contig	Length (bp)	Gene bank	Annotation	Organism	Score	E value	Identity (%)
00068	526	ZP04062464	catenin, alpha	*Streptococcus salivarius* SK126	57.4	3e-07	72
00103	635	ZP01052431	lipid A export ATP-binding/permease protein MsbA	*Polaribacter sp.*	152	1e-29	51
00150	861	ZP01732662	hypothetical protein CY011000950	*Cyanothece sp.* CCY0110	265	3e-63	60
00232	500	ZP04850522	conserved hypothetical protein	*Bacteroides sp.*	165	1e-39	48
00263	537	YP002561532	binding-protein-dependent transport system membrane protein	*Streptococcus uberis* 0140J	34.3	3.1	40
00279	525	ZP04292608	hypothetical protein bcere000954610	*Bacillus cereus* R309803	166	6e-25	54
00311	534	ZP02032374	hypothetical protein PARMER02386	*Parabacteroides merdae* ATCC	56.6	6e-07	78
00375	525	ZP04876635	isochorismatase family protein	*Thermococcus barophilus* MP	137	3e-31	50
00435	515	ZP05856451	putative DNA-binding protein	*Prevotella veroralis* F0319	36.6	0.63	31
00504	516	ZP06415165	acyltransferase 3	*Frankia sp.*	52.4	1e-05	31
00526	501	YP003574723	MazG family protein	*Prevotella ruminicola* 23	270	3e-71	86
00545	518	YP003122785	glycosyl transferase	*Chitinophaga*		2e-37	46

			group 1	*pinensis* DSM 2588	157		
00552	504	YP003090464	TonB-dependent receptor	*Pedobacter heparinus* DSM 2366	37.4	0.37	28
00584	536	ZP04569195	conserved hypothetical protein	*Fusobacterium sp*	33.9	4.1	52
00619	516	YP001212506	threonyl-tRNA synthetase	*Pelotomaculum thermopropionicum* S1	81.3	2e-14	33
00633	517	ZP01855831	hypothetical protein PM8797T17212	*Planctomyces maris* DSM 8797	55.8	1e-06	27
00636	507	ZP02030931	hypothetical protein PARMER00907	*Parabacteroides merdae* ATCC	176	7e-43	51
00655	501	YP001322784	N-6 DNA methylase	*Methanococcus vannielii* SB	250	2e-65	71
00666	517	ZP_01050878	hypothetical protein MED13403699	*Dokdonia donghaensis* MED134	192	2e-41	55
00697	510	YP003641626	ATP synthase F0, A subunit	*Thermincola sp.* JR	54.7	2e-06	44
00774	529	ZP06865580	ABC transporter substrate binding protein	*Starkeya novella* DSM 506	35.8	1.1	29
00777	501	ZP01886720	DNA repair protein	*Pedobacter sp.* BAL39	100	4e-20	54
00780	518	ZP06256965	arylsulfatase-activating protein AtsB	*Prevotella oris* F0302	214	6e-48	100

00807	526	ZP03008390	hypothetical protein BACCOP00231	*Bacteroides coprocola* DSM	182	9e-45	53
00851	520	ZP04745424	2-isopropylmalate synthase	*Roseburia intestinalis* L1-82	217	3e-55	65
00895	509	ZP02033023	hypothetical protein PARMER03044	*Parabacteroides merdae* ATCC	169	5e-41	68
00899	514	ZP06391526	acyl-ACP thioesterase	*Dethiosulfovibrio peptidovorans* DSM 11002	101	2e-20	43
00919	522	ZP04818483	lipoprotein	*Staphylococcus epidermidis* M23864	43.5	0.005	36
00944	503	ZP04062464	catenin, alpha	*Streptococcus salivarius* SK126	79.3	9e-08	76
00968	514	ZP03709146	hypothetical protein CLOSTMETH	*Clostridium methylpentosum*	152	6e-36	47
00977	509	YP336158	benzoate 1,2-dioxygenase beta subunit	*Burkholderia pseudomallei* 1710b	36.6	0.63	52
00989	514	YP339793	Bax protein	*Pseudoalteromonas haloplanktis* TAC125	36.2	0.82	29
00991	566	ZP06825402	short-chain dehydrogenase/reductase family oxidoreductase	*Streptomyces sp.*	45.8	0.001	33
01001	507	YP003574519	6phosphofructokinase	*Prevotella ruminicola* 23	214	1e-54	85
01009	500	ZP02074641	hypothetical protein CLOL25001412	*Clostridium sp.* L2-50	184	1e-45	53

01016	516	ZP03207027	hypothetical protein BACPLE00643	*Bacteroides plebeius* DSM	244	8e-57	68
01022	546	ZP03798012	hypothetical protein COPCOM00265	*Coprococcus comes* ATCC	100	4e-20	54
01023	502	ZP06286140	conserved hypothetical protein	*Prevotella buccalis* ATCC 35310	217	3e-55	66
01034	541	YP003574140	hypothetical protein PRU0786	*Prevotella ruminicola* 23	161	1e-38	76
01038	518	ZP04452917	hypothetical protein GCWU00018202230	*Abiotrophia defectiva* ATCC	63.2	6e-09	54
01047	506	YP001525801	outer membrane autotransporter barrel protein	*Azorhizobium caulinodans*	172	3e-05	44
01048	505	YP003574930	phosphoglycerate kinase	*Prevotella ruminicola* 23	221	1e-56	88
01049	535	ZP05346882	capsular polysaccharide biosynthesis protein Cps4E	*Bryantella formatexigens*	212	8e-54	61
01056	857	ZP04292677	hypothetical protein bcere000955340	*Bacillus cereus* R309803	60.5	4e-08	66
01063	786	YP003369670	hypothetical protein Psta1128	*Pirellula staleyi* DSM 6068	37.4	0.37	24
01066	507	ZP06717877	radical SAM domain protein	*Ruminococcus albus* 8	201	1e-50	74
01091	502	YP003575468	glutamate 5-kinase	*Prevotella ruminicola* 23	139	7e-32	61

01119	500	ZP01960340	hypothetical protein BACCAC01954	*Bacteroides caccae* ATCC 43185	188	1e-46	56
01131	539	ZP06201563	protein phosphatase PrpC	*Bacteroides sp.* D20	125	1e-27	57
01141	521	YP001556800	hypothetical protein Sbal1954382	*Shewanella baltica* OS195	37.7	0.28	31
01176	530	YP002276802	Tail Collar domain protein	*Gluconacetobacter diazotrophicus* PAl 5	96.3	8e-13	66
01184	521	YP003369588	DNA gyrase, B subunit	*Pirellula staleyi* DSM 6068	196	4e-49	63
01186	521	ZP02424505	hypothetical protein ALIPUT00622	*Alistipes putredinis* DSM 17216	256	5e-67	68
01191	525	ZP06864841	hypothetical protein NpolA408531	*Neisseria polysaccharea* ATCC	165	2e-33	58
01197	519	YP001669393	polar amino acid ABC transporter, inner membrane subunit	*Pseudomonas putida* GB-1	36.2	0.82	23
01206	508	YP003249814	exporter of the RND superfamily protein-like protein	*Fibrobacter succinogenes*	229	4e-59	98
01214	522	ZP05616052	conserved hypothetical protein	*Faecalibacterium prausnitzii* A2-165	48.1	2e-04	45
01221	510	ZP04793125	histidine kinase	*Desulfovibrio vulgaris* RCH1	37.4	0.37	35
01223	517	YP425047	hypothetical protein RruB0011	*Rhodospirillum rubrum* ATCC 11170	33.9	4.1	40
01237	733	ZP03642701	hypothetical protein BACCOPRO_01059	*Bacteroides coprophilus* DSM 18228	276	2e-66	66

01238	509	YP002937691	DNA topoisomerase I	*Eubacterium rectale* ATCC 33656	246	2e-57	71
01256	508	ZP02424000	hypothetical protein ALIPUT00115	*Alistipes putredinis* DSM 17216	108	1e-22	52
01259	513	YP001510150	hypothetical protein Franean15900	*Frankia sp.*	38.9	0.13	32
01270	507	ZP06719272	anaerobic ribonucleoside-triphosphate reductase	*Ruminococcus albus*	278	1e-73	81
01272	506	ZP01728235	Putative transmembrane protein	*Cyanothece sp.* CCY0110	32.7	9.1	44
01279	533	ZP06489452	hypothetical protein XcampmN07730	*Xanthomonas campestris pv*	38.5	0.17	34
01286	501	ZP05736650	hypothetical protein GCWU00032502764	*Prevotella tannerae* ATCC	159	1e-14	34
01297	526	YP715633	hypothetical protein FRAAL5473	*Frankia alni* ACN14a	57.8	0.15	88
01299	502	ZP06419154	AP endonuclease, family 2	*Prevotella buccae* D17	158	1e-37	55
01323	511	ZP05391231	acetate kinase	*Clostridium carboxidivorans* P7	50.4	4e-05	81
01331	548	ZP05291780	N-carbamoylputrescine amidase	*Acidithiobacillus caldus* ATCC	68.6	1e-10	35
01341	511	ZP01037748	hypothetical protein ROS21707894	*Roseovarius sp.* 217	53.5	5e-06	31

01343	541	ZP02435069	hypothetical protein BACSTE01306	*Bacteroides stercoris* ATCC	249	5e-65	69
01354	509	YP003574211	hypothetical protein PRU0858	*Prevotella ruminicola* 23	209	2e-46	68
01365	528	ZP01728785	type II restriction-modification enzyme	*Cyanothece sp.* CCY0110	271	1e-71	71
01366	513	YP002475101	transporter, drug/metabolite exporter family	*Haemophilus parasuis*	32.7	9.1	33
01373	521	ZP06245074	dihydrodipicolinate synthase	*Victivallis vadensis* ATCC BAA-548	224	2e-57	69
01377	539	YP003548908	glutamine amidotransferase class-I	*Coraliomargarita akajimensis*	37.4	0.37	32
01380	521	YP002508655	UDP-N-acetylmuramyl-tripeptide synthetase	*Halothermothrix orenii*	93.2	6e-18	38
01381	505	ZP02425964	hypothetical protein ALIPUT02122	*Alistipes putredinis* DSM 17216	120	4e-26	80
01382	509	ZP02423859	hypothetical protein EUBSIR02741	*Eubacterium siraeum* DSM 15702	150	4e-35	49
01398	515	ZP05403519	LL-diaminopimelate aminotransferase	*Mitsuokella multacida* DSM 20544	146	4e-34	58
01399	516	ZP02037176	hypothetical protein BACCAP02789	*Bacteroides capillosus* ATCC	290	2e-77	84
01407	510	YP001320138	amino acid carrier protein	*Alkaliphilus metalliredigens* QYMF	38.9	0.13	52

01419	505	ZP03208382	hypothetical protein BACPLE02026	*Bacteroides plebeius* DSM 17135	284	8e-69	76
01425	526	ZP06112077	putative HD domain protein	*Clostridium hathewayi* DSM 13479	70.1	5e-11	46
01426	528	ZP02206655	hypothetical protein COPEUT01438	*Coprococcus eutactus* ATCC	35.8	1.1	41
01430	527	ZP06201078	conserved hypothetical protein	*Bacteroides sp.* D20	157	2e-37	50
01433	562	ZP06864231	hypothetical protein NpolA405309	*Neisseria polysaccharea* ATCC	105	8e-22	54
01434	516	ZP01733412	beta-hexosaminidase precursor	*Flavobacteria bacterium* BAL38	62	1e-08	35
01443	506	ZP05031712	tetratricopeptide repeat domain protein	*Brevundimonas sp.* BAL3	33.5	5.3	45
01449	501	YP001038064	hypothetical protein Cthe1648	*Clostridium thermocellum* ATCC	115	8e-25	50
01451	514	YP003573910	hypothetical protein PRU0539	*Prevotella ruminicola* 23	210	2e-53	79
01452	511	ZP05734648	fibronectin type III domain protein	*Prevotella tannerae* ATCC	49.7	7e-05	30
01460	528	ZP06005446	phosphoenolpyruvate carboxykinase	*Prevotella bergensis* DSM	137	3e-31	84
01466	525	ZP04847555	alpha-rhamnosidase	*Bacteroides sp*	169	6e-41	55
01471	519	YP003197044	hypothetical protein Dret0164	*Desulfohalobium retbaense* DSM	35.4	1.4	30

01486	514	ZP06416734	Lanthionine synthetase C family protein	*Frankia sp.*	33.1	6.4	40
01491	509	ZP06865019	hypothetical protein NpolA409445	*Neisseria polysaccharea* ATCC	74.3	2e-06	46
01494	504	ZP06268569	valine--tRNA ligase	*Prevotella bivia* JCVIHMP010	328	1e-88	89
01496	504	YP288338	surface protein	*Thermobifida fusca* YX	118	0.006	35
01503	514	YP001359553	DNA primase DnaG	*Sulfurovum sp.* NBC37-1	112	8e-24	42
01508	511	YP001529924	parallel beta-helix repeat-containing protein	*Desulfococcus oleovorans* Hxd3	74.3	0.069	47
01509	520	ZP06268374	putative membrane protein	*Prevotella bivia* JCVIHMP010	149	6e-35	46
01519	501	ZP04743806	response regulator/phosphatase	*Roseburia intestinalis*	176	4e-43	54
01522	520	ZP04450981	hypothetical protein GCWU00018200261	*Abiotrophia defectiva* ATCC	180	3e-44	56
01523	503	ZP03626308	Fibronectin type III domain protein	*Eubacterium Ellin514*	132	9e-13	47
01534	533	ZP03488851	hypothetical protein EUBIFOR01437	*Eubacterium biforme* DSM 3989	242	7e-63	72
01536	500	YP866596	filamentous haemagglutinin outer membrane protein	*Magnetococcus sp.* MC-1	42.4	0.011	26
01539	511	ZP01967247	hypothetical protein RUMTOR00793	*Ruminococcus torques* ATCC	191	2e-47	74

01550	527	YP910270	pyridoxal biosynthesis lyase PdxS	*Bifidobacterium adolescentis* ATCC 15703	172	9e-42	83
01553	561	YP001876206	glucosamine/fructose-6-phosphate aminotransferase, isomerizing	*Elusimicrobium minutum* Pei191	215	3e-48	56
01558	514	YP003574621	MORN repeat protein	*Prevotella ruminicola* 23	1014	9e-63	75
01565	515	ZP05346079	cellobiose-phosphorylase	*Bryantella formatexigens* DSM 14469	285	3e-69	83
01569	534	YP001307094	dihydrodipicolinate synthase	*Thermosipho melanesiensis* BI429	172	9e-42	48
01574	506	ZP02164258	hypothetical protein KAOT1_00785	*Kordia algicida* OT-1	82.8	7e-15	25
01576	514	YP827375	Ig family protein	*Solibacter usitatus Ellin* 6076	111	0.024	30
01583	514	ZP02426408	hypothetical protein ALIPUT02574	*Alistipes putredinis* DSM 17216	66.6	4e-04	38
01586	514	ZP05918372	YngK protein	*Prevotella sp.*	255	8e-67	68
01590	528	YP003250349	DNA internalization-related competence protein ComEC/Rec2	*Fibrobacter succinogenes*	58.9	1e-07	34
01592	526	ZP03391603	glycosyl transferase, group 1 family protein	*Capnocytophaga sputigena*	101	2e-20	67
01594	501	ZP04546024	transposase	*Bacteroides sp.*	124	2e-27	60
01603	500	YP003576167	3-oxoacyl-(acyl-carrier-protein) synthase III	*Prevotella ruminicola*	113	5e-24	85

01604	503	ZP02425197	hypothetical protein ALIPUT01340	*Alistipes putredinis* DSM 17216	70.1	5e-11	34
01605	519	ZP02180681	hypothetical protein FBALC113647	*Flavobacteriales bacterium*	33.1	6.4	44
01606	528	ZP06184981	toxin-antitoxin system, antitoxin component, HicB family	*Mobiluncus mulieris* 28-1	93.6	4e-18	68
01609	500	YP001436234	hypothetical protein ESA00093	*Cronobacter sakazakii*	36.6	0.58	33
01610	548	ZP06286985	A/G-specific adenine glycosylase	*Prevotella buccalis* ATCC 35310	264	2e-69	75
01616	511	ZP03762256	hypothetical protein CLOSTASPAR_06294	*Clostridium asparagiforme*	83.2	5e-15	37
01623	524	ZP06256667	putative outer membrane protein	*Prevotella oris* F0302	219	5e-56	61
01625	523	YP003510900	hypothetical protein Snas2113	*Stackebrandtia nassauensis* DSM	37.7	0.26	25
01627	514	ZP05735213	conserved hypothetical protein	*Prevotella tannerae* ATCC 51259	155	9e-37	49
01629	536	YP001416808	hypothetical protein Xaut1906	*Xanthobacter autotrophicus* Py2	33.1	6.4	31
01630	505	ZP05254937	conserved hypothetical protein	*Bacteroides sp*	38.9	0.12	60
01633	540	ZP06142951	LPS biosynthesis protein	*Ruminococcus flavefaciens* FD-1	155	7e-37	44
01635	536	ZP05059680	PAS fold family	*Verrucomicrobiae bacterium* DG1235	35.8	0.99	32

01640	506	YP003547906	Polynucleotide adenylyltransferase region	*Coraliomargarita akajimensis* DSM 45221	198	1e-49	56
01643	500	YP332514	hypothetical protein BURPS1710b_1103	*Burkholderia pseudomallei*	36.2	0.76	28
01645	518	ZP01287219	Glycosyl transferase, group 1	*delta proteobacterium* MLMS-1	70.1	5e-11	44
01654	510	ZP02908261	hypothetical protein BamMEX5DRAFT3615	*Burkholderia ambifaria*	37.4	0.34	33
01655	500	YP001179586	hypothetical protein Csac0776	*Caldicellulosiruptor saccharolyticus*	45.8	0.001	29
01661	506	YP001605666	hypothetical protein YpAngolaA1120	*Yersinia pestis angola*	32.7	8.4	46
01663	506	ZP05507059	hypothetical protein StreC15170	hypothetical protein	37	0.44	38
01665	515	YP003461703	heat shock protein Hsp20	*Thioalkalivibrio sp.* K90mix	65.5	1e-09	35
01673	513	ZP02421382	hypothetical protein EUBSIR00206	*Eubacterium siraeum* DSM 15702	153	4e-36	50
01684	515	NP769091	hypothetical protein blr2451	*Bradyrhizobium japonicum* USDA 110	38	0.44	37
01688	507	YP337025	hypothetical protein BURPS1710b_A1869	*Burkholderia pseudomallei*	41.2	0.024	33
01690	516	ZP02036779	hypothetical protein BACCAP02390	*Bacteroides capillosus* ATCC	104	2e-21	60
01697	505	YP001681524	DNA methylase	*Heliobacterium modesticaldum* Ice1	197	3e-49	53

01701	520	ZP02486958	hypothetical protein Bpse737830	*Burkholderia pseudomallei* 7894	36.2	0.76	41
01709	513	YP561640	coenzyme F390 synthetase	*Shewanella denitrificans* OS217	35	1.7	29
01713	514	ZP06393308	glycosyl transferase, WecB/TagA/CpsF family	*Dethiosulfovibrio peptidovorans*	32.7	8.4	25
01714	509	YP003573622	nucleotidyl transferase family protein	*Prevotella ruminicola* 23	268	7e-71	83
01727	527	YP003575309	electron transfer flavoprotein subunit beta	*Prevotella ruminicola* 23	213	3e-54	100
01729	536	ZP05082976	autotransporter	*Pseudovibrio sp.*	41.2	0.024	30
01733	503	ZP05347683	putative efflux ABC transporter, permease protein	*Bryantella formatexigens*	60.5	4e-08	36
01745	504	YP003575272	hypothetical protein PRU1997	*Prevotella ruminicola* 23	37	0.44	31
01746	518	ZP01302597	beta-lactamase	*Sphingomonas sp.* SKA58	94.7	2e-18	41
01754	505	YP003575682	succinate dehydrogenase/fumarate reductase flavoprotein subunit	*Prevotella ruminicola* 23	320	2e-86	94
01767	528	ZP03681601	hypothetical protein CATMIT00213	*Catenibacterium mitsuokai* DSM	242	5e-63	64
01769	532	ZP05856235	DNA topoisomerase III	*Prevotella veroralis* F0319	245	6e-64	70

01780	522	ZP04787260	PHP domain protein	*Thermoanaerobacter brockii subsp*	38.9	0.12	34
01790	504	YP003153196	Cna B domain protein	*Anaerococcus prevotii* DSM 20548	36.6	0.58	35
01792	500	YP003167680	acriflavin resistance protein	*Candidatus Accumulibacter*	151	1e-35	50
01810	501	YP587879	diguanylate cyclase	*Cupriavidus metallidurans* CH34	35.4	1.3	35
01815	536	ZP02917307	hypothetical protein BIFDEN00585	*Bifidobacterium dentium* ATCC 27678	197	3e-49	55
01833	523	ZP05282570	putative epimerase	*Bacteroides fragilis*	293	1e-71	81
01840	504	ZP04855986	surfactin synthetase B	*Ruminococcus sp.*	126	9e-10	28
01854	525	YP003104182	hypothetical protein Amir6536	*Actinosynnema mirum* DSM 43827	37	0.44	37
01868	516	ZP02405280	carbohydrate diacid regulator	*Burkholderia pseudomallei* DM98	37	0.44	29
01878	501	ZP06418844	beta-ketoacyl synthase domain protein	*Prevotella buccae* D17	107	4e-22	49
01880	507	ZP05483515	putative sensor with HAMP domain	*Streptomyces sp.* AA4	37.7	0.26	31
01884	516	YP305509	cell surface protein	*Methanosarcina barkeri str*	693	3e-19	35
01899	519	ZP06077382	hydroxysteroid dehydrogenase 12	*Bacteroides sp.*	99	1e-19	43

01906	514	ZP06256287	glutathione peroxidase	*Prevotella oris* F0302	82.4	9e-15	79
01931	512	ZP04455933	hypothetical protein GCWU00034201970	*Shuttleworthia satelles*	202	8e-51	65
01935	534	YP003575445	PadR family transcriptional regulator	*Prevotella ruminicola* 23	42	9e-31	86
01964	502	ZP06241952	protein of unknown function DUF935	*Victivallis vadensis* ATCC BAA	114	2e-24	47
02020	710	ZP01721985	hypothetical protein BB1490516165	*Bacillus sp.* B14905	36.6	0.58	30
02031	828	ZP04292608	hypothetical protein bcere000954610	*Bacillus cereus* R309803	219	1e-18	65
02035	594	ZP02544188	hypothetical protein cdiviTM7_00470	candidate division TM7 single	243	2e-50	78
02045	854	ZP00047507	COG3979: Uncharacterized protein contain chitin-binding domain type 3	*Magnetospirillum magnetotacticum* MS-1	35	1.7	33
02067	660	ZP04292608	hypothetical protein bcere0009_54610	*Bacillus cereus* R309803	113	1e-11	64
02080	897	YP003496752	membrane protease subunit HflC	*Deferribacter desulfuricans*	37.4	0.34	26
02090	518	YP432066	DNA-dependent helicase II	*Hahella chejuensis* KCTC 2396	35.8	0.98	31
02097	959	ZP04292608	hypothetical protein bcere000954610	*Bacillus cereus* R309803	196	5e-35	62
02109	632	ZP04292677	hypothetical protein	*Bacillus cereus*	129	1e-05	90

Table 8.5: Summary of blastX results of T4 assembled contigs.

Contig	Length (bp)	Gene bank	Annotation	Organism	Score	E value	Identity (%)
00005	567	ZP04062464	catenin, alpha	*Streptococcus salivarius* SK1261	77.8	3e-07	86
00043	526	NP688215	hypothetical protein SAG1206	*Streptococcus agalactiae* 2603V	34.3	3.8	22
00060	503	ZP04292608	hypothetical protein bcere000954610	*Bacillus cereus* R309803	76.2	8e-07	54
00065	702	YP001938607	putative conjugative transfer protein TraN	*Orientia tsutsugamushi*	34.7	2.9	45
00107	505	YP001922224	integral membrane sensor signal transduction histidine kinase	*Clostridium botulinum* E3 str. Alaska E43	33.9	5.0	29
00134	555	YP476125	cation transport ATPase	*Synechococcus sp.* JA-3-3Ab	34.7	2.9	56
00174	514	YP425047	hypothetical protein Rru_B0011	*Rhodospirillum rubrum* ATCC 11170	33.9	5.0	40
00176	503	ZP04055951	hypothetical protein PORUE0001_0884	*Porphyromonas uenonis* 60	33.1	8.5	29
00186	522	YP003575274	phosphoserine phosphatase SerB	*Prevotella ruminicola* 23	254	1e-47	100
00194	515	YP516851	putative fumarate reductase flavoprotein subunit	*Desulfitobacterium hafniense* Y51	68.2	2e-10	50

00202	528	ZP03717680	hypothetical protein EUBHAL02765	*Eubacterium hallii* DSM 3353	57.4	4e-07	42
00238	523	YP001093797	enoyl-CoA hydratase/isomerase	*Shewanella loihica* PV-4	35	2.2	38
00254	531	YP003304495	PAS sensor protein	*Sulfurospirillum deleyianum* DSM 6946	34.7	2.9	35
00256	516	ZP03166961	hypothetical protein RUMLAC00618	*Ruminococcus lactaris* ATCC	60.5	5e-08	55
00273	531	ZP05545124	phosphoglycerate kinase	*Parabacteroides sp.* D13	221	2e-56	77
00280	710	ZP01732662	hypothetical protein CY011000950	*Cyanothece sp.* CCY0110	235	9e-61	56
00286	523	ZP02033680	hypothetical protein PARMER03715	*Parabacteroides merdae* ATCC	113	6e-24	37
00290	502	YP943051	hypothetical protein Ping1659	*Psychromonas ingrahamii* 37	34.3	3.8	34
00301	515	ZP06242250	lipolytic protein G-D-S-L family	*Victivallis vadensis* ATCC BAA-548	55.8	1e-06	26
00315	510	ZP03850035	outer membrane protein	*Chryseobacterium gleum* ATCC 35910	50.8	4e-05	57
00322	513	ZP01961149	hypothetical protein BACCAC_02775	*Bacteroides caccae* ATCC 43185	281	1e-74	77
00326	503	ZP02423148	hypothetical protein EUBSIR02006	*Eubacterium siraeum* DSM 15702	77.4	4e-13	37

00340	544	YP002994761	hypothetical protein TSIB1360	*Thermococcus sibiricus* MM 739	35	2.2	33
00351	537	NP953758	hypothetical protein GSU2713	*Geobacter sulfurreducens* PCA	65.1	2e-09	40
00361	511	ZP04057624	Sua5/YciO/YrdC/YwlC family protein	*Capnocytophaga gingivalis* ATCC	65.9	1e-09	49
00364	500	ZP06596088	oxalate:formate antiporter	*Bifidobacterium breve* DSM 20213	197	6e-43	63
00365	531	YP003575297	hypothetical protein PRU2023	*Prevotella ruminicola* 23	179	3e-37	97
00370	531	YP003574684	hypothetical protein PRU1372	*Prevotella ruminicola* 23	314	7e-78	84
00371	527	YP001301479	hypothetical protein BDI0062	*Parabacteroides distasonis* ATCC 8503	161	2e-38	42
00377	518	ZP02041468	hypothetical protein RUMGNA02237	*Ruminococcus gnavus* ATCC	55.5	2e-06	37
00378	532	ZP04156057	hypothetical protein bmyco000310050	*Bacillus mycoides* Rock3-17	82.8	0.006	48
00393	575	YP001757434	hypothetical protein Mrad28314791	*Methylobacterium radiotolerans*	34.3	3.8	35
00425	513	ZP02536162	Lytic transglycosylase, catalytic	*Endoriftia persephone* 'Hot961+Hot962	35.8	1.3	33
00441	518	ZP03968895	conserved hypothetical protein	*Sphingobacterium spiritivorum* ATCC 33300	71.2	3e-11	49

00489	866	YP747193	hypothetical protein Neut0968	*Nitrosomonas eutropha* C91	38.1	0.26	28
00528	826	ZP04292608	hypothetical protein bcere000954610	*Bacillus cereus* R309803	168	1e-40	55
00535	672	ZP04292677	hypothetical protein bcere000955340	*Bacillus cereus* R309803	89	1e-16	44

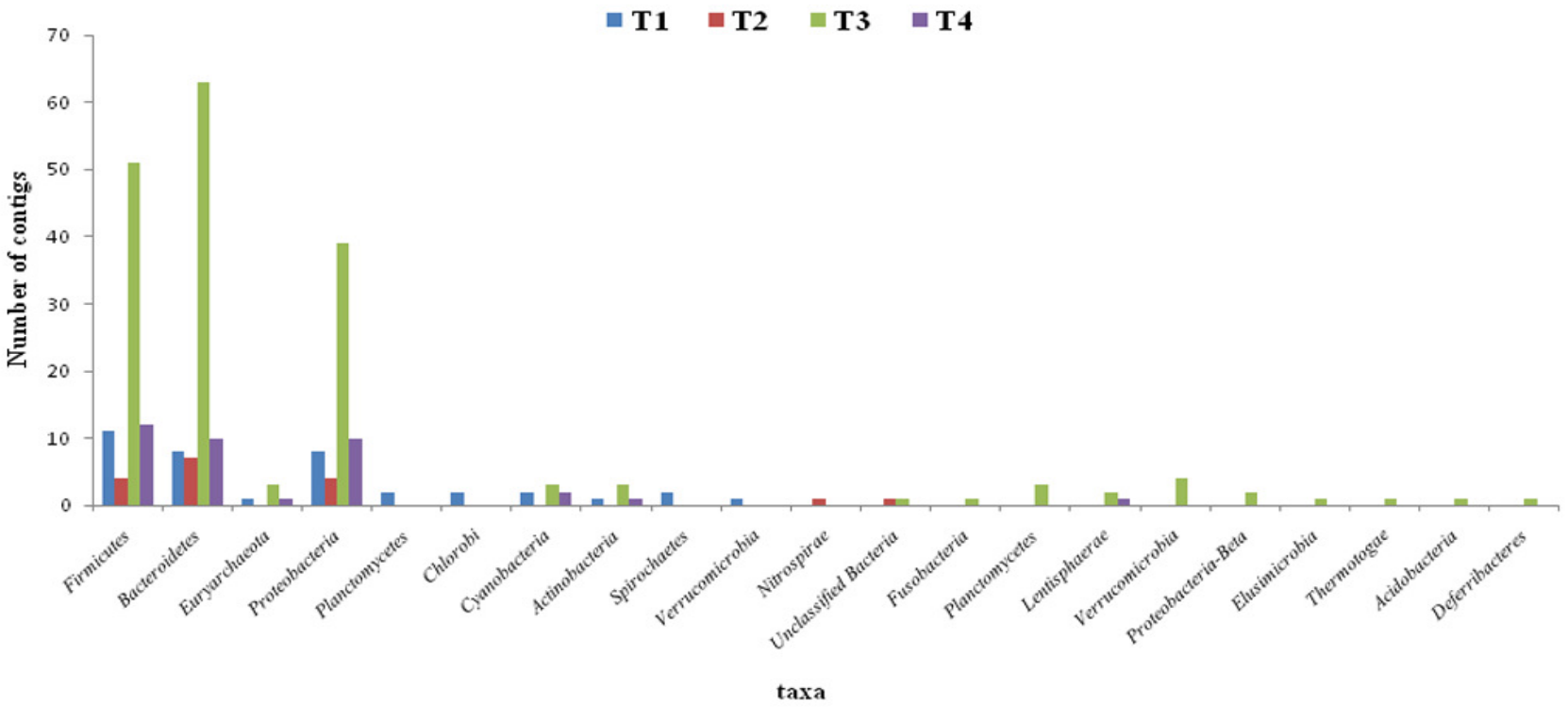


Figure 8.4. Distribution of Translations of assembled contigs (EGT; > 500 nucleotides) of four pyrosequnced Surti buffalo rumen microbiome based on BLASTX result.

Chapter 9. Summary and conclusion remarks

The rumen is one of the most complicated microbial ecosystems in nature, containing mainly three groups of anaerobic microorganisms: bacteria, protozoa and fungi. These groups with environmental components constitute the 'rumen microbial ecosystem', and ferment plant carbohydrates and digest nitrogenous compounds. In the analysis of this ecosystem, microbial makeup, interaction between the microbe and their environment are of paramount importance.

The techniques outlined in this thesis are improvements over current methods to study microbial makeup and interactions between ruminal microorganisms in response to different feeding regimes. Culture-independent molecular biology techniques based on analysis of 16S/18S rDNA allow rapid profiling of microbial population in samples from complex ecosystems such as the rumen.

Present study focuses, the combination of better sampling and molecular-based microbial profiling techniques can allow a better quantification and community structure of microbes available in the ruminants.

The Surti buffalo (*Bubalus babalis*) has a diverse range of bacteria in the rumen. The sequences of cellulolytic bacteria (*Ruminococcus callidus*, *Acetovibrio cellulolyticus*, non fibrolytic bacteria (*Streptococcus sp.*), proteolytic bacteria (*Prevotella ruminicola*), acid utilizers (*Succiniclasticum ruminis*), and pectin utilizers (*Treponema sp.*) were recovered in the present study. Where as many of sequences remain unidentified.

The rumen of *Bubalus babalis* is dominated by sequences related to members of hydrogenotrophic methanogens (methanomicrobiales and methanobacteriales), which indicate that in rumen the main reaction may be H_2- dependent CH_4 production. The presence of plants material (cell wall) adapted to rumen conditions and influences the quality of substrate entering anaerobic environment. Present finding is of great importance in characterizing methanogenic pathway in rumen ecology.

The protozoa community in *Bubalus babalis* rumen is dominated by entodiniomorphid protozoa. Among holotrich, two species are also identified as *Dasytricha ruminantium* and *Isotricha prostoma*. The investigation of protozoa-bacterial interactions should greatly advance the state of mechanistic understanding of the interface between the dairy buffalo and their ruminal microbial ecosystem. This

can in turn improve feeding practices and allow minimization of the environmental impact of dairy production systems.

Four major representative groups of ruminal microbes covering fibrolytic, non-fibrolytic bacterial communities, methanogens and protozoan were detected using real time PCR assay. Results revealed that *R. albus* was the most dominant fibrolytic among the all detected fibrolytic species. In non-fibrolytic, *S. bovis* and *S. ruminantium* were also detected with abundance in rumen fluid. The high magnitude of non-fibrolytic bacteria followed by plant fiber rich diet suggests the development of mutual relationships between fibrolytic and non-fibrolytic bacterial communities. The population size of Methanomicrobiales and Methanobacteriales was significance higher compare to the Methanococcales. Among ciliate protozoa, *D. ruminantium* was found to be the predominant over *Entodinium sp*. and probably may help in fiber digestion.

In the roughage:concentrate study, two major representative groups of ruminal microbes covering fibrolytic and non-fibrolytic bacterial communities were demonstrated. *R. flavefaciens* and *F. Succinogenes* were the most dominant fibrolytic species in high level of roughage diet (wheat straw), which may forms a multi-enzyme cellulosome complex that could play an integral role in the ability of this bacterium to degrade plant cell wall polysaccharides. Non-fibrolytic, *S. ruminantium* and *T. bryantii* were also detected with abundance in rumen fluid. Present finding could be useful in manipulating feeding regimes for Indian buffalo population.

To gain a greater understanding of the ecology and metabolic potential of *Bubalus babalis* rumen microbiome, we applied comparative metagenomics (phylotype analysis and SEED subsystems-based annotations) to examine randomly sampled pyrosequence data from four different microbiomes. The distribution of phylotypes and environmental gene tags (EGTs) detected within each rumen sample were primarily the bacteroidetes/chlorobi, firmicutes and proteobacteria groups. This observation could be help in better understanding of mechanistic process altering the production and uptake of amino nitrogen will help the livestock nutritionists to improve the overall conversion of dietary nitrogen into microbial protein. Also, the sequences captured from the Surti rumen metagenomics contain new enzymes could be potential for industrial and bioenergy applications

Future Directions

The ruminal habitat has provided microbiologists with a rich bounty of information pertaining to microbial schemes of anaerobic metabolism, and the interactions among ruminal microorganisms and the host animal have been well described. In the past, much of the information has been derived by cultivation of a particular microorganism either axenically, or as part of a restrictive subset of the rumen microbial consortia. Molecular technologies now provide the potential to more completely examine a microbe's response to its growth environment, through the rapidly emerging fields of molecular ecology, genomics and proteomics. In the future these technologies assist with the overall goal of optimizing ruminant production and help in minimized methane production

Genomics and metagenomics are providing powerful new tools for exploring rumen microbes. Currently, genome projects are being conducted on cultivated representatives of the rumen microbiome, while the uncultivated majority of organisms are beginning to be accessed via metagenomics. The ability of these molecular approaches to gather gene information, from both pure cultures and uncultivated organisms, means that they are likely to discover many new features of rumen microorganisms that would otherwise never have been detected. This will contribute significantly to an improved understanding of how rumen microorganisms mediate digestion for the ruminant. From an applied standpoint, these genomic approaches will undoubtedly identify a much greater array of genes encoding plant-degrading hydrolytic enzymes. Functional analysis of individual enzymes and enzyme complexes should give an improved choice of enzymes as additives for improving lignocellulose digestion in the rumen. Comparison of these enzymatic components is also likely to uncover common characteristics and strategies used by fibre-degrading organisms, which may lead to opportunities for altering and improving fibre digestion in the rumen via the addition of enhanced levels of the appropriate microbial enzymes.

The future for rumen metagenomics will depend heavily on advances in sequencing technology. Function-based metagenomic studies will undoubtedly discover many new enzymatic activities, but without the context of the genome of the organism producing that activity and knowledge of the organism's abundance in the

rumen, the significance of the activity to rumen function will not be apparent. In the shortterm, metagenomics studies targeting phylogenetic markers (such as ssrRNA or mcrA genes) are likely to be more informative, and will spread further light on the true microbial diversity in the rumen. Studying how the microbial community is affected by the type of ruminant species, by differences in breed or by the variation in diets encountered in production systems within or between countries will begin to define the rumen microbiome in specific terms. Metagenomic libraries of less complex, but functionally relevant, subsets of the rumen microbiome are also likely to be informative. Hopefully these metagenomic approaches will unravel some of the complexities of the rumen microbial environment and begin to define the microbial and ecological principles that control rumen function.

To gain a greater understanding of the ecology and metabolic potential of this microbiome (Indian buffalo rumen) will further enhance the efficiencies of this process towards its successful application. Determining the role of rumen microbes and their enzymes in plant polysaccharide breakdown is fundamental to understanding digestion and maximising productivity in ruminant animals. Application of metagenomics and over expression of genes is expected to enter the field of plant degradation (co-metabolism) by biological means, with significant impact on it. Thus, the role of molecular ecology has yet to feature prominently in this vital area of rumen microbiology.

The basic information so generated will be helpful to take up further studies on various aspects like: (i) Identifying microbes resistant to incriminating factors like Tannins in feeds, fodder, agro industrial byproducts and non conventional feeds which will be helpful in better utilization of these feeds, (ii) Exploring the regulatory mechanism for production of volatile fatty acids in rumen, (iii) Altering fermentation pattern to reduce the methanogenesis by increasing propionate production, (iv) To evaluate the contribution of bacteria and protozoa in supplying nitrogen to the host animal, (v) The data obtained here with could be used in manipulating feeding regimes for Indian buffaloes.

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Appendix A

Important buffers and reagents

Some of the important buffers and reagents used in the present study are given here as under:

a) TAE (50X)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Deionized water up to	1000 ml

b) TBE (5X)

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
Deionized water up to	1000 ml

c) Agarose gel loading buffer (6X)

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Ficoll	15% (w/v)
(Type 400; Pharmacia)	
Dissolved in appropriate volume of deionized water	

d) LMT Elution buffer

Tris base	0.12 g
EDTA	0.0075 g
Deionized water up to	50 ml

e) TE buffer (pH 8.0)

Tris base	0.06 g
EDTA	0.0075 g
Deionized water up to	50 ml

f) 2% X-gal solution

Dissolved 0.1 g of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in a final volume of five ml of dimethyl formamide (DMFO), aliquoted in one ml and stored at –20^{o}C. Solution was protected from direct sun light.

g) 20% IPTG solution

Dissolved 0.4 g of IPTG (Isopropyl- β -D-thiogalactopyranoside) in a final volume of two ml of sterile MiliQ® water, aliquoted in one ml and stored at –20^{o}C.

h) 1M $CaCl_2.2H_2O$

7.35 g of CaCl2.2H2O was disssolved in final volume of 50 ml of sterile MiliQ® water; the solution was sterilized by passing through 0.22µ membrane filter and stored in aliquots of one ml at 4 ^{o}C.

i) 1M $MgCl_2.6H_2O$

10.16 g of $MgCl_2.6H_2O$ was disssolved in final volume of 50 ml of sterile MiliQ® water, the solution was sterilized by autoclaving at 15 lb psi for 15 min and stored in aliquots of one ml at room temperature.

j) Glycerol (60%)

Diluted 60ml of 100% glycerol to a final volume of 100ml in deionized water and sterilized by autoclaving at 15 lb psi for 15 min.

Bacteriological Media

Luria-Bertani Broth

Tryptone	1.0%
Yeast Extract	0.5%
NaCl	1.0%

For LB plates 1.5% agar was added and autoclaved at15 lb psi for 15 min

Appendix B

High Throughput Metagenomic DNA Sequencing

The double stranded, non degraded, pure and RNA free genomic DNA samples devoid of any particulate matter and having an $OD_{260/280}$ ratio of approximately 1.9 with quantity of minimum 100 ng/µl and total concentration of 10-15 µg/µl were used as the starting material.

Metagenomic DNA Library Preparation

Four metagenomic DNA samples were processed into a library of random sets of single stranded DNA fragments that are flanked with amplification and sequencing primer sequences which represent the whole span of sample sequence using the GS FLX Titanium General Library Preparation Kit.

Metagenomic DNA Fragmentation (Nebulization)

Nebulizer Assembly and DNA Samples Dilution

a) 10µg of each DNA sample was pipetted to the bottom of the Nebulizer cup separately and TE Buffer was added to make final volume of 100µl.
b) 500µl of Nebulization Buffer was added in each cup and swirled up and down.
c) The entire nebulizer assembly was assembled, capped tightly and transferred to the externally vented hood and connected to the nitrogen tank.

DNA Nebulization and Purification of Fragmented DNA

a) Nitrogen at 30 psi (2.1 bars) pressure was applied to the nebulizer assembly to fragment the DNA for 1 min.
b) The nebulization assembly was tapped up and down to collect the droplets in the bottom and it was ensured that the sample volume was minimum 300µl.
c) It were purified by Qiagen MinElute spin columns as follows:
    - Nebulized samples (750µl) were added in MiniElute spin columns and centrifuged at 13,000 rpm for a min.
    - The flow-through was discarded and the above step was repeated till the samples were exhausted.
    - To it, 750µl of Buffer QG was added and centrifuged at 13,000 rpm for 1 min and the flow-through was discarded.
    - The columns were washed twice by addition of 750µl of PE buffer and centrifugation at 13,000 rpm for 1 min.

- The flow-through was discarded and the columns were given dry spin at 13,000 rpm to collect any remaining flow-through.
- MiniElute columns were placed in clean 1.5 ml micro-centrifuge tubes.
- DNA was eluted by adding 20µl Buffer EB to the center of the membrane, columns were kept stand still for 5 min and then centrifuged for 2 min at 13,000 rpm.

Metagenomic DNA Fragment Size Selection

The Gel Cut option was used to select the DNA fragments within an upper and lower range of sizes. For the Genome Sequencer FLX Titanium chemistry, the recommended size range is 500-800 bp.

a) Agarose gel @ 0.8 % (w/v) (GTG SeaKem® LE agarose, Lonza, USA) was prepared in 1X TAE Buffer by adding EtBr (1% w/v) to the gel @ 5µl/ 100 ml gel.
b) The entire 20µl of the eluate was loaded into the well along with 4µl of 6X loading dye along with 1 Kb ladder in the separate well.
c) The electrophoresis was done at 100 V for 1.5 h.
d) Under UV illumination, the library fragments between 500 to 800 bp in size, was cut with a sharp, sterile BP blade.
e) The DNA fragment was transferred into a clean pre-weighted 15 ml centrifuge tube.
f) The DNA fragment was purified using QIAquick Gel Extraction Kit (Qiagen) as follows:
    - To the One volume of the gel slice, three volumes of Buffer QG were added.
    - The tube was incubated at room temperature till the slices of gel dissolved completely.
    - Equal volume of isopropanol was added to the sample and mixed by inverting the tubes several times.
    - Sample was then applied to the QIAquick column, and centrifuged for 1min at ≥ 13,000 rpm.

- Flow-through was discarded and QIAquick column was placed back in the same collection tube. The above steps were repeated till the sample was exhausted.
- 500µl of Buffer QG was added to the spin column and centrifuged again at 13,000 rpm for 1 min. Flow-through was discarded.
- MiniElute column was washed twice with 750µl of Buffer PE by centrifuging at 13,000 rpm for 1 min after incubation for 2-5 min before spinning.
- Flow-through was discarded and QIAquick column was again centrifuged at 13,000 rpm for 1 min to remove residues of ethanol.
- QIAquick column was then placed in a clean, new 1.5 ml micro-centrifuge tube.
- DNA was eluted by adding 24µl of Buffer EB to the center of the membrane, column was kept stand still for 5 min and then centrifuged at 13,000 rpm for 2 min.

DNA Sample Quality Assessment

a) One µl of the size-selected purified DNA material was run on DNA 7500 LabChip in Agilent 2100 Bioanalyzer (Agilent Technologies, USA) as per the manufacturer's.
b) The average mean size of the DNA fragments was recorded.

Fragments End Polishing

a) In a microcentrifuge tube, the following reagents, were added in the order indicated to make up 50µl final volume:
    - ~23µl nebulized, size-selected DNA fragments
    - 5µl 10X Polishing Buffer
    - 5µl BSA
    - 5µl ATP
    - 2µl dNTP Mix
    - 5µl T4 PNK
    - 5µl T4 DNA polymerase
b) The components were mixed well and incubated for 15 min at 12^0 C.
c) Immediately, incubation was done at 25^0 C for an additional 15 min.

d) The polished fragments were purified using MinElute PCR Purification Kit, according to the manufacturer's instructions as follows:
    - To the column, the polished DNA fragments and 250µl of Buffer PBI was added and spun at 13,000 rpm for 1 min. The flow-through was discarded.
    - MiniElute column was washed twice with 750µl of Buffer PE by centrifuging at 13,000 rpm for 1 min after incubation for 2-5 min before spinning.
    - Flow-through was discarded and MinElute column was again centrifuged at 13,000 rpm for 1 min to remove traces of ethanol.
    - MiniElute column was then placed in a clean, new 1.5 ml micro-centrifuge tube.
    - DNA was eluted by adding 10µl of Buffer EB to the center of the membrane and the column was incubated at RT for 5 min.
    - The same was then centrifuged at 13,000 rpm for 2 min.

Adapter Ligation

a) In a microcentrifuge tube, the following reagents were added, in the order as indicated to make the final volume of 40µl:
    - ~10µl Polished DNA
    - 20µl 2X Ligase Buffer
    - 5µl Adaptors
    - 5µl Ligase
b) The components were mixed well and the ligation reaction was incubated at 25^0 C for 15 min.

Small Fragment Removal

a) The volumes of the eluate of ligated materials were measured and Buffer EB was added to make a final volume of 100µl.
b) The appropriate amount of AMPure beads determined as per the calibration of the lot in use was added.
c) It was then vortexed to mix and incubated for 5 min at RT.
d) Using a Magnetic Particle Concentrator (MPC) (Invitrogen, USA), the beads were pelleted against the wall of the tube to remove the supernatant.

e) The beads were washed twice with 500µl of 70 % Ethanol and then dried.
f) The tube was removed from the MPC and 25µl of 10 mM Tris-HCl, pH 8.0 (or Qiagen's Buffer EB) was added and vortexed to resuspend the beads.
g) The beads were pelleted with MPC and supernatant containing purified adapter ligated DNA was transferred to a fresh microcentrifuge tube.

Library Immobilization

a) Fifty µl of Library Immobilization Beads were washed twice with 100µl of 2X Library Binding Buffer, using the MPC.
b) The beads were resuspended in 25µl of 2X Library Binding Buffer.
c) The 25µl purified, ligated DNA was added to the tube of washed Library Immobilization Beads.
d) The tube was mixed well on a tube rotator for 20 min at RT.
e) The immobilized Library was washed twice with 100µl of Library Wash Buffer using MPC.
f) The beads were pelleted using MPC and the Library wash buffer was removed.

Fill in Reaction

a) The following fill in reaction mix was prepared with 50µl final volume:
    - 40µl Molecular Biology Grade water
    - 5µl 10X Fill-in Polymerase Buffer
    - 2µl dNTP Mix
    - 3µl Fill-in Polymerase
b) The 50 µl of fill-in reaction mix was added to the tube containing the library-carrying beads and mixed and incubated at 37^0 C for 20 min.
c) Using the MPC, the immobilized library was washed twice with 100µl of Library Wash Buffer.
d) All the remaining Library Wash Buffer was removed from the pelleted beads.

Single Stranded DNA Library Isolation

a) The Neutralization Solution was prepared by mixing 500 µl of Buffer PBI with 10µl of 3 M Sodium Actetate (pH 5.2).
b) Melt Solution was prepared by adding 0.125 ml of 10 N NaOH to 9.875 ml of Mol. Bio. Grade Water.
c) Fifty µl of Melt Solution was added to the washed library beads and vortexed.

d) The beads were pelleted (MPC) and the supernatant was transferred to the Neutralization Solution.
e) The above step was repeated and the two melts were pooled into the same tube of Neutralization Solution.
f) An extra 5µl of the 3 M sodium actetate pH 5.2 was added if the pH indicator in Buffer PBI did not quickly return to its neutral / acidic yellow color after the addition of each Melt Solution wash and mixing.
g) The same was purified on MinElute PCR Purification Kit as follows:
    - To the column, the sample and 250µl of Buffer PBI was added and spun at 13,000 rpm for 1 min. The flow-through was discarded.
    - MinElute column was washed twice with 750µl of Buffer PE by centrifuging at 13,000 rpm for 1 min after incubation for 2-5 min before spinning.
    - Flow-through was discarded and the column was then centrifuged at 13,000 rpm for 1 min to remove residues of ethanol.
    - MinElute column was then placed in a clean, fresh 1.5 ml micro-centrifuge tube.
    - DNA was eluted by adding 15µl of Buffer EB to the center of the membrane, the column was kept stand allow for 5 min and then centrifuged at 13,000 rpm for 2 min.

DNA Library Quality Assessment and Quantitation

a) Library quality and quantity assessment was done by running 1µl of single stranded DNA library on a RNA Pico 6000 Labchip in Agilent 2100 Bioanalyzer.
b) The library stock concentration in molecules/ µl was calculated as follows:

$$\text{Molecules/µl} = \frac{(\text{Sample conc.; ng/µl}) \text{ X } (6.022 \text{ X } 10^{23})}{(328.3 \text{ X } 10^{9}) \text{ X (avg. fragment length)}}$$

c) The library was diluted to 1 X 10^8 molecules/ µl in TE buffer and stored at -15^0 C to -25^0 C.
d) The working aliquots were prepared from 1 x 10^8 molecules/µl stock solution. Typical working aliquots may be 100µl for Large Volume Emulsions (LVE);

25µl if for Small Volume Emulsions (SVE), at a concentration of 1 x 10^6 molecules/µl.

Emulsion PCR (emPCR) Amplification

The emulsion (emPCR) were conducted to perform *in vitro* amplification of the single stranded library of quantitated DNA fragments to generate library of clonally amplified, bead-immobilized, single-stranded DNA fragments representative of the entire genomic DNA by using GS FLX Titanium emPCR Kit (Roche)

To estimate the correct volume of Diluted DNA library (1 X 10^6 molecules/ µl) to be added to each tube of DNA capture beads, which would give best sequencing results, first, the library titration was performed using **small volume emulsion (SVE)** and amplification. The titrations which gave best recoveries for the enriched beads i.e. range between 5 to 15% (best ~ 8%) of the original bead input were selected for the **large volume emulsion (LVE)** which results in clonally amplified library ready for sequencing.

Preparation of the Live and Mock Amplification Mixes

a) The Live Amplification Mix was prepared as given in table B.1 as per the size and number of emulsions required for the experiment.

Table B.1: Preparation of Live Amplification Mix

S. No.	**Reagent**	**LV Kit (µl)**		**SV Kit (µl)**		
		1 cup	**2 cup**	**4 tubes**	**8 tubes**	**16 tubes**
1.	emPCR additive	-	-	360	720	1440
2.	Mol. Bio. Grade water	2700	5400	280	560	1120
3.	5 X Amplification Mix	780	1560	190	380	760
4.	Amplification Primer	230	460	55	110	220
5.	emPCR Enzyme Mix	200	400	50	100	200
6.	PPiase	5	10	2	4	8
7.	Total (µl)	3915	7830	937	1874	3748

b) The mix was votexed for 5 sec and stored at 4^0 C

c) In a 15 ml falcon tube, 2ml for LVE (1ml for SVE) of 5X Mock Amplification Mix was diluted with 8 ml for LVE (4 ml for SVE) of Mol. Bio. Grade water and mixed properly and stored at + 2^0 C to + 8^0 C until used.

DNA Library Capture

a) The 10X Capture Bead Wash Buffer TW was diluted to 1X (1 ml Buffer + 9 ml water) using sterile MilliQ water.
b) The amount of DNA Capture Beads required were aliquoted as per requirement as follows:
    - For LVE, one emulsion cup requires one tube of beads.
    - For SVE, one emulsion tube requires 80µl of bead suspension.
c) The aliquot tubes were spun for 10 sec, rotated at 180 and spun again. The supernatant was discarded.
d) Each tube of beads was washed twice with 1 ml of 1X Capture Bead Wash Buffer TW (200µl for SVE) and the supernatant discarded.
e) For SVE only, the washed DNA Capture Beads were distributed into single emulsion aliquot size in the appropriate number of 1.7 or 0.2 ml tubes (usually 16 tubes; 40µl per tube) and the beads were pelleted to discard the supernatant.
f) An aliquot of the DNA library was thawed and the correct volume of DNA library (per library preparation and the equation below) was added to each tube of Capture Beads by using the following formula:

$$\text{µl of library needed per tube} = \frac{\text{molecules per bead desired} \times \text{no. of beads per tube}^*}{\text{library concentration (molecules/µl)}}$$

where,

* 35×10^6 for LVE; 2.4×10^6 for SVE

For library titration, (SVE), the following amount of library diluted to 1×10^6 molecules/µl was used:

- Tube 1: 1.2µl of diluted DNA library (= 0.5 molecule/bead)
- Tube 2: 2.4µl of diluted DNA library (= 1 molecule/bead)
- Tube 3: 4.8µl of diluted DNA library (= 2 molecules/bead)
- Tube 4: 9.6µl of diluted DNA library (= 4 molecules/bead)

Emulsification

a) The Emulsion Oil was premixed on the TissueLyser (Tissuelyser II, Qiagen, Germany) at 28 Hz for 2 min for LVE (25 Hz for 2 min for SVE in the outer rows of the TissueLyser tube racks).

b) Five ml of 1X Mock Amp Mix was added to each cup of Emulsion Oil for LVE (290µl per tube of Emulsion Oil for SVE) and inverted 2-3 times to mix.
c) In the TissueLyser, they were shaken at 28 Hz for 5 min (25 Hz for 5 min for SVE).
d) The same was vortexed for 5 sec.
e) The DNA Capture Beads with library DNA mixes for individual emulsion reactions were prepared as follows:

   a. For LVE only:

   - Each captured library mix was transferred to a clean 15 ml falcon tube.
   - One ml of Live Amp Mix was added to each of the microcentrifuge tubes, rinsed thoroughly and added to 15 ml falcon tube.
   - Another 2.75 ml of Live Amp Mix was added to each 15 ml tube (total about 3.75 ml).

   b. For SVE only:

   - To each aliquot of captured library mixes, 215µl of Live Amp Mix was added (Total about 55µl).

f) The captured library preparations were added as follows:

   - For LVE, the content (~ 3.75 ml) was first vortexed properly.
   - It was then poured from a 15 ml tube of captured library into each cup of pre-emulsion.
   - For SVE, the content (~ 255µl) was vortexed properly.
   - It was then poured from a 1.7 ml tube of captured to each tube of pre-emulsion.

g) The cups (or the tube racks) were inverted 2-3 times to mix.
h) The cups were then shaken in the TissueLyser at 12 Hz for 5 min for LVE (15 Hz for 5 min for SVE).

emPCR Amplification

a) After emulsification, the emPCR amplification mixes were pipetted into 96-well thermocycler plates @ 100µl per well and any air bubbles were dislodged by shaking. The wells were capped and sealed properly.
b) The plates containing the emulsified amplification reactions were placed into the thermocycler (Gene Amp® PCR System 9700, Applied Biosystems, USA).

c) The amplification was carried out as follows:
    - 4 min at 94^0 C
    - 50 X (30 sec at 94^0 C; 4.5 min at 58^0 C; 30 sec at 68^0 C)
    - 10^0 C until halted

Bead Recovery for SVE

Emulsion Collection and Initial Washes for SVE

a) The 1X Enhancing Fluid TW and 1X Annealing Buffer TW was prepared from their concentrates as follows:
    - To the 62.5 ml of 4X Enhancing Fluid TW stock, 187.5 ml of Molecular Biology Grade Water was added (in its 250 ml container) and swirled to mix and kept on ice.
    - To the 8 ml of 10X Annealing Buffer TW stock (in its 250 ml container), 72 ml of Molecular Biology Grade Water was added and swirled to mix and kept on ice.
b) The emulsion was drawn from each well using a 16 gauge blunt, flat tip needle attached to a 10 ml syringe.
c) About 100µl of isopropanol was added to each well and then drawn into the same syringe.
d) The collected material that drained from the well walls was drawn again, the syringe was inverted to draw 3 ml of air and the needle was discarded.
e) A blue filter was attached (without over tightening) to the syringe.
f) A Kimwipe was placed on top of the vortex platform and the syringe was vortexed for 5 sec at maximum speed, with the filter pointing toward the Kimwipe.
g) The contents of the syringe were expelled gently through the filter, into a waste jar containing bleach.

Bead Washes and Recovery for SVE

a) The beads were washed three times with isopropanol as follows:
    - The syringe was inverted and 3 ml of air was drawn.
    - Around 3-5 ml of fresh isopropanol was drawn without drawing additional air and vortexed for 5 sec (with a Kimwipe on the vortex).

- The contents were gently expelled through the filter, into a waste jar containing bleach.

b) The beads were washed three times with 1X Enhancing Fluid TW:
   - The syringe was inverted and 3 ml of air was drawn.
   - Three ml of 1X Enhancing Fluid TW was drawn without drawing additional air and vortexed for 5 sec (with a Kimwipe on the vortex).
   - The contents were gently expelled through the filter, into a waste jar containing bleach.

c) The washed beads were collected from the filter by carrying out the following steps twice:
   - The syringe was inverted and 3 ml of air was drawn and 1X Enhancing Fluid TW was drawn to the 0.5 ml mark of the syringe without drawing additional air and vortexed for 5 sec (with a Kimwipe on the vortex).
   - Promptly, the syringe was inverted and another 3 ml of air was drawn to pull the resuspended beads away from the filter.
   - The filter was removed from the syringe.
   - The bead suspensions from both collections were dispensed into a single fresh 1.7 ml tube (kept the filter after the first collection; discarded after the second collection).

Bead Recovery for LVE

Vacuum assisted Emulsion Breaking

a) The solutions - 1X Enhancing Fluid TW and 1X Annealing Buffer TW was prepared from their concentrates as described above.
b) The 50 ml conical tubes (saved the caps) were affixed to each of the two lids of the emPCR Breaking Kit LV and the blue connector inserted into the top opening of the 8-pronged transpette.
c) The other end of the tubing was connected to a vacuum source (with liquid trap).

Emulsion Collection and Initial Washes for LVE

a) The emulsions were aspirated from 8 wells at a time, using the transpette and collected into the two 50 ml tubes.

b) The PCR plates were aspirated twice with 100µl of isopropanol per well.
c) Then, 5 ml of isopropanol was aspirated to collect the beads from the tubing.
d) The two 50 ml tubes containing the amplified DNA beads were then capped after turning off the vacuum.

Bead Washes and Recovery for LVE

a) The 50 ml tube contents in pairs were mixed and isopropanol was added to a final volume of 40 ml in each tube and vortexed.
b) The beads were pelleted in a centrifuge at 930 X g for 5 min (2000 rpm in Allegra^{TM} 25 R centrifuge, rotor SX4750, Beckman Coulter^{TM} Germany).
c) The supernatant was discarded and the bead pellets were rinsed twice with 35 ml of isopropanol.
d) The bead pellets were rinsed with 35 ml of 1X Enhancing Fluid TW and the supernatant was poured slowly until the white bead pellet started moving from the bottom of the tube.
e) The beads were transferred to 2 ml tubes for each emulsion cup processed and centrifuged to remove the supernatant.
f) Each 50 ml collection tube was rinsed with 600µl of 1X Enhancing Fluid TW and was added to 2 ml tubes and centrifuged to remove the supernatant.
g) The tubes were spun for 10 sec, rotated and spun again. The supernatant was removed and the tubes were rinsed twice with 1 ml of 1X Enhancing Fluid TW and pellets were resuspended in 1 ml of 1X Enhancing Fluid TW.

DNA Library Bead Indirect Enrichment

Preparation for Indirect Enrichment

a) The heating dry-block was set to 65^0 C.
b) A stock of Melt Solution was prepared by mixing 125µl of NaOH (10 N) in 9.875 ml of MilliQ water.
c) The beads were centrifuged for 10 sec, rotated and spun again. The supernatant was discarded.
d) The ss DNA beads was prepared by melting the ds DNA amplification products by following the protocol twice:
    - One ml of Melt Solution was added per tube of beads and vortexed and incubated for 2 min.
    - The beads were pelleted in a bench top minifuge, as above.

- The supernatants were discarded.

e) The bead pellets were washed twice by centrifugation with 1 ml of 1X Annealing Buffer TW per tube each time.

f) 1X Annealing Buffer TW (45µl per tube for LVE; 30µl for SVE) and 25µl per tube for LVE (6µl per emulsion tube equivalent, in each tube of SVE beads) of Enrichment Primer were added and vortexed.

g) The tubes were placed in a heat block at 65^0 C for 5 min and then promptly cooled on ice for 2 min.

h) 800µl of 1X Enhancing Fluid TW was added and vortexed.

i) The beads were pelleted by centrifugation as above and the supernatants were removed.

j) The bead pellets were washed twice by centrifugation with 1 ml of 1X Enhancing Fluid TW per tube each time.

k) The supernatant was removed.

l) Each bead pellet was then resuspended in 800µl of 1X Enhancing Fluid TW and vortexed.

Preparation of the Enrichment Beads

a) The tube of Enrichment Beads were resuspended by vortexing for 1 min as indicated below:

- 160µl per LVE emulsion cup processed
- 20µl per SVE emulsion tube processed

b) The Enrichment Beads were pelleted in a Magnetic Particle Concentrator (MPC) and the supernatant was removed.

c) The pelleted beads (MPC) were washed and vortexed twice with 500µl of 1X Enhancing Fluid TW.

d) The supernatant was decanted.

e) Then, 320µl (LVE)/ 20µl (SVE) of 1X Enhancing Fluid TW was added and vortexed.

Enrichment of the DNA Carrying Beads

a) Eighty µl for LVE (20µl for SVE) of washed Enrichment Beads was added to each tube of amplified DNA beads.

b) They were vortexed and rotated at ambient temperature (+ 15 to + 25º C) for 5 min.

c) The tubes were placed in the MPC and incubated for 3–5 min to pellet them.
d) The tubes were capped and inverted several times.
e) The supernatants would appear milky.
f) Without drawing off any enrichment beads, the supernatants were removed from each tube.
g) The beads were washed with 1 ml of 1X Enhancing Fluid TW per tube, until there were no visible beads remaining in the supernatants (usually 6-10 washes for LVE; 3 – 6 washes for SVE) as follows:
    - 1 ml of 1X Enhancing Fluid TW was added per tube.
    - The tubes were removed from the MPC and vortexed.
    - The tubes were placed back into the MPC to pellet the beads.
    - Carefully, the supernatant was decanted from each tube.
h) The enriched washed beads were then collected.

Collection of the Enriched DNA Beads

a) The tubes were resuspended in 700µl of Melt Solution and vortexed for 5 sec and the tubes were placed back into the MPC to pellet the Enrichment Beads.
b) The supernatants, containing enriched DNA beads from two tubes (one cup for LVE) (one tube for SVE) were transferred to a separate 1.7 ml microfuge tube.
c) For LVE only: The beads were spun for 10 sec, rotated and spun again and the supernatant was discarded.
d) The steps above were repeated for better DNA bead recovery, pooling the melts from each pair of tubes (from each single tube for SVE) and discarded the tubes of spent Enrichment Beads.
e) The beads were spun for 10 sec, rotated and spun again and the supernatant was discarded.
f) The bead pellets were washed three times with 1 ml of 1X Annealing Buffer TW to completely neutralize the Melt Solution.
g) The beads were resuspended in 200µl for LVE (30µl per SVE emulsion tube equivalent) of 1X Annealing Buffer TW.

Sequencing Primer Annealing

a) 48µl for LVE (6µl per SVE emulsion tube equivalent) of Sequencing Primer was added and vortexed.

b) The tubes were placed in a heat block at 65^0 C for 5 min and then placed promptly on ice for 2 min.

c) Then, 800µl of 1X Annealing Buffer TW for LVE (500µl for SVE) was added to pellet the beads and the supernatant was removed.

d) Each of the bead pellets were washed twice with 1 ml for LVE (500µl for SVE) of 1X Annealing Buffer TW.

e) It was then resuspended in 1 ml for LVE (100µl for SVE) of 1X Annealing Buffer TW.

f) The % bead enrichment was calculated by counting 3-5µl aliquot of the beads with a particle counter using Leica Microscope (Leica DM 2500, Germany) using the formula:

$$\% \text{ Bead Enrichment} = \frac{\text{Number of enriched beads X 100}}{\text{total input beads*}}$$

where,

* 35 X 10^6 beads for LVE; 2.4 X 10^6 beads for SVE per emulsion

g) Percent enrichment values between 5 and 15% (best ~8%) are indicative of libraries yielding good sequencing results.

h) Store the beads at 4^0 C for sequencing.

The Pre-Wash the instrument

a) The Pre-wash cassette was prepared by placing the GS FLX Pre-wash Tube Holder and the Pre-wash Tubes on top of the Reagents cassette.

b) The same was loaded into the fluidics area on the sequencing instrument and the sipper manifold was lowered carefully and exterior fluidics door was closed.

c) The prewash was launched by selecting PreWash Run in the instrument.

Preparation of Bead Buffer 2 (BB2)

a) To the 200 ml of pre-chilled Titanium Bead Buffer, 1.2 ml of Titanium Supplement CB and 34µl of Apyrase were added.

b) They were mixed by gentle inversion between each addition and kept on ice.

Preparation of Pico Titer Plate (PTP) and Bead Deposition Device (BDD)

a) The PTP ID number was noted and the same was submerged in BB2 and left for at least 10 min at RT.

b) The bead loading gasket and the BDD were washed with MilliQ water and air dried.

Preparation of the Packing Beads

a) The stock (3X) solution was washed thrice with 1 ml of BB2 by spinning at 9300 X g for 5 min in a refrigerated centrifuge (Allegra^{TM} 25 R centrifuge).
b) It was resuspended in 550µl of BB2 after third wash.

Preparation of Sample and Control DNA beads

a) The number of sample DNA beads required for the gasket was determined as per the table B.2 (column 3).
b) The volume of DNA library bead suspension to use was calculated, based on the number of beads needed and the concentration of the library, in beads/µl (determined at the end of the emPCR Amplification procedure).
c) The proper volume of sample DNA library was aliquoted into clean tubes of the appropriate size (e.g. 2 ml tubes for large regions, 1.7 ml tubes for medium or M/S regions, and 0.2 ml tubes for small regions).
d) The proper amounts of control DNA Beads were added as per the table B.2 (column 4).
e) The tubes were spun for 1 min, rotated and spun again.
f) The volume of supernatant to be removed to leave the proper target volume for the gasket was calculated as per the below table B.2 (column 5).
g) The volume calculated was dispensed out from each tub

Table B.2: Preparation of DNA Beads for each bead loading gasket configuration

Loading Region Size	**PicoTiterPlate Size**	**No. of DNA Library Beads per region**	**Volume of control DNA per region**	**Target Final volume, after Centrifugation (µl)**
Medium	70 X 75 mm	790,000 (X4)	10 (X4)	30 (X4)

h) The DNA Bead Incubation Mix (DBIM) was prepared by mixing 1570µl of BB2, 150µl of Polymerase cofactor, 300 µl of DNA polymerase to yield the total volume of 2020µl.
i) The DBIM was added to each tube of DNA beads as given in table B.3.

Table B.3: Dilution of DNA Beads in DNA Bead Incubation Mix

Loading Region Size	**PicoTiter Plate Size**	**DNA beads (µl)**	**DBIM (µl)**	**Total volume (µl)**
Medium	70 X 75 mm	30 (X4)	320 (X4)	350 (X4)

j) The samples were incubated in the lab rotator for 15 mins at RT.

Preparing the Enzyme and PPiase Beads (Bead Layers 1, 3 and 4)

a) The Enzyme and PPiase Beads were vortexed and pelleted using MPC to remove the supernatants and were washed 3 times with 1 ml of BB2.
b) The beads were resuspended by addition of BB2 (1000µl) to (each tube) for the Enzyme Beads and 500µl for the PPiase beads and mixed.
c) The beads for layers 1, 3 and 4 were prepared as per below table B.4.

Table B.4 : Dilution of Enzyme and PPiase Beads for bead layers 1, 3 and 4

Bead Layer	**Kit**	**BB2 (µl)**	**Enzyme beads (µl)**	**PPiase beads (µl)**	**Total volume (µl)**
Layer 1		3250	550	-	3800
Layer 3	XLR70	2500	1300	-	3800
Layer4		3340	-	460	3800

Combining the DNA and the Packing Beads (bead layer 2)

a) After DNA bead incubation in DBIM, the appropriate volumes of washed Packing Beads and BB2 were transferred to the tubes containing the DNA beads as indicated in table B.5 and vortexed to mix the beads.
b) The tubes were placed in a lab rotator for atleast 5 min at RT.
c) The unused packing beads were discarded.

Table B.5: Preparation of bead layer 2

Loading Region Size	**PicoTiter Plate Size**	**DNA beads (µl)**	**Packing beads (µl)**	**BB2 (µl)**	**Total volume (µl)**
Medium	70 X 75 mm	350 (X4)	100 (X4)	210 (X4)	660 (X4)

Assembly of the BDD with the PTP Device and the Bead Loading Gasket

a) The soaking PTP device was removed from the tray by handling with the edges.
b) The excess BB2 was poured off and the back of the PTP device was wiped properly.
c) The BDD was assembled with the PTP device.

Depositing the Four Layers of Beads on the PTP Device

Deposition of Bead Layer 1: the Enzyme Beads Pre-Layer

a) The tube of bead layer 1 was vortexed for 5 sec.
b) Then, 660µl of bead layer 1 was loaded into each of the 4 regions of the PTP device through the port holes on the BDD top.
c) It was ensured that no air bubbles were incorporated.
d) The loaded PTP device was sealed with BDD port seals (or strips of MicroSeal).
e) The PTP along with BDD was spun at 1620 X g for 5 min (Allegra^{TM} 25 R centrifuge) using a counterweight to deposit the beads into the wells.

Deposition of Bead Layer 2: the DNA and Packing Beads

a) The supernatant of layer 1 was removed from the PTP with a pipette through the port holes on the BDD top after removing the seals.
b) The Tube of Layer 2 beads were removed from the lab rotator and 660µl of bead layer 2 was loaded into each of the 4 regions of the PTP device.
c) The loaded PTP device was sealed with BDD port seals (or strips of MicroSeal).
d) The same was spun at 1620 X g for 10 min (Allegra ^{TM} 25 R centrifuge) using a counterweight to deposit the beads into the wells.

Deposition of Bead Layer 3: the Enzyme Beads Post-Layer

a) The supernatant of layer 2 was removed from the PTP with a pipette through the port holes on the BDD top after removing the seals.
b) The tube of bead layer 3 was vortexed for 5 sec and 660µl of bead layer 3 was loaded into each of the 4 regions of the PTP device through the port holes on the BDD top (ensuring that no air bubbles were incorporated).
c) Loaded PTP device was sealed with BDD port seals (or strips of MicroSeal).

d) The BDD containing PTP was spun at 1620 X g for 5 min (Allegra ™ 25 R centrifuge) using a counterweight.

Deposition of Bead Layer 4: the PPiase Beads

a) The supernatant of layer 3 was removed from the PTP with a pipette through the port holes on the BDD top after removing the seals.
b) The tube of bead layer 4 was vortexed for 5 sec and 660µl of bead layer 4 was loaded into the regions of the PTP device through the port holes.
c) Loaded PTP device was sealed with BDD port seals (or strips of MicroSeal).
d) The same was spun at 1620 X g for 5 min (Allegra ™ 25 R centrifuge) using a counterweight to deposit the beads into the wells.

The Sequencing Run

Removing the Pre-Wash Cassette and Cleaning the Fluidics Area Deck

a) The pre-wash Reagents cassette was removed and the pre-wash tubes were discarded and the outside of the cassette were wiped.
b) The fluidics area deck was wiped with 50% ethanol and allowed to air dry.

Preparing and Loading Sequencing Reagents Cassette

a) Titanium Supplement CB (6.6 ml) and 1000µl of DTT were added to each of the 4 bottles of Titanium Buffer CB provided in the kit.
b) The bottles were mixed by gentle inversion.
c) The bottles of supplemented Titanium Buffer CB and the Sequencing Regents tray (cold) were placed in the Reagents cassette.
d) Five µl of the PPiase reagent was diluted in 45µl the Inhibitor TW reagent (position 9 of the Reagents tray).
e) The reagent supplements were added to one tube at a time, in the order shown in table B.6.

Table B.6: Supplementing Sequencing Reagents with Sodium Chlorite, PPiase, Apyrase, and dATP

Kit	**Sodium Chlorite tablet to "Post Run Wash" tube**	**Volume of Diluted PPiase to "Inhibitor TW" tube**	**Volume of Apyrase to "Buffer for Apyrase" tube**	**Volume of dATP to "Buffer for dATP (A)" tube**
XLR70	1 tablet	13.2µl	260µl	3000µl

f) The entire sequencing tray was mixed by inverting atleast 20 times till no undissolved particles could be seen.
g) The caps from all the reagent bottles and tubes were removed.
h) The reagent cassette was loaded into the instrument and the exterior fluidics door was closed
i) The spent PTP device and cartridge seal were removed from the PTP cartridge after opening the camera door.
j) The surface of the cartridge was wiped with 50% ethanol using Kimwipe and air dried.

Loading and Setting the Run Script and Other Run Parameters

a) On the instrument computer, start button was clicked to open the Run Wizard and Custom Sequencing Run Option was selected and the Next button was clicked whereby the PicoTiterPlate ID was entered in the second Run Wizard window and next button prompt was clicked.
b) The Pico TiterPlate region was selected in the Run's fifth window and next button prompt was clicked to open sixth window, to select the number of cycles appropriate for the run whereby 200 cycles (produces reads of about 350 - 450 bp) were selected.

Inserting the PTP Device and Launching Sequencing Run

a) The cartridge seal was installed after opening the camera door.
b) When centrifugation of Bead layer 4 was completed, the supernatant was removed with the help of pipettor, through the port holes in the BDD top.
c) The PTP was removed from the BDD.
d) The back was wiped clean with 50 % ethanol and allowed to air dry.
e) The PTP device was then slided into the PTP cartridge frame, face down and the camera door was closed.
f) The Start Button in the Run's Wizard last window was clicked to start the run immediately.

Data Processing during Sequencing Run

a) The GS Sequencer governs the sequencing run itself, whereby the raw images were acquired as digital images (PIF files). The raw data was processed by the GS Run Processor, which was composed of two parts: image processing (generation of pixels/ image in Composite Wells Format (CWF) Files) and

signal processing (generation of flowgrams containing read basecalls and per-base quality scores in Standard Flowgram Format (SFF) files).

b) The time taken for the completion of run was about 8-10 h.

Appendix C

Publications originated from current studies

International

1. **Singh, K. M.,** Pandya, P. R., Parnerkar, S., Tripathi, A. K., Rank, D. N., Kothari, R. K. and Joshi, C. G. Molecular identification of methanogenic archaea from Surti buffaloes (*Bubalus bubalis*) in Gujarat, India reveals more hydrogenotrophic methanogens phylotypes. *Braz J Mcicrobiology*. 2010, (In Press).
2. **Singh, K. M.,** Pandya, P. R., Parnerkar, S., Tripathi, A. K., Ramani, U., Koringa, P. G., Rank, D.N., Joshi, C.G and Kothari, R.K. Methanogenic Diversity Studies within the Rumen of Surti Buffaloes Based on Methyl Coenzyme M Reductase A (mcrA) Genes, Point to Methanobacteriales. *Pol. J. Microbiology.* 2010, **59** (3): 175-178.
3. Pandya, P. R., **Singh, K. M.,** Parnerkar, S., Tripathi, A. K., Mehta, H. H., Rank, D. N., Kothari, R. K. and Joshi, C. G. Bacterial Diversity in the rumen of Indian Sutri buffalo (*Bubalus bubalis*), assessed by 16S rDNA Sequences analysis**.** *J Appl Genetics*. 2010, **51** (3): 395-402.

Paper presented in International conference/ symposium

1. **Singh, K. M.,** Pandya, P. R., Tripathi, A. K., Koringa, P. G., Rank, D.N., Kothari, R.K. and Joshi, C.G. Quantification of Uncultured Bacteroides in buffalo rumen ecology: a possible indicator for fermentation process. An International Conference on Fluorescence in Biology. 2009, TIFR, Mumbai. March 16-19.
2. **Singh, K. M.,** Pandya, P. R., Parnerkar, S., Tripathi, A. K., Mehta, H. H., Ramani, U., Koringa, P. G., Rank, D.N., Kothari, R.K. and Joshi, C.G. Development of an Assay to Quantify Anaerobic Protozoa in Rumen Ecology. International Conference on Biomedical and Genomic Research. 2009, Gujarat University, Ahmedabad. January 29- 31.
3. **Singh, K. M.,** Pandya, P. R., Parnerkar, S., Tripathi, A. K., Mehta, H. H., Ramani, U., Koringa, P. G., Rank, D.N., Kothari, R.K. and Joshi, C.G. Diversity of Bacteria and Archaea from Ruminal Fluid of Buffalo through

Molecular Genetic Analysis of Structural and Functional Genes. International Symposium on Microbial Biotechnology: Diversity, Genomics and Metagenomics. 2008, Delhi University, New Delhi. November 18-20.

4. **Singh, K. M.,** Pandya, P. R., Parnerkar, S., Mehta, H. H., Rank, D. N., Kothari, R. K. and C. G. Joshi. Examining the Bacterial diversity in ruminal fluid of buffalo using 16S rDNA Clone Libraries. International Conference on Trends in Cellular and Molecular Biology. 2008, JNU, New Delhi. January 6 - 8.