ADDIS ABABA UNIVERSITY
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IDENTIFICATION AND QUANTIFICATION OF RUMINAL MICROORGANISMS IN BLACKHEAD OGADEN SHEEP (BHS) USING REAL TIME PCR

BY
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JUNE -23- 2009
DEBRE ZEIT, ETHIOPIA

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ACKNOWLEDGEMENTS

I am very much indebted to my advisor Dr Kelay Belihu (PHD, Associate professor) for his guidance starting from research title selection to active support of the entire work. Secondly I thank my co advisor Dr Birhan Tamir (PHD, Associate professor) for his advice and correction of paper work. I am also highly grateful to my brother Temesgen Getahun for his all round support of my success and indirectly as the output of this scientific work is attributed to him as well. I also would like to thank Faculty of Veterinary Medicine (FVM), Bureau of Agriculture and Rural Development of Amhara Regional State, National Veterinary Institute (NVI) and National Animal Health Research Centre (NAHRC) for their financial and laboratory facility contribution. Individually, Dr Gelagay Ayelet from NVI and Mr Abera Kebede from NAHRC are also credited for their coordination of the work process. Last but not the least, I forward my acknowledgment to my colleague Dr Tefera Sime for his genuine assistance of logistics during sample collection; Dr Tewodros, Tesfaye and Mr Abebe Lega for their friendly help, particularly in solving computer application; and others who provided me good encouragement while I was working this study.

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LIST OF ABBREVIATION

ARF	anaerobic rumen fungi
BHS	Blackhead Ogden Sheep
CT	Threshhold cycle
DM	Dry Matter
FAB	Fluid Associated Bacteria
IAA	International Atomic Agency
LSS	Large Sub Unit
MPN	Most Probable Number
NDF	Neutral Detergent FIBER
MPT	Tropical multi purpose trees
MT	Melting temperature
Ng	Nano Gram
OD	Optical density
PAB	Particle associated Bacteria
PCR	Polymerization Chain Reaction
rDNA	Ribosomal Deoxyribos Neuclic Acid
RF	Rumen fluid
RRNA	Ribosomal Riboxy Neuclic Acid
SSU	Small Sub Unit

ABSTRACT

This study was carried out in Jijiga and Gursum Weredas (Jijiga Administrative Zone) of the Somali Regional State of Ethiopia with the objectives of identifying and quantifying functional rumen microboal groups of Blackhead Ogaden Sheep under natural grazing system and analyzing variability of relative quantity of identified microbial groups of Blackhead Ogaden Sheep in different grazing lands and seasons. Rumen fluid samples were collected from Black Head Ogaden sheep kept in shrubs covered grazing land (Group1) and open grazing land (Group 2). Dry and wet season representing samples were also collected from G2 (DG2 and WG2). Total genomic DNA was isolated from each sample using DNeasy Blood and Tissue Kit. Using DNA Master Mix SYBR Green reaction and Bio Rad RT-PCR technique, amplification was performed for targeted ruminal microbes of general bacteria, *F. Succinogens*, *R. flavifaciens*, methanogens, total protozoa and anaerobic fungi from extracted genomic DNA. All targeted ruminal microbes were identified in every study groups except missing 1 methanogen from a single sample of WG2. Based on mean's comparison of microbial relative proportion against general bacteria, quantity of general bacteria, anaerobic fungi and *F.succinogens* were higher in sheep kept in shrubs covered grazing land than open grazing lands ($p<0.05$). In seasonal comparisons, sheep grazing during dry period exhibited higher proportion of anaerobic fungi than sheep grazing in wet period. Although statistical measurement didn't show significant variation in means, all identified ruminal microbes except methanogens were found in a little bit greater quantity in sheep grazing in shrubs covered land than those in open grazing land. Reduction of total protozoa and *F. succinogens* was also observed for shifting of sheep grazing from dry to wet season. It can be concluded from this study that shrubs covered grazing land favored abundance of general bacteria, *R. flavifacience* and anaerobic fungi in the rumen of Blackhead Ogaden sheep population reared dominantly in natural grazing system. Seasonal shifting from wet to dry increased the quantity of ruminal anaerobic fungi significantly.

Key words: Black head Ogaden, Rumen, Microbes, Characterization, Molecular, Season, Grazing land location

1. INTRODUCTION

The bovidae family (3,000 million heads) is the most diverse group (155 species) and includes the African antelope and buffalo, cattle, sheep and goats. Nutritional strategy and adaptation to feed resources have played a major role in the evolution of modern herbivores (Van Soest, 1994).

The ruminant animals play an important role especially in the livelihood of farmers in the developing world, providing sustenance as milk and meat, animal traction, manure for crop production, energy, cash income from sales of their products and a safety net of capital assets to face risks and misfortune in harsh environments (Delgado *et al*., 1999)

The major feed resources for ruminants, particularly in arid and semi-arid areas are fibrous feed stuffs of grasses, trees and shrubs and crop residuals (Leng, 1991). In ruminants, more than 60% of the total digestion and in some diets such as straw, as much as 90% takes place in the rumen (Jouany, 1994). The rumen is a highly specialized, pre-peptic adaptation of the digestive tract that facilitates the storage and microbial processing of a large quantity of plant material (Hungate, 1988). The digestion of plant material and subsequent conversion of primary ruminal products such as volatile fatty acids (VFA) for energy requirements to the host ruminant and compounds incorporated into microbial cells are performed through a synergistic act and a complex symbiotic relationship of billions of microbes within the rumen (Mackie, 1997). The situation has also opened to the ruminant food resources not available to other animals and rendered the ruminant free from the need for external sources of B-vitamins and amino acids (McDonald *et al*., 1995).

The genotypic and phenotypic diversity of the major functional groups of ruminal microorganisms is identified in different ruminant species and geographical regions (Hespell *et al*., 1997). Highly diversified rumen microbial ecosystem consisting of bacteria (1010–1011 cells/ml and representing more than 50 genera); ciliated protozoa (104–106/ml, from 25 genera); anaerobic fungi (103–105 zoospores/ml, representing 5 genera) and bacteriophages (108–109/ml) (Hobson, 1989).

In ruminal feed digestion and nutrient conversion process, bacteria and fungi contribute for some 80% of the digestion activity and protozoa balance the rest (Dijkstra and Tamminga, 1995). *R. flavefaciens, Fibrobacter succinogenese, Ruminococcus albus,* and *Butyrivibrio fibrisolvens,* are regarded as the primary fiber degrading bacteria in the rumen (Krause *et al.*, 1999c; Weimer *et al.*, 1999).

On the other hand, methane production by methane-producing *archaea* (known as methanogens) is another determinant phenomenon in rumen microbial ecosystem. Approximately 95% of methane emitted by ruminants is produced in the rumen (Australian Greenhouse Office, 2003). Mainly due to this enteric and slurry methanogenesis, livestock is the single largest source of agricultural greenhouse gas emissions, which accounts for about 16% of total atmospheric methane emissions (Boadi, D *et al*, 2004). Moreover, methanogenesis in the rumen represents a 2–12% energy loss of intake (Giger-Reverdin and Sauvant, 2000). Apart from this, greater than 50% of the dietary fiber passes through the digestive tract in an undegraded form (Cherney *et al.*, 1991).

Aiming at improving efficiency of post-ingestion digestion of feeds and ultimately to improve productivity in ruminants, several direct physico-chemical treatment of the feed (Chesson, 1993; Lopez-Guisa and Satter, 1992; Schwab, 1995) and various indirect biotechnological manipulation of rumen fermentation are currently being employed to modify the composition and activities of the rumen microbial population, increasing propionate concentration in the rumen, depression of methane production and decreasing dietary protein degradation by bacteria (Jouany, 1994; Van Nevel and Demeyer, 1977; Flint and Scott, 2000).

To bring such interventions into effect, characterization of microbes and various ruminal parameters under different dietary conditions is crucially important task (Zoetendal *et al.*, 2004). In the early time of rumen microbiology, microbial diversity and enumeration enquiries were monitored by cultivation based techniques. But due to limitations inherent in the technique (Stewart *et al.*, 1997), molecular-based approaches have been adapted recently and resulting in much more comprehensive description of microorganisms. Researchers have shown that this technique can be used successfully on samples extracted from rumen contents to monitor populations in the rumen (Tajima *et al.*, 2001; Klieve *et al.*, 2003).

However, since the issue is yet growing and the infancy stage of new techniques, studies have been yet limited to some advanced regions (countries). Scope of most studies is focusing on few (one or two) microorganisms separately than including more major functional groups or microbial community at once; and the monitoring is with respect of testing specific dietary trials on station farm ahead of establishing thorough knowledge of rumen under natural condition in different geographical regions. The microbiota of rumen is highly responsive to changes in diet including additives, host, and health of the host animal, geographic location and season (Dehority and Orpin, 1997)

In the production systems of the semi-arid areas, ruminant animals in general and particularly Blackhead Ogaden sheep graze extensively on low quality naturally growing forages as the basal diet (Wilkins, 2000). The quality and quantity of these forages become more critical in the dry seasons and thereby imposing more serious constraint to the development and productivity of these animals (Topps, 1992). Their digestion in the rumen is slow and a prolonged retention-time limits the feed intake (Ørskov, 1995). Different from diets in temperate climates, tropical diets are typically limited in protein and fermentable energy content resulting in suboptimal microbial growth (Leng, 1990). The nutritional status of ruminants depends mainly on the ability of rumen fermentation by microbes (Preston and Leng, 1987).

A wide variety of shrubs and trees species are found being the most visible forms in many of these desert landscapes (McKell, 1980). Their importance increases in arid areas (Getachew, 2002). According to Belaynesh (2006) rangeland assessment of jijiga zone, 76% of the individual trees and shrub species in the sample sites belonged to the lowest height classes (i.e. < 5m), which are accessible for small ruminants. In areas with low stocking rate, sheep rely on browsing up to 20% of their consumption demand (Wilson *et al.*, 1975). The utilization of many is rather opportunistic (Kallah *et al,* 2000). Many of the shrubs and trees are problematic as feed supplements because they often contain anti-nutritional compounds such as tannins, saponins and non-protein amino acids, which are either toxic to rumen microbes or to the animal by themselves or their metabolic products (D'Mello, 1992; Lowry *et al*., 1996).

On the other hand, this is good fate since plant extracts with high concentration of secondary metabolites are good candidates for achieving one or more of the objectives of manipulation of rumen microbial ecosystem by mitigating methane emission and by enhancing nitrogen excretion by ruminants (Teferedegn, 2000). However, some studies show that the ruminants like the feral goats, sheep and camel, which continuously feed upon tanniferousus diets usually develop tolerant microflora (Wiryawan *et al.*, 1999; Teferedegne *et al.*, 1999; Odenyo and Osuji, 1997). BHS survive under scares, lignified and anti-nutritive enriched plants by providing substantial uses for the livelihood of pastoralists for long period of time. Based on these marvel situations, it inspires to hypothesize that Blackhead Ogaden Sheep could have developed different microbial diversity and composition for the sake of adaptation to constraints mentioned above.

The objectives of this study were thus:

- To identify and quantify functional rumen microboal groups of Blackhead Ogaden Sheep under natural grazing system;
- To analyze variability of relative quantity of identified microbial groups of Blackhead Ogaden Sheep in different grazing lands and seasonal classes.

2. LITERATURE REVIEW

2.1 Rumen ecology

The stomach consists of non secretary fore stomach and a secretary stomach abomasum). The fore stomach (reticulum, rumen and omasum) serves as a microbial fermentation vat of ingesta mainly by hydrolysis and anaerobic oxidation. Abomasum is largely concerned with hydrolysis of protein by pepsin and acid medium (Kellems and Church ,1998). The rumen is an open, dynamic, stable, a very diverse and complex microbial ecosystem. As of the genotypic and phenotypic mounting evidence, diversified major functional groups of ruminal microorganisms are hosted in different ruminant species and geographical regions. (Hespell et al., 1997

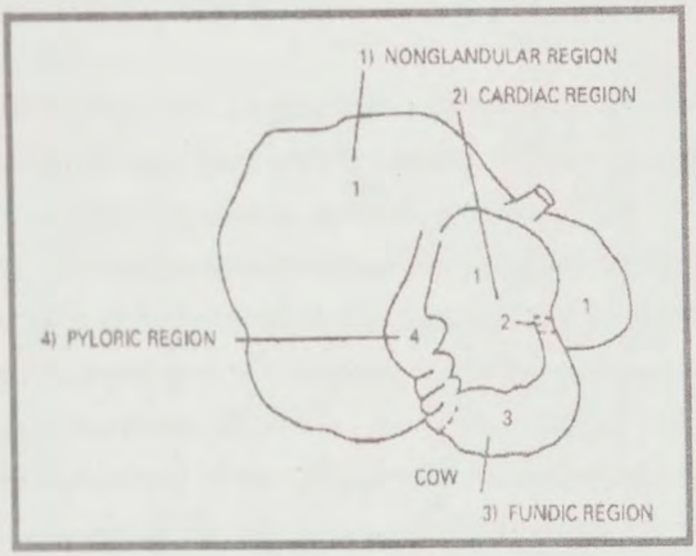

Figure 1: Schematic diagram of the chambered stomach (Allison.1993)

The rumen ecosystem comprises a diverse population of obligately anaerobic bacteria, fungi and protozoa and it is defined by the intense selective pressures of the ruminal environment. These microbial symbionts are adapted to survival under conditions of anaerobiosis, high dilution rates, high cell densities and protozoal predation, and have evolved the capacity for efficient utilization of complex and recalcitrant plant polymers such as cellulose and hemicelluloses. Degradation and utilization of ingested plant material by ruminants is regulated by plant, animal and microbial factors (Forsberg C.W. and Cheng K.-J. 1992).

Studies have shown that during rumen development, ingested microbes colonize and establish in a defined and progressive sequence of obligatory anaerobic, facultative anaerobic and aerobic bacteria (Morvan .B, *etal.* ,1994) .Microbial colonization of the digestive systems of newborn ruminants follows a typical ecological succession in that bacteria proliferate in the fluid phase immediately after birth, colonize the digestive tract tissue and facilitate the subsequent sequential colonization of the fluid by fungi and protozoa (Cheng *et al.*,1991).

As the rumen develops the levels of facultative anaerobes and aerobes decrease and the populations of obligate anaerobes increase. As a result, the composition of the ruminal microflora in adult animals is quite different to that in pre-ruminants (Krause D.O *et al*, 2000).The formation of complex multi-species consortia is a prerequisite to the microbial digestive activities in mature ruminants (Fonty *et al.*, 1988;). Hungate, *et al*. (1988) described the cooperative host-microbial symbiosis in ruminants in which the microbes produce and gain access to fibre with enzymes not produced by the host animal (Faichney G J.,*et al*, 1997).

Microbial digestion and synthesis of microbial components in the rumen requires certain conditions provided by the host. These include retention of digesta and ruminal microbes for prolonged periods of time, anaerobiosis, constant temperature (39 c^0), neutral to slightly acidic pH (5.5-7.0), and removal of end products (Stevens *et al.*, 1980). Reduction of carbon dioxide to methane by using hydrogen as the electron donor 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). In most circumstances, this environment is closely controlled by mechanisms such as the type and quantity of food consumed, saliva secretion during eating, mixing via ruminal contractions, diffusion/secretion of materials (urea, bicarbonate) into the rumen; absorption of end products (VFA, NH3) and passage of undigested residues and microbial cells out of the rumen (Leng, 1973).

In ruminants, more than 60% of the total digestion and in some diets such as straw as much as 90% takes place in the rumen (Jouany, 1994). Ruminal microbes are well adapted to ferment a variety of carbohydrates including sugars, starches, and the complex polysaccharides in plant cell walls (cellulose, hemicelluloses, pectin etc.). The end products of fermentation of carbohydrates by the mixed population of ruminal microorganisms are the three primary VFA (acetic, butyric and propionic acids) and by products such as H_2, methane and carbon dioxide too (Van Soest, 1994).

A portion of the dietary protein is also degraded and fermented by ruminal microbes with the resultant production of additional VFA and of smaller amounts of branched chain fatty acids (isovalerate, isobutyrate and 2-methylbutyrate) and ammonia (Van Soest, 1994). From the symbiotic degradation of injesta process, both mirobes and hosting animal benefits for nutrients (Faichney GJ.et al, 1997).

In general, about 75% of the energy in the fermented substrate (carbohydrate) is recovered as VFA (Ulyatt *et al.*, 1975) and provide 50-80% of the total metabolisable energy available to the host being absorbed in the rumen. (Merchen and Bourquin, 1994).Digestion by fermentation is a costly since microbial metabolism of carbohydrate to produce VFA is accompanied by significant losses of energy as methane and heat (Reid *et al.*, 1980). Methanogenesis in the rumen represents a 2–12% energy loss of intake (Giger-Reverdin and Sauvant, 2000). Apart from the above methanogenesis !ose, greater than 50% of the dietary fiber passes through the digestive tract in an un degraded form (Cherney *et al.*, 1991) Several methods are currently employed to manipulate rumen fermentation to enhance post-ingestion nutritive value of fibrous forages through use of biotechnology including inoculants of native and recombinant rumen microorganisms, natural adaptation and microbial feed enzymes (Flint and Scott, 2000)

2.2 Functional microbial groups in the rumen

The diversity of microorganisms in the rumen is extensive and comprises bacteria, fungi, protozoa (Hespell et al, 1997), and even phage (Klieve A V, Bauchop T, 1993).

2.2.1 Cellulolytic Bacteria

The major cellulolytic bacteria in the rumen are *Butyrivibrio fibrisolvens, Ruminococcus albus, Ruminococcus. flavefaciens*, and *Fibrobacter succinogenes*. The first three species are Gram positive but *F. succinogenes* is Gram-negative and together with the ruminococci are the most fibrolytic and can potentially be manipulated genetically, or ecologically, for increased ruminal cellulolysis. (Chen J, *et al*, 2004). The number Gram-positive bacteria tend to increase on increasing high energy (concentrated) diets in the ration.

In cellulose degrading bacteria, the presence of maximum number of were represented by *Ruminococcus albus* and *R. flavefaciens* (59.8%) *Bacteroides succinogenes (Fibrobacter succinogenes)* (19.2%), *Butyrivibrio fibrisolvens* (11.1%), *Clostridium lochheadii* (3.8%) and *C. longisporum* (1.3%). The study on buffaloes showed that the cellulose-degrading bacteria get stimulated (57.4% higher numbers than that in control) when buffaloes are fed *Saccharomyces cerevisiae* within their diets (Kumar U. *et al.*, 1997). In the study of Krause, *et al* (1999), the total combined signal from *R. albus, R. flavefaciens*, and *F. succinogenes* was 4.0% of the total bacterial 16S rRNA. These data were in general agreement with total cellulolytic counts (5.2% of total culturable counts or 3.1% of direct counts). In the study of Michalet-Doreau *et al* (2001), *F. succinogenes* and *R. flavefaciens* were more enriched in PAB and firmly adherent PAB populations when compared with the total bacteria (which included FAB), whereas rRNA from R. albus was comparable in all phases

Ruminococcus species

Cellulolytic ruminococci are gram-positive and can be divided into two mains species; *R. albus and R. flavefaciens* (Hespell R B. *et al., 1997*). It is difficult to divide these two species on the basis of morphology or phenotype but the ability of R. flavefaciens to produce succinate as a major end-product seems to be consistent with their phylogenetic position (Krause D O *.et aL.*, 1998). the ability to digest dry matter (DM) or neutral detergent fibre (NDF) of rhodes grass (*Chloris gayana*), spear grass or lucerne (*Medicago sativa*) of ruminococci strains was found varied greatly (Krause D O . *et a.l*, 1998). The "true" ruminococci is the type of cellulolytic strain *R. flavefaciens C94* (Krause DO. *et al.*, 1998). They are placed within cluster IV of the *colostridia* (Collins *et al.*, 1994) with *R. albus*, and *R. callidus*. But, several apparently non-cellulolytic ruminococci (R. *torques, R. gnavus, R. obeum*, and *R. productus*) are in cluster XIV. Total cellulolytic Ruminococcus have been estimated to be between 4% (Latham M. J. *et al.*, 1971), and 11% (Latham M. J. *et al.*, 1972) of the total ruminal population. Hybridization to rRNA from sheep fed a predominantly tropical grass diet (Rhodes grass) indicated that ruminococci were approximately 5% of the population.

Fibofacter succinogenes

Universal bacterial primers amplified the 16S rDNA sequence of *F. succinogenes* from pure cultures of this species consistently (Tajima *et al.*, 1999; Larue *et al.*, 2004). *Fibrobacter succinogenes* had a higher threshold cycle (even above15th cycle) from real-time polymerase chain reaction (PCR) than other bacteria. Based on Michalet-Doreau *et al* (2001), F. succinogenes was detected using specific primers, but *F. flavefaciens* have been poorly represented or virtually devoid in clone libraries constructed using universal primers (Larue *et al*, 2004).

The absence of *F. succinogenes*-like sequences in clone libraries is not due to the lack of this species in the rumen sample or due to mismatches of primers. The under-representation of these microbes, particularly *F. flavefaciens*, in many clone libraries of rumen fluid would be that fluid associated bacteria (FAB) to be less enriched with fibrolytics than would the particle associated bacteria (PAB) (Firkins J.L and Yu Z., 2007).

Butyrivibrio fibrisolvens

Butyrivibrios were first isolated by Hungate R.E (1950) and fermented cellulose poorly. Bryant M.P and Burkey L.A (1953) subsequently characterized similar isolates as gram-negative, butyrate producing rods. As of more recent 16S rDNA analysis, the DNA-DNA hybridization results that *B. fibrisolvens* is polyphyletic (Forster R.J *et al.*, 1996) and all strains sequenced so far fall within cluster XIV of the *colostridia* (Collins *et al.*, 1994) of which most are non cellulolytic. When hybridized with rRNA from sheep, deer and cattle consuming high forage diets, *B. fibrosolvens* made up less than 1% of the population (Forster *et al., 1997*). Recently these same probes showed that *B. fibrisolvens* was less than 2% of the ruminal population in sheep eating a tanniniferous diet (Forster *et al.*, 1996). Traditional microbiological enumeration estimates of *B. fibrisolvens* in rumen vary from 2% on a high concentrate diet to as high as 38% on high forage diet (Latham M J.*etal*., 1972). However, culturable numbers have probably been overestimated and studies have used growth on glycerol tributyrate (Latham M J. *et al.*, 1971) or motility (Van Gylswyk N.O, 1970) to presumptively identify *B.fibrisolvens*. When a group of 22 *B. fibrisolvens* strains were assessed for their ability to digest spear grass (*Heteropogon contortus*) few could digest more than 10% of the dry (DM) (McSweeney C. S.,1998)which indicate their lower role in fibrolytic activity.

2.2.2 Methanogrns

Methane-producing Achaea (known as methanogens) is a distinct group of organisms which are a normal component of the animal gut microbial ecosystem. Methanogens residing in the animal gastrointestinal tract belong to the genera Methanobacterium, Methanobrevibacter, Methanosphaera, Methanomicrobium, Methanogenium, and Methanosarcina (Miller and Lin, 2002). The most predominant species of methanogens in the animals' gut are related to the genus Methanobrevibacter (Garcia *et al.*, 2000). In Australian Sheep grazing at pasture more diversified methanogens had been found than sheep fed either the oaten hay or lucerne hay diet (Andre´-Denis G. W. *et al.*, 2003)

A cellulolytic bacterium strain of Ruminococcus flavefaciens was found to form syntrophic associations with methanogens by producing H_2 (Wolin *et al.*, 1997). A few non-methanogenic species of group *Achaea* have also been isolated from animals (Boopathy, 1996). Population densities reached around 104 methanogens per gram at 1 week of age and increased in an exponential manner to a maximum of 108–109 per gram at 3 weeks of age. Methanogens comprise approximately 0.5 to 3% of the total population of microflora in steers, cows, sheep, pigs, and goats (Lin *et al.*, 1997). Most methanogens obtain energy by reduction of carbon dioxide, to methane by using hydrogen as the electron donor. Methanogenesis accounts for 2 - 12% of the dietary energy loss in ruminants (Giger-Reverdin and Sauvant, 2000).

2.2.3 Rumen protozoa (ciliate)

✓ The ciliate protozoa are the dominant class of total protozoa and naturally occurring ones in all domesticated ruminants (Williams and Coleman, 1997). Ciliate protozoa become established in the rumen of young animals in the first weeks of life through maternal contact and by transfer from other members of the flock or herd (Cheng, *et al.*, 1991).

Protozoa are less numerous than the bacteria but account for 50% of the total rumen microbial mass, owing to their large size (Jouany and Ushida, 1999). Ruminal ciliate protozoa are categorized as entodiniomorphs and holotrichs based on their physical properties and metabolism. The entodinomorphs form the majority of the protozoal community in the rumen (about93.3% the protozoa population) feed principally by engulfment of particulate matter like bacteria (which serve as the major protein source). They have also enzymes that attack cellulose, hemicelluloses. Holotrichs depend on nonstructural polysaccharides, especially, starches and soluble sugars (Allison, *et al.,* 1993).

Approximately one-quarter to one-third of fibre degradation in the rumen of normally faunated animals is due to protozoa (Orpin, 1984).The protozoa are considered to have an important role in determining the density of some microbial groups like methanogens and cellulolytic bacteria and the transformation state of dietary in the rumen ecosystem through either directly their own enzymes or indirectly as a consequence of their effects on engulfment of other fibrolytic microorganisms present. (Williams and Withers, 1991)

2.2.4 Fungi

Five genera of anaerobic chhytridomycetous fungi have been isolated from the rumen: *Anaeromyces*, *Caecomyces*, *Neocallomastix*, *Orpinomyces* and *Piromyces* (Ho and Barr, 1995) and are considered to be involved in fibre refractory degradation (Hespell *et al.,* 1997). Cellulases and xylanases are the enzymes produced by these fungi and are the most active fibrolytic enzymes (Trinci *et al.,* 1994). Remarkably high activity *xylanases* have been isolated from *Neocallimastix patriciarum* and *from Orpinomyces joyonii* (Gilbert *et al*., 1992)

Fungi are strictly anaerobic and their life cycle includes a motile phase (as a zoospore) and a vegetative phase tallus (sporangium) (Orpin, 1977). During the vegetative phase, they become established on feed particles using rhizoids, which can penetrate cell walls (Orpin and Joblin, 1997). Rumen fungi account for approximately 8% of the microbial biomass in the rumen based on zoospores count (Orpin and Joblin, 1988). Based on the oligonucleotide probes developed for fungi (Faichney *et al*., 1997) analysis, the rumen anaerobic fungi constituted only 1-4% of microbial biomass, which is less than it was originally thought to be (8%). Real time PCR performed on a10 nanogram (ng) of fungal genomic DNA at each time point produced a consistent cycle threshold (Ct) value of 13.82 ± 0.09 (mean ± SEM) for the polycentric isolate and 14.74 ± 0.27(mean ± SEM) for the monocentric isolates (Stuart E. Denman and Christopher S. McSweeney,)

The unique functional role of fungi appears to be their capacity to degrade the plant cuticle, which is a protective barrier on the outside of the surface of leaves and stems (Akin and Rigsby, 1987). This presumably enables ruminal fungi to gain access to plant polysaccharides not available to cellulolytic bacteria. Ruminal fungi possess extremely active cellulases and hemicellulases (Mountfort and Asher, 1989). Fungi attack the recalcitrant cell walls of plant fibre using rehizoids (monocentric species) or rhizomycelia (polycentric species) that penetrate the fibre and extract cell wall components (Akin and Benner, 1988). The rumen fungi also possess proteolytic activity. The proteolytic action of fungi facilitates the bacterial penetration of proteinaceous plant tissue by the expense of fungal rhizoids (Wallace and Joblin, 1985); this layer is known to prevent the cellulolytic bacteria from gaining access to the secondary cell wall (Engels and Brice, 1985). The ability to penetrate and colonize fibre and the production of highly degradative enzymes strongly suggests that ruminal fungi could have an important role in the digestion of fibrous feed of ruminants. The contribution of the rumen fungi to the fermentation of feed in vivo has not yet been quantified; however, the presence of anaerobic rumen fungi was found to increase digestibility of fibre by up to 40% compared to animals without rumen fungi (Gordon and Phillips, 1993).

2.3 Interaction of microbes

It has been known for some time that protozoa predate upon and compete for substrate with the Bacteria as well as having synergistic relationships with the methanogenic *Archaea* (Williams & Coleman, 1992), but the complexity of interactions among these microbes has not been well characterized in vivo. Based on in vitro experiments, some protozoa exhibit selective predation on different species of bacteria (Dehority, 2003) including preference toward cellulolytics (Koenig *et al*, 2000). Defaunation of sheep, though, did not significantly change numbers of culturable cellulolytic bacteria, whereas refaunation decreased the cellulolytics below those observed in the original faunation period (Koenig *et al.*, 2000). The FAB has been proposed to be physically more susceptible to predation than PAB (McAllister *et al.*, 1994), which tend to contain more adherent cellulolytics. So interactions between protozoa and bacteria might be more complex in vivo than in vitro. Harboring prokaryotic populations both extra- and intra-cellularly, protozoa have well established syntrophic interactions with bacteria and especially methanogens (Hegarty, 1999).

The metabolic and Predator-prey interactions between different rumen microbes are essential in sustaining the rumen microbial community and its collective activity (Wolin, 1990). In "real ecosystems" microorganisms exist and interact as members of consortia, and it is the phenotypes expressed as members of consortia that ultimately determine functionality (Caldwell D.E. *et al.*, 1997).

As the rumen is inhabited with a dense and diverse group of microorganisms that have different metabolic capabilities, the interactions between the organisms is complex. The nature of interspecies interactions and the extent of mutual dependence vary enormously, ranging from simple metabolically cross-feeding to very specialized total dependence for nutrients, such as vitamins, N, carbon and energy sources (Wolin *et al.*, 1997). The engulfment and digestion of bacteria by protozoa is the most obvious predator -prey interaction in the rumen (Williams and Coleman, 1992).

The fibrolytic activity of rumen fungi is enhanced by hydrogen-utilizing methanogens (Joblin, 1990), which decrease the repressive effect of hydrogen pressure in the rumen (Orpin and Joblin, 1988). A cellulolytic bacterium strain of Ruminococcus flavefaciens was found to form syntrophic associations with methanogens by producing H_2 (Wolin *et al.*, 1997). The slow generation time and repressive effect of some bacteria like Ruminococcus species on the growth of fungi (Stewart *et al.*, 1992) might be the reason for their lower numbers in rumen. A correlation was observed between the numbers of methanogens and those of cellulolytic microorganisms in fresh rumen samples of sheep, cattle, deer, llama, and cecal contents from horses (Morvan *et al.*, 1996).

2.4 Factors affecting composition of ruminal microbes

The composition and proportion of microbes are influenced by external factors such as diet, feeding frequency, age, geographical location, season and ruminant host interaction and others (Tajima *et al.*, 2001).

2.4.1 Feed

Type and status feed influences composition and proportion of microbial community. Sequences clustering of *Treponema* (*Spirochaetes* phylum) were found only in cows fed a hay diet, and those clustering with *Ruminobacter amylophilus* (*Proteobacteria* phylum) were greatly increased in cows fed a grain-based diet (Kocherginskaya *et al.*, 2001). et al (2000) noted increased frequencies of sequences similar to known lactate-producing and lactate-utilizing bacteria three days after a shift to a high-grain diet. several sequences associated with cellulolytic ruminococci in were detected prior to the diet shift, but none were detected thereafter or their number decreases as Tajima reported. Quantity of these fibrolytic bacteria is higher during fibrous feeds and lower in shifting to concentrate rich feed stuffs. *F. succinogenes* (formerly *Bacteroides succinogenes), R. flavefaciens* and *R. albus* generally considered to be primarily responsible for degradation of plant cell walls in the rumen (Cheng K. J. *et al.*, 1991). The phase of rumen content, being solid or fluid also influences distribution of some FAB or PAB bacteria due to substrate affinity. In in vitro experiment of fibre fermentaton, Tajima et *al.*, (1999) noted lower prevalence of *Firmicutes* in rumen fluid where as *Prevotellas* were found fairly and equally distributed between solid and liquid phases. Additionally, only solids revealed *R. flavefaciens*, and only the fluid revealed *S. ruminantium* and *Proteobacteria.* In the trend of feeding roughages increasingly resulted in the detection of more Ruminococcus and *Butyrivibriosome* secondary colonizers apparently use degradation products from primary colonizers as substrate (Wells *et al.*, 1995).

In various studies, foliage (Newbold *et al.*, 1997;Odenyo *et al.*, 1997 and Teferedegne *et al.*,1999) as well as fruits of tropical multipurpose trees(MPT) (Hess *et al.*, 2003 and Patra *et al.*, 2006) have been reported to be able to suppress ruminal protozoa population due to result of their plant secondary metabolites(Hu *et al.*,2006). A potential methane-suppressing effect of specific tropical plants could result from direct effects on the methane-forming microbes, the methanogenic *archaea*, or from indirect effects on the ruminal protozoa population which are, besides the cellulolytic bacteria, the major hydrogen suppliers to the methanogens (Ushida andJouany, 1996). Wang *et al.*, (2000), additionally found that cellulose digestion by Ruminococcus spp. and *Fibrobacter succinogenes* is inhibited by sapponins. Another potentially very important observation is that the anaerobic rumen fungi, *Neocallimastix frontalis* and *Piromyces rhizinflata,* are highly sensitive to *Yucca schidigera* (Wang *et al.*, 2000).

Recent molecular approaches have revealed that the composition of methanogens in animal gastrointestinal can vary with different diets. By observing 16S rRNA sequences, pasture-grazing sheep had been found greater methanogen diversity than sheep fed either an oaten hay or lucerne hay (Wright *et al.*, 2004). Dicarboxylic acids, including aspartate, malate and fumarate, which are potential precursors of propionate, or butyrate may act as electron sinks competing with methanogens for the available H_2 (Newbold, C.J. *et al.*, 2005).

A decrease in protozoal numbers has been reported in the rumen of sheep infused with pure lucerne saponins (Odenyo *et al.*, 1997) and Enterolobium cyclocarpum (Navas-Camacho *et al.*, 1993). In contrast to these findings, Van Nevel & Demeyer (1990) have found no indication of any toxic effects or effects of sarsaponin on protozoa growth or protein breakdown in vitro. In the presence of lucerne saponins the bacterial number remained unaffected, but the population is changed from a morphologically-diverse one to fewer morphotypes (Lu *et al.,* 1987). In other study,Lucerne saponins have been found to cause a decrease in the efficiency of microbial protein synthesis in sheep, because the growth of bacteria as well as that of protozoa is depressed (Lu & Jorgensen, 1987).

In vitro experiment of *Sasbania sesban* foliage fermentation by adding rumen fluid from sheep of UK and Ethiopian breed, inhibition of protozoan activity occurred in the former but not in the latter (Teferedegne *et al.*, 1999). They have demonstrated clearly the time dependence of the detoxification process showing that adaptation occurs in animals receiving dietary *S. sesban* such that the antiprotozoal component is destroyed more quickly than in control sheep. Factors such as feeding frequency might influence in vivo responses (Oldick & Firkins, 2000). It was found that anaerobic rumen fungi (ARF) were not detectable on Sulpher (S) deficient *Digitaria sp* (Gordon *et al.*, 1984). Calcium seems to be essential for the growth of *Fibrobacter* (formerly known as *Bacteroides) succinogenes* and to stimulate growth of other species particularly the fibrolytic organisms by supporting a greater production of the isoacids, isobutyrate and isovalerate.(North Carolina State University Report, 2004-2005)

2.4.2 Host factor (genotype, species, age)

Host specificity has been demonstrated to affect the colonization ability of certain microflora since one microorganism or strain from one animal may fail to colonize another animal. In spite of same breed, some individual rats in one study resist methanogenic colonization (Florin *et al.*, 2000).The negative or positive response of protozoal defaunation to *s.sesban* in Ethiopian and British breeds respectively could indicate host factor (Teferedegne *et al.*, 1999). Some compounds that are present in gastrointestinal tracts are necessary for growth of methanogens. For example, Methanomicrobium mobile requires a heat-stable factor that is available in bovine rumen fluid (Tanner *et al.*, 1988). Taylor *et al.*, (1974) also found that coenzyme M, a growth factor presenting in rumen fluid, is essential for growth of *Methanobrevibacter ruminantium.* Age difference also affects colonization of rumen by different microorganisms.

2.6 Isolation and Quantification Methods

2.6.1 Traditional enumeration of ruminal microbes

Microbiologists in the 1950's to 1980's worked out cultivation procedures to grow bacteria from the rumen (Dehority, 2003) and specific growth factors, products formed, and other landmark types of information have been gleaned using this approach. Many types of cross-feeding and synergistic relationships have been identified (Wolin *et al.*, 1997). But most examples of community ecology really can't be mimicked using standard isolation procedures (Tajima *et al.*, 1999). Sampling from the rumen is probably even more difficult than for bacteria because of the cyclic chemotaxis of some protozoa, especially *isotrichids*, toward sugars after the animal is fed, followed by settling to the ventral rumen or reticulum (Dehority, 2003), the site from which sampling is harder, particularly from sheep owing to have smaller rumen size. Despite the standardization of counting procedures, variation in protozoal counts among animals is high. Morphology-based speciation and enumeration is also very difficult as enormous but functionally minor microbial species can be found this method. Some protozoa species may simply to have only morphological variants of the same operational taxonomic unit (Dehority, 1994). Sample dilution to yield a targeted number of cells might result in inaccurate and imprecise enumeration of minor species because the latter are often so diluted out that they are infrequently detected per microscopic field resulting in reduced and quite variable in protozoa cell number to biomass ratio. Hence, protozoa enumeration in the rumen still is problematic

Enumeration of bacterial species or groups based on selective media (Dehority, 2003) has several limitations too, including 1) viable but non-dividing cells of culturable species (Leedle *et al.*, 1982), 2) colony-forming units that were actually initiated by clumps of cells (Dehority, 2003), 3) inability to grow some or perhaps the majority of species in laboratory media (Wells & Russell, 1996), 4) the statistical variation inherent with cultivation-based procedures.

Many authors have assumed that the difference between total direct counts and total viable counts of bacteria is largely attributed to nonculturable bacteria in the samples (Wells & Russell, 1996; Zoetendal *et al.*, 2004) because of potentially confounding factors like not long enough incubation prior to growth scoring (colony counting) (Dehority, 2003) and lyses due to inappropriate amounts of substrate (Wells & Russell, 1996) Estimates of the extent of ruminal bacterial diversity cultured is as little as 10% (Krause D.O., and Russell J.B.,1996).

In contrast, Krause *et al.*, (1999) argued that cellulolytic most-probable number (MPN) probably overestimates the truly cellulolytic population because of the presence of cellulodextrin degraders that cannot degrade polymeric cellulose. MPN enumeration, while being much more agreeable for use in animal experiments, had less precision than actual counting methods (Dehority *et al.*, 1989) and can lead to inaccuracy in quantification of bacterial populations, depending on the type of MPN statistics used (McBride *et al.*, 2003).

Although much useful information has been generated from co-cultures of bacteria, extrapolation of data to in vivo situations might not be appropriate (Dehority, 2003). For polymeric cellulose as substrate, bacterial growth rate is probably dependent on the surface area for bacterial adhesion (Fields *et al.*, 2000), which increases and then decreases after provision of substrate. The growth rate (μ) depends on the substrate concentration (S) and the affinity toward that substrate (K_s) besides to the maximum growth rate (μ_{max}) (Dijkstra *et al.*, 1998) indicating fluctuation of microbial number consistency due to growth media factor. Apart from this, a small portion of the ruminal bacteria can only be grown in laboratory media (Stewart *et al.*, 1997). The inability to grow all ruminal microbes has therefore limited understanding of ruminal microbial communities, such as diversity, population composition, and functionality

2.6.2 Quantification of microbial populations by molecular biology

The circumvention of cultivation limitations requires culture-independent methods. Recently, molecular approaches have provided an alternative means of investigating the gastrointestinal ecosystem without bacteriological culture methodology (Eckburg *et al.*, 2005). Roselló-Mora and Amann (2001) argued that "chemotaxonomy" should be used in conjunction with genomic information for a more complete "phylo-phenetic" characterisation of microbial populations. Concerning phylogeny sorting, the large subunit (LSU) ribosomal RNA (rRNA) and the intergenic spacer region between the LSU and small subunit (SSU) rRNA probably contain more phylogenetic information than SSU rRNA (Roselló-Mora and Amann, 2001). But, the latter received much more attention because of earlier sequencing limitations and subsequent accumulation of large numbers of SSU rRNA sequences deposited in public databases. The large amount of SSU rRNA or the corresponding gene (rDNA) sequence information continues to drive current research approaches. The combination usage of molecular biology techniques by cloning and random selection of clones for sequencing of SSU rRNA genes with other emerging technologies (Polz *et al.*, 2003; Zoetendal et al, 2004), has been used more frequently to date to uncover diversity of microbes in the rumen (Firkins J.L.,Yu Z., 2006).

The majority of microbes in the rumen elude pure culture, and their existence is identified only from phylogenetic marker sequences (almost exclusively rRNA or its coding sequence, rDNA). Sequencing of SSU rRNA genes has become a standard procedure for the identification of isolates and it is now impossible to adequately describe microbial communities without SSU rRNA sequence data. Dendrograms from rRNA or rDNA sequences generally provide relatively stable clusters, although the relative degree of branching depends on the method used (Rosselló-Mora & Amann, 2001). A species has been defined anywhere between 95 and 99% SSU rRNA sequence identity (Zoetendal *et al.*, 2004). Rosselló and Amman (2001) discussed standardization of a species at 70% DNA similarity, which corresponds with approximately 97% rDNA similarity, although they cautioned that a species should not be distinguished solely on rRNA or rDNA sequence information. Relative abundance of some microbe species is variable when comparing rRNA and RDNA data among studies. Differences among studies could be explained by the various PCR conditions (Tajima *et al.*, 2001), including choice of primers or cloning vector (Von Wintzingerode *et al.*, 1997), or from host factors that differ among experiments.

An increasingly large amount of SSU sequence data are available in public databases to facilitate identification of rRNA sequences retrieved from rumen samples; the problem is that almost all sequences derived from their study were >90% identical to those in the databases, but many of these database sequences were derived from uncultured bacteria(Tajima et al 2000) . As of Krause *et al.*, (1999) genotypic and phenotypic diversity evaluation among Ruminococcus strains of *R. albus* and *R. flavefaciens*, 4 of the 23 strains significantly changed the grouping when the data were based on digestibility of cellulose highlighting the need for characterization of integrated techniques for uncultured strains.

By genes sequencing and random selection of clones, selected data are reported showing the diversity and community composition (Krause and Russell, 1996). rDNA phylogenic clusters are constructed from Rumen fluid (Whitford *et al.*, 1998),and from fractionated rumen fluid and particle samples using clone libraries (Larue *et al.*, 2004).

Various RNA- and DNA-based approaches have been used and are continuing to evolve to provide the tools to change and improve our perspectives about ruminal microbial communities in vivo. The particle-associated bacteria (PAB) are considered the most important group of bacteria mediating fibrolysis in the rumen but also are the hardest from which to obtain a truly representative sample, which is needed to accurately quantify bacterial N flows to the duodenum (Volden, 1999). Molecular-based procedures can help verify that the PAB samples collected using various buffers truly represent the entire PAB population, including those that won't detach without significant lysis (Balcells *et al.*, 1998)

Table 1: Table showing clustering of cloned 16S rDNA sequences from rumen samples.

Host/Diet	Rumen phase	procedure	Target bacteria	Results
3 sheep fed Rhopdes hay and protein every 3 hours	Total rumen content	rRNA probe	F. succinogenes Ruminococcus flavefaciens + albus	1.8% Of bacterial 16S rRNA 2.7% Of bacterial 16S rRNA
15 lambs fed alfalfa hay and milk or milk replacer	FAB from stomach tube	rRNA probe	F. succinogenes Rflavefaciens + albus	2 to 4% Of bacterial 16S rRNA 3 to 5% Of bacterial 16S rRNA
4 dairy cows fed mixed diets varying in alfalfa and corn silage levels	PAB extracted by chilling	rRNA probe	F. succinogenes R flavefaciens R albus	0.22% Of bacterial 16S rRNA 0.16% Of bacterial 16S rRNA 1.1% Of bacterial 16S rRNA
4 sheep fed 40% alfalfa hay/60% barley concentrate	PAB by filtration and washing	rRNA probe	F. succinogenes R. flavefaciens R. albus	2.1% Of bacterial 16S rRNA 1.4% Of bacterial 16S rRNA 1.1% Of bacterial 16S rRNA
4 sheep fed alfalfa hay	Total bacteria	rRNA probe	F. succinogenes R. flavefaciens R. albus	4. 3% Of bacterial 16S rRNA 6%3% Of bacterial 16S rRNA 0.81 Of bacterial 16S rRNA
4 sheep fed alfalfa hay	PAB	rRNA probe	F. succinogenes R. flavefaciens R. albus	5.8% Of bacterial 16S rRNA 2.5% Of bacterial 16S rRNA 0.93% Of bacterial 16S rRNA
4 sheep fed alfalfa hay	Firmly adherent PAB by screening and washing	rRNA probe	F. succinogenes R. flavefaciens R. albus	6.3% Of bacterial 16S rRNA 2.7% Of bacterial 16S rRNA 0.73% Of bacterial 16S rRNA
1 sheep fed 80/20.50//50, and 20/80 alfalfa hay/concentrate diets	Total bacteria	Competitive pCR	F. succinogenes R. flavefaciens R. albus	0.6 to 4.5 x 10^{7} cells/ml 3 to 6 x 105 cells/ml 3 to 5 x 104 cells/ml
10 steers fed paspalum hay and gradually switched to 75% rolled barley	FAB	Real time PCR	B. fibrisolvens S. Bovis Megasphaera elsdenii	107 to 10^{4} cells/ml 108 to 10^{7} cells/ml 104 to 10^{8} cells/ml

Bacterial samples were collected from fluid-associated bacteria (FAB) or particle- associated bacteria (PAB).

2.6.3 Quantification of microbial populations by molecular techniques

Quantification of important groups or species using molecular based procedures can help explain their role in ruminal metabolism but also to provide perspective for more qualitative approaches than gene sequencing (Rosello-Mora and Aman, 2001). In particular, methodological limitations of polyphylogeny (Zoetendal *et al.*,, 2004) and the need for hundreds of thousands of rRNA sequences for adequate characterization of the true microbial diversity and community composition (Larue *et al.*, 2004), the need of effective utilization of molecular-based procedures for quantitative purposes in ruminant nutrition studies is essential

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Although the technique is new, some projects on the characterization of rumen microbial diversity and related rumen ecology parameters have been carried out by international atomic agency (IAA) in collaboration with different countries using real time PCR. But still these activities are yet limited to specific issues of methanogens and to some extent about cellulytic microbes and they are done discretely. As of proceedings of IAA(,2007) Reported from Brazil, Colombia and Ethiopia indicated effects of tannin rich plants and coconut oil can result in reduction of quantity of protozoa ,methanogens , *F.succinogens* ,anaerobic fungi as indicated in table 2.

Table 2: Relative proportion of microbial community of rumen by real time PCR amplification technique presented being summarized and converted in %.

Origin of genomic DNA	*General bacteria*	*Methanogens*	*F.succinogens*	*R.flavifaciens*	*Anaerobic fungi*	*Total protozoa*
Colombian[1] cattle		0.045	0.58	0.0088	0.012	0.0065
Colombian cattle with coconut oil treatment		0.015	0.013	0.0084	0.0027	0.0035
P value		0.0126	0.9467	<0.1172	<0.0001	0.8309
Ethiopian sheep[2]	17.13 (0.7)	0.0122 (1.44)	0.4787(0.74)	0.122(0.54)		
Ethiopian sheep with coconut oil treatment	20.39 (0.65)	0.0695 (2.00	3.57(2.96)	0.33(2.0)		
Brazil sheep fed LEU[3]		0.00011	0.0095	0.0001	0.00013	

1= sheep animals with superscript numbers were used as control group.1 cattle fed with urea and molasses treated hay, 2= sheep fed with-hay and wheat bran and 3= sheep provided with- *Leucaena leucocephala* (LEU) treatment.

Real time PCR or quantitative PCR is a variation of the standard PCR technique used to quantify DNA or messenger RNA (mRNA) in a sample. Over the last 10 year several molecular tec niques have been developed for to analyse natural microorganisms (Wintzingerode *et al,* 1997). The analysis of microbial ecosystems has changed more and more toward molecular biological methods.RNA and their genes rDNA are used as reliable *polygenetic markers for relatedness between isolated and uncultured.the 16s rDNA can be* directly amplified from extracted DNAs with a set of primers binding to conserved regions of 16srDNA

Using sequence specific primers, the relative number of copies of a particular DNA or RNA sequence against housekeeping gene can be determined. Quantification of amplified product is obtained using fluorescent probes and specialized machines that measure fluorescence while performing temperature changes needed for the PCR cycles. A standard curve for real-time PCR is prepared by serially diluting a known amount of rDNA copies (Sylvester *et al.*, 2004). When added to a PCR reaction, each successive dilution requires a longer cycle to reach the threshold signal (fluorescence units above background units). The first cycle above this threshold is the threshold cycle. PCR is the most sensitive technique to detect sequences that are present in very low concentrations in the environment. It is possible to determine quantitatively SSU rDNA or rRNA of the target microbe using PCR. Although RT-PCR still needs to be proven, suitable for analyzing complex rumen microbial communities, this application looks promising because microbial targets in very low concentration can be quantified, which are difficult using other approaches .When added to a PCR reaction, each successive dilution requires a longer cycle to reach the threshold signal (fluorescence units above background units).

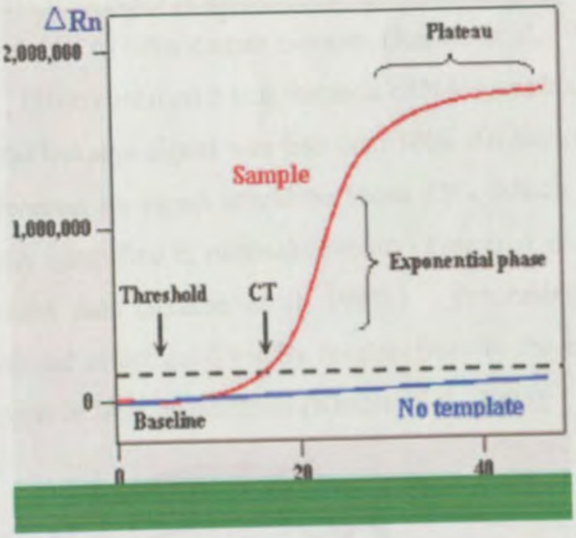


Figure 1: Amplification curve

The threshold cycle (The first cycle above the threshold) in regular samples (as opposed to standard curves) is affected both by initial conditions that influence amplification of starting templates (e.g., copy number per cell or molecules associating with DNA) and by amplification efficiency (P) of new copies, which increasingly predominate in PCR product produced from previous PCR product although the slope is determined only from later PCR cycles. The PCR efficiency = 10(-1/slope) – 1 (Stratogene Mx3000PTM Real-time PCR System Instruction Manual, La Jolla, CA, USA). In reality, PCR product accumulation decreases and then ceases during later cycles when the reagents become limiting (Polz & Cavanaugh, 1998). Many factors can influence the amplification reaction, and fingerprinting techniques alone do not provide quantitative data (Wintzingrode *et al.*, 1997). Polz and Cavanaugh (1998) noted the increasingly bias of the frequency of PCR product relative to initial template as PCR cycles progresses to higher replications level. They suggested that pooling of PCR replications might provide enough PCR products from only 5 cycles (with less bias than more cycles) while also reducing variation associated with replicate amplifications. Similarly, assuming a constant efficiency of amplification of DNA templates, the product accumulation can be estimated using the formula $(1 + P)^{n.}$ as logarithmic bacterial growth can be assessed mathematically as 2^x, where x represents the number of division cycles, 1 represents the proportion of initial copies and P is the fractional amplification efficiency of copies of each cycle (n) (Dehority, 1998).

Quantitative amplification of RNA is difficult (Krause *et al.*, 1999b) and variable, depending on its concentration relative to other carrier biomass (Raskin et al, 1997) demanding usage of rDNA., Krause *et al.*, (2000) estimated that Bacteria rRNA was about 60 to 70% of the total rRNA signal. The total Eukarya signal was less than 10%. Archaea signal was not measured directly, but, by difference, its signal would be about 25%, which is much higher than the *Archaea* signal directly quantified in ruminal contents (Ziemer *et al.*, 2000) or even expected from microscopic count data (Krause et al, 1999b). . Processing method prior to DNA extraction has a profound effect on diversity results. Despite the established importance of only a few main bacteria in fiber degradation (Krause *et al.*, 2003),

2.9 Non-16S rRNA-based profiling

Most culture-independent techniques used to describe bacterial communities have focused on the sequence diversity of 16S rRNA. However, determining G+C content has been used successfully (Holben *et al.*, 2004). The advantage of these types of profiling is that they are direct without any amplification step. However, the identification of bacteria causing the shifts in the profiles is difficult, because the data lacks phylogenetic information.

3. MATERIAL& METHODS

3.1 Study area

The survey was conducted in Jijiga and Gursum weredas (Jijiga administrative Zone) of the Somali Regional State of Ethiopia (Figure 3). The Somali Regional State is situated in the eastern part of the country. It is located 4° to 11° north latitude and 40° to 48° east longitude. The altitude ranges between 500 and 1,600 meters above .sea level. The total area of the regional state is estimated to be around 325 thousand km^2. The topography of the region is mainly lowland. However, there are some relatively high spots. The temperature of the area is related to altitude. At Jijiga, area of higher elevation, the average monthly temperature varies between 16 to 20 C^O whereas at Degahabur with lower altitude, it is about 22 to 26 C^O. The rainfall is erratic and bimodal with an annual average range of 300mm to 500mm. The population leads it's livelihood on livestock under pastoral or agropastoral farming system (MOARD-PADS 2004).

Jijiga administrative zone borders Shinilie administrative zone to the north, the Hararghe highlands of Oromia Region to the west, Dagahabur to the south, and Somalia to the east (SCF-UK *et al.*, 2001). Jijiga administrative zone can be divided into three separate food economy zones (FEZs), namely, sedentary agriculturalists, agro pastoralists and pastoralists. Agropastoralism is a dominant production system in Jijiga Zone. The agro-ecological classification of the Zone includes A1 (arid zone one), A2 (arid zone two), SA1 (semiarid one), and SA2 (semiarid two) (MOARD-PADS, 2004). Gursum is bordered on the south by Babille, on the west by the Oromia Region, and on the north and east by Jijiga.

The region consists of about 38% grassland coverage and 54% bush and shrubs Afro plane associated land (IAR, 1993). The vegetation is mainly of a sub-desert range type consisting of dominantly a sparse cover of shrubs and closed thickets of bush cover in some areas. *Acaylpha fruticosa*, *Acaylpha volkansii*, *Acacia bussei*, *Acacia brevespica*, *Acacia etbaica*, *Acacia nilotica*, *Acacia nubica*, *Acacia seyal*, *Acacia tortilis*, *Aloe spp*., *Balanites glabra* and

Calpurnia aurea are some of muli-purpose shrubs and trees in the region (Annex 2) (Belaynesh, 2000).

The forages browsed by the livestock can be classified into two main groups: ephemeral annual plants, which germinate and remain green for only a few weeks after rain and perennial shrubs and tree fodders (Calabrò *et al.*, 2007).

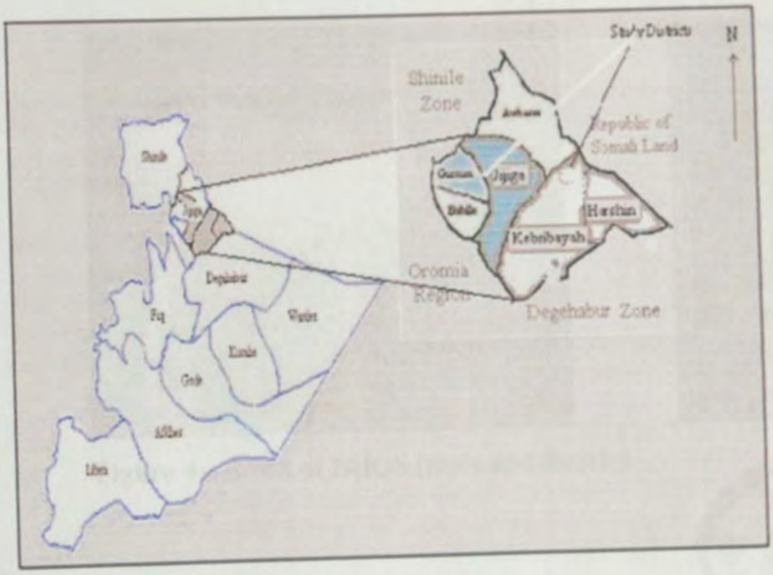


Figure 3: Map of the study area

3.2. Study population

Blackhead Somali/Ogaden Sheep found in the study areas were the study population. The breed is indigenous to the natural habitat (Ogaden area) in the Somalli Regional State of Ethiopia. The breed is also found in surrounding areas of southeast of Ethiopia and as far west as in the Gemu Gofa zone of Ethiopia (Galal, 2003). It is also found in neighboring Somalia and Kenya and they are known as the Black Head Somalia (Osman, 1985). The sheep stand second in number next to camels and contributes a great deal to the national economy as it has special merits in the Middle East and Arabian countries (FAO, 2007). This is probably the most internationally known East African breed of sheep.

As showed in figure 5, the Black Head Ogaden (BHO) sheep are distinguished by a blackhead with small and rather short and usually polled appearance; short and outward-forward inclined ears; short stiff shiny white hair body and limbs coverage; a well developed dewlap extending from the chin to the chest with fat deposits under the chin and the chest and short and fat-rump with its thin tip sticking straight backward or somewhat hanging down (Galal, 2007).

Figure 4: Breed of BHOS (male and female)

3.2 Sample collection

Tow study locations namely: bush covered grazing land around Gursum and open grazing land in Jijiga, hereafter named as Group 1 (G1) and Group 2 (G2), respectively, were selected based on vegetation coverage, agro ecology type and BHS population density. Group 1 is characterized by more than 25% shrubs coverage, fragile (ups and down hills) landscape, lying below 1750 meters above sea level, and medium BHS population size. Whereas, Group 2 have features of less than 3% shrubs coverage, plane land, lying above 1570 meters above sea level and higher BHO population size. Besides to location difference, seasonal factors were also considered for sample collection in G1 (coded as DG1 and WG1).

In group 1, 18 HHs were selected, from which 9 were for dry season and another 9 were for wet season. Similarly from Group 2, 9 BHOS owning households (HHs), residing far apart were randomly selected only for.wet season sampling. One mature male above 18 months age Black Head Ogaden Sheep was selected from each household for rumen fluid collection. The dry season samples were collected in the beginning of August, 2008 and the wet season sampling was done at the end of October, 2008. A total of 27 rumen fluid samples (aliquot), each measuring about 2ml, were taken from center of the rumen using needle aspiration and vacutainer tube. Rumen fluid samples were collected in morning time (9-10AM) and hold in ice cooling during sampling collection and then under -20^0C and -70^0C until microbial amplification.

3.3 DNA extraction

About 50µl rumen fluid was pipetted from filtrate (aliquot) of each sample and total genomic DNA was isolated separately by employing DNeasy Blood & Tissue Kit (Spin-Column Protocol) (QIAGEN, 2006, CA, USA). Some modification was done to compromise the amount of nucleated and non nucleated microbes as the whole microbial community was treated together as described in annex 1. Isolated genomic DNA was preserved under -70 until DNA was extracted for microbial population analysis. Quality of the samples was approved at A260/A280 and DNA concentration was adjusted to 30ng /µl.

3.4 Real time PCR amplification

Real-time PCR assays were performed using Fast Start DNA Master SYBR Green Light Cycler®). The set of specific primers used for amplification was targeting for general bacteria, Methanogens, *R. flavifaciens*, *F. succinogens*, total Protozoa and anaerobic fungi. General bacteria primer-template Inter run (universal primer) was used as positive control too. The designed primers (provided by IAEA,Thermo electron Corporation ,GmbH,Germany) used for the real time PCR are described in annex 2. A total of 189 rotor wells filled with PCR mixture of 3 study groups and negative control were run in 4 rounds with real time PCR.

Amplification was performed using personal opticon version 3.1 software programmed Bio Rad-Thermocycler-(PCR instrument) (Roche Applied Science, 2005). The threshold was set to 0.1. The temperature transition rate was set to 20 c^0/ second. Optimization of assay conditions was performed for primer s melting temperature (mT) and hold time in pre incubation period and annealing segment as indicated in Table 5.PCR program was set to 40 cycles.

Table 3: Real time PCR mixture

Component	*Concentration*	*Volume (μl)*
SYBR-Master mix	10x	2μ
F-primer	0.5x	1
R-primer	0.5x	1
MgCL2	3Mm	1.6
H_2O (PCR grade)		12.4
Template	30ng	2
Final volume		20(μl)

Product of each target microbial group was amplified or quantified using fluorescent probes and specialized machines (thermal cycler platform mini opticon apparatus and opticon monitor3 soft ware) that measure fluorescence while performing temperature changes needed for the PCR cycles. Amplicon specificity was not performed since amplification run was done for different targets at once. Melting curve analysis was omitted since it was impossible to apply for mixed microbial community. The cycle conditions were conditioned as indicated in Table 4. Fluorescence detection was performed at the end of each denaturation and extension step.

Table 4: Setup of a PCR amplification process

Analysis mode	*Program/segment*	*Temperature (^{0}C)*	*Hold time (seconds)*	*Slope Co/second*
Pre-incubation		95	15	
	Denaturing	95	10	
Amplification	Annealing	56*^{1}	5*^{2}	20
	Extension	72	20	
Cooling		40		0.1

^{1} annealing temperature is dependant of nucleotide content of primers and melting temperature was optimized to possible level accordingly.^{2} incubation time was limited to 5 second to increase specificity of primer binding.

3.4. Data management and analysis

Threshold cycle (ct), optical density of florescence signals (od), amplification efficiency and pattern of amplification curve data of each target microbial group was recorded from amplification. The relative proportion of specific microbial groups was computed as relative percentage to the estimated abundance of general bacterial 16S ribosomal gene (which served as gene housekeeping). Thus, the relative proportion of targeted microbial groups was calculated by subtracting their ct values from respective ct of general bacteria:

$\blacktriangle \text{ct X} = \text{ct X} - \text{ct Gb}$, and then the result was calculated from $100(2^{-\blacktriangle \text{ct X})}$ Where X stands for targeted microbe and Gb stands for general bacteria.

Direct change of ct of general bacteria was also used in comparison of study groups. Data were entered in the Microsoft excel (2007) for descriptive statistics and preparation of graphs. SPSS (version 15, 2006) was used to test statistical significance of differences among the different groups by independent t-test.

4. RESULT

4.1 Identification of ruminal microorganisms

From a total of 189 rotor wells filled with PCR assay mixture and negative control run in 4 rounds, 188 amplicons or products of DNA templates were identified. One methanogen amplicon from G2 (wet shrubs covered grazing land) was not detected. In all samples, florescence signal of general bacteria was detected at very early ct stage followed mostly by total protozoa.

As displayed in the amplification curve in Figure 6, florescence signals of general bacteria, *F. succinogens* and *R. flavifaciens* elevated up more exponentially per cycle than others. General bacteria and total protozoa reached plateau phase before the program was completed.

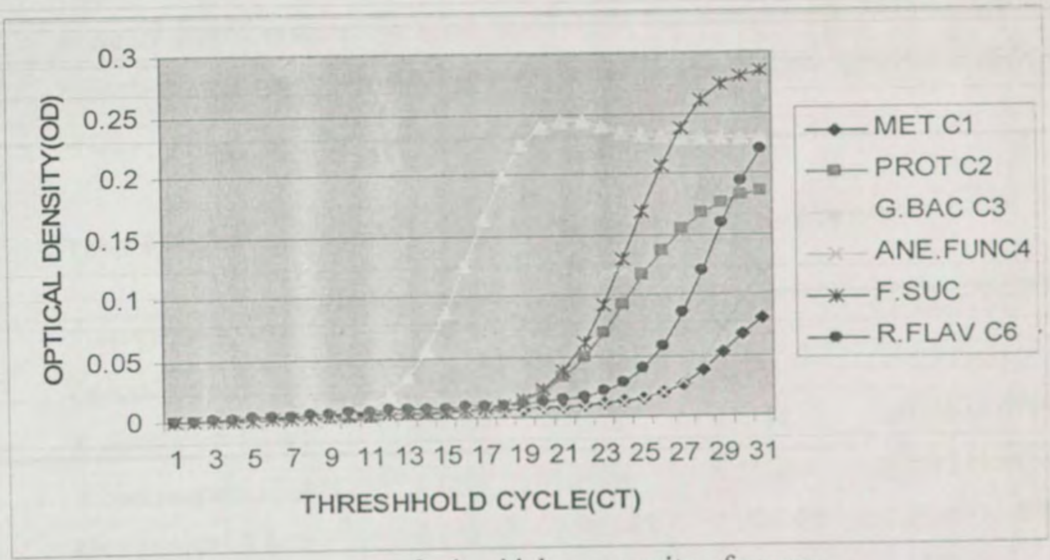


Figure 5: Amplification curve of microbial community of rumen

The amplification efficiency was above 20% for all DNA copies or amplicons of targeted microbial groups except 8.66% and 16% efficiencies observation in 1 protozoa and 1 methanogens belonging to WG2samples. Amplicons like general bacteria, anaerobic fungi, *R. flavifacience* and *F. succinogens* showed better amplification efficiency with lower variability (SE) while protozoa and *methanogens* were expressed comparatively with lower efficiency and fluctuation (Table 7).

Table 5: Amplification efficiency of ruminal microbes of BHS

Ruminal microbes	*Mean efficiency (%)*	*Standard error*
Protozoa	27.09	12.14
Methanogen	22.79	6.16
General bacteria	43.21	6.71
Anaerobic fungi	42.20	5.51
R. flavifacien	37.43	5.95
F. succinogens	32.82	3.35

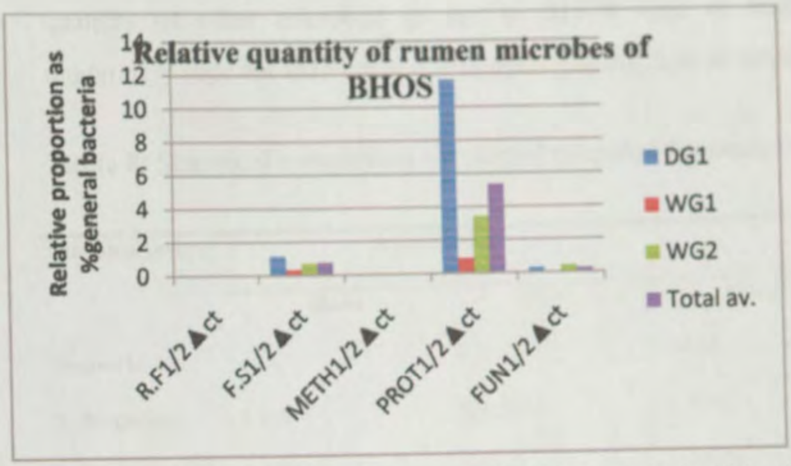
4.2. Quantification of the identified ruminal microbes

The relative proportion of targeted microbial community from the whole study sample or study population showed the presence of 5.33 % (±6%) Protozoa, 0.74 % (±1.2%),*F.succinogens* 0.25% (±0.27%), anaerobic fungi 0.036% (±0.07%), *R.flavifaciense* and 0.0022 % (±0.0018%) methanogens in BHOS (Table 6 and Figure 5). Relative proportion of general bacteria was not computed in terms of exponential value because of lacking reference genetic material for it. But based on the fact that genomic DNA sample with general bacteria emerged early meant to represent better quantity, relative proportion of DG1> WG1> WG2.

Table 6: Relative proportion of microbial community of rumen in BHO

Ruminal microbes	*DG1*	*WG1*	*WG2*	*Overall (SE)*
General bacteria*	14.4	14.6	16.7	15.25 (1.6)
R. flavifacien 1/2▲ct	0.0161	0.0318	0.0588	0.0356 (0.0714)
F. succinogens 1/2▲ct	1.1562	0.3428	0.7186	0.7392 (1.1785)
Methanogen1/2▲ct	2.11E-03	2.72E-03	1.84E-03	2.2E-03 (1.87E-03)
Protozoa 1/2▲ct	11.6891	0.9064	3.4165	5.3373 (6.)
Anaerobic fungi 1/2▲ct	0.284	0.0414	0.4166	0.2473 (0.2707)

* General bacteria is represented by ct



Representation of R.F=*Ruminococcus flavifaciens;* F.S=*Fibrofacter succinogens;* METH=Methanogens; PROT= Total protozoa; FUN= Anaerobic fungi and 1/2$^{▲ct}$ stands for computed microbial quantity in %

Figure 5: Relative proportion of rumen microbes in mixed study groups

4.3. Comparison of ruminal microbes by study groups

Comparison of microbes by study groups indicated that samples from BHS reared in dry season of open grazing land showed 12, 6.9 and 3.4 folds higher abundance of total protozoa, anaerobic fungi and *F.succinogens*(respectively) than that of similar microbial groups of BHS managed in wet season. Reversely, methanogens and *R.flavifaciens* in dry season were found 1.2 and 1.5 folds lower than that of identical microbes in wet season.

Table 1: Ratio of relative proportion of microbial community of BHS by paired study groups

Ruminal microbes	*DG1:G1*	*WG1:WG2*
R. flavifacien 1/2▲ct	0.506708	0.540438
F. succinogens 1/2▲ct	3.372582	0.47706
Methanogen1/2▲ct	0.776625	1.479429
Protozoa 1/2▲ct	12.89554	0.265317
Anaerobic fungi 1/2▲ct	6.8623	0.099334

Comparison between locations, open grazing land samples expressed 1.5 folds more methanogens than shrubs covered grazing land of similar wet season. Except methanogens, quantity of other microbial groups in BHOS kept in bush covered grazing land was moderately superior than the sheep in open grazing land to respective microbes.

Table 8: Statistical comparison of ruminal microbes between dry group 1 and wet group1

Microbial groups	*DG1(JIJIGA)*		*WG1(JIJIGA)*		**P-value**
	Mean	**SEM**	**Mean**	**SEM**	
General bacteria	14.4	0.45	14.63	0.53	0.627
R. flavifaciens	0.016	0.00392	0.0318	0.0063	0.119
F. succinogens	1.1562	0.1215	0.34283	0.05731	0.510
Methanogens	0.0021	0.0006	0.0027	0.0004	0.841
Total protozoa	11.689	1.7563	0.9064	0.5976	0.086
Anaerobic fungi	0.2839	0.1168	0.0414	0.0066	0.020

In comparison of different season but falling in the same locality (DG1 and WG1), only relative proportion of anaerobic fungi showed significant difference ($p< 0.05$), where as mean's of others were not significantly different ($p>0.05$).

Table 9: Comparison of ruminal microbes between locations of the same season

Microbial groups	*WG1(JIJIGA)*		*WG2(GURSUM)*		*P. vale*
	Mean	SE	Mean	SE	
G.bacteria	14.63	.535	16.72	.225	0.025
R. flavifacies	0.0318	0.006	0.0588	0.04	0.024
F.succinoges	0.3428	0.0573	0.7186	0.7	0.063
Methanogens	0.0027	0.0004	0.0018	0.0008	.482
Totalprotoza	0.9064	0.5976	3.4164	1.3	0.070
Anaerobicfui	0.0414	0.0066	0.4166	0.061	0.000

Comparison of microbes quantity between locations indicated significant variability of anaerobic fungi, *R. flavifaciens* and general bacteria ($p<0.05$) with intensity of very high to optimal significance level, respectively.

5. Discussion

The manner of fluctuation of amplification efficiency between amplicons may be related to reaction conditioning. Better amplification efficiency was resulted for those targeted microbes set at ideal reaction condition. The poor and fluctuation of amplification efficiency in these samples could be occurred by some deviation of recommended annealing temperature and air bubble trapping in the capillary tip respectively.

The reaction was performed involving different primers (multi primers) demanding heterogonous annealing temperature at once to create average working situation for all targeted members of microbial community. The recommended annealing temperature for primers of total protozoa and methanogens was a little bit deviated for the sake of compromising temperature reqirement of others This would be due to their higher starting genomic DNA concentration as the assay is quickly out competed by higher concentration of starting genomic DNA and due to too high number of cycles than that could be set for them.

Threshold of g.bacteria of BHS emerged at (15.25±1.6) in whole sample; at 14.4 in DG1 at 14.63 in WG1 and at 16.7 in WG2.The greater ct values of open grazing land and the lower ct values in shrubs area of general bacteria may indicate as there could present more proportion of bacterial spicies and non *arceal* methanogens in the first group but with lower quantity in the second group probably being inhibited by plant metabolites. Expressed quantity of methanogens in the groups is indeed supporting as they could be one sort of factors for the general bacterial quantity difference.

There is scarcity of documented report to compare ct values and relative proportion of most functional ruminal microbes quantified with Real Time PCR technique. An experimental trial tested on Ethiopian highland sheep by Aschalew (2007) reported diurnal average ct values of (17.13±0.7) from s control group (n=2) fed with hay and wheat bran and 20.39(±0.65) from coconut oil treated sheep (n=2) for general bacteria. Quantity of general bacteria in G1 of BHOS is higher than that of highland sheep kept on station while quantity of general bacteria in WG2 is closer to value of the control groups of highland sheep. This may happen for existence of more and divers bacterial species in BHOS grazing extensively in open grazing land. And nearness of bacterial quantity between G2 of BHOS and highland sheep may reflect declination of bacterial population and as it would be attributed to inhibitory effect of plant metabolites. Or, apart from treatment variation in experimental procedure may have brought this difference. Aschalew used 10ng/μl genomic DNA concentration whereas we used 30ng/μl. The sample size of his work was very small from ours sample size.

Based on observation of descriptive results of samples and even though not statistically supported probably because of smaller sample size or other external errors, quantity of total protozoa particularly, and *F.succinogens* as well decreases in wet grazing season of BHOS. With the same interpretation, Shrubs covered grazing land seems to exhibit presence of more all targeted rumen microbes except methanogens in BHOS as compared to open grazing land as long as both locations are in raining season. The growing of seasonal shrubs, trees and forbs which are enriched with antinutritve factors, in open grazing land during raining period may suppress some rumial microbes like total protozoa,*F.succinogens* anaerobic fungi and *R.flavifaciens.*where as presence of shrubs throughout most months in shrubs endowed grazing land seems to be adopted by those favored(as mentioned early) ruminal microbial groups.

Reduction of total protozoa may be occurred because of feeding of sheep on seasonally emerged antinutritive enriched shrubs ,trees and forbs in raining time and as consequence of their suppress effect. various studies indeed reported that foliage (Newbold *et al.*, 1997;Odenyo *et al.*, 1997 and Teferedegne *et al.*,1999) as well as fruits (Hess *et al.*, 2003 and Patra *et al.*, 2006) of tropical multipurpose trees(MPT) could suppress ruminal protozoa population due to result of their plant secondary metabolites(Hu *et al.*,2006). Reduction of and anaerobic rumen fungi may also be due to inhibition effect of antinutritive factors like saponins containing plants as reported by Wang *et al.*, (2000). Or the reduction of *Fibrobacter succinogenes* and increasing of *R. flavefaciens* and vice versa trend result in this study may be happened because of potential competition interactions among cellulolytic bacteria as *R. albus* and *R. flavefaciens* produce inhibitors that suppress growth of *R. flavefaciens* and *F. succinogenes*, respectively (Junqin Chen). Intensity of competition among cellulolytic bacteria is also influenced by presence of non-cellulolytic species like *Sel. ruminantium* . Additionally, higher quantity of anaerobic fungi and *F. succinogenes* in dry grazing time may be attributed to their competitiveness to fibrous feedstuffs. Competitiveness of anaerobic fungi to plant polysaccharides by its unique functional role of active cellulases and hemicellulases enzymes and penetration of the fibre by rehizoids or rhizomycelia may help anaerobic fungi to exist in larger quantity in dry-open and shrubs covered grazing environment. The previous studies also explained such phenomena (Mountfort and Asher, 1989) and (Akin and Benner, 1988). The reverse trend of relative proportion of protozoa verses cellulolytic bacteria and methanogens in this study may be because of predator–prey interaction of effect of the former and the latter as it was reprted by Williams and Coleman (1992). The reason of quantity fibrolytic bacteria being higher during dry grazing season could be as a result of its fibrolytic capacity of fibrous feeds as it was revealed by Cheng K. J. *et al.*, (1991) experiment.

Shrubs covered grazing land promotes presence of all targeted rumen microbes except methanogens in BHOS as compared to open grazing land as long as both locations are in raining season. The growing of seasonal shrubs, trees and forbs which are enriched with antinutritve factors, in open grazing land during raining period may suppress some rumial microbes like total protozoa,*F.succinogens* anaerobic fungi and *R.flavifaciens.* Whereas presence of shrubs throughout most months in shrubs endowed grazing land may be adopted by ruminal microbial groups.

Some studies also show that the ruminants like the feral goats, sheep and camel, which continuously feed upon tanniferousus diets usually develop tolerant microflora (Wiryawan *et al.*, 1999;_Teferedegne *et al.*, 1999;_Odenyo and Osuji, 1997). Absence or presence of protozoan inhibition in Ethiopian sheep and UK sheep breed and rate of detoxification process in time progression between animals also indicated the adaptation respons of animals for early exposure of antiprotozoal component (Teferedegne *et al.*, 1999) frequent feeding of BHOS on feedstuffs containing metabolites may develop adaptation responses to the rumeinal microbes and it is confirmed by in vivo experiment(Oldick & Firkins, 2000).

Although most identified microbes was seen to vary by their quantity between study groups when it is looked based on descriptive point of view, only general bacteria, *R.flavifacience* and anaerobic fungi were found significantly in different quantity between BHOS sheep population groups varying for grazing land type. Seasonal shifting from wet to dry period increases ruminal anaerobic fungi in BHOS significantly. The smaller the sample size being less and possible presence of experimental errors in our study can attribute to missing means difference at significance level. We suggest that increasing sample size and attempts to reduce experimental errors would confirm statically variability of those microbes indicating quantity variation in samples.

6. CONCLUSION AND RECOMMENDATION

It can be concluded from this study that shrubs covered grazing land favored abundance of general bacteria, *R. flavifacience* and anaerobic fungi in the rumen of Blackhead Ogaden sheep population reared dominantly in natural grazing system. Seasonal shifting from wet to dry increased the quantity of ruminal anaerobic fungi significantly.

Based on the above conclusion, the following are recommended:

- Further research should be carried out in different breeds of sheep kept under different feeding system using Real Time PCR technique. On station (controlled) and on farm experiments shall be done to generate more information;
- Characterization of rumen ecology and microbial diversity works should be performed using standardized methodologies;
- Evaluation on interaction of a range of various forage shrubs / trees and functional rumen microbes including parameters of rumen ecology shall be broadly studied;
- Useful forage species that enhance efficiency of rumen fermentation and reduce methane emission shall be screened and properly utilized for productivity improvement of hardship resistant ruminants like Blackhead Ogaden sheep.

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8-ANNXES

Annex 1. Procedures for Genomic DNA extraction
Protocol: Purification of Total DNA from animal Blood or cells (Spin –Column protocol)

1. For nonnucleated: pipet 20 µl Proteinase K into a 1.5 ml or 2 ml microcentrifuge tobe (not provided). Add 50-100 µl anticoagulated blood. Adjust the volume to 220 µl with PBS. Continue with step 2.
2. Add 200 µl buffer AL (without added ethanol). Mix thoroughly by vortexing, and incubate at 56^{0}c for 10 min.
3. Add 200 µl ethanol (96-100%) to the sample and mix thoroughly by vortexing.
4. Pipet the mixture from step 3 in to the DNeasy ini spin column placed in a 2 ml collection tube (Provided). Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tub.*
5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.*
6. Place the DNeasy Mini spin column in a new 2 ml collection tube (Provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000x g(14,000 rpm) to dry the DNeasy membrane.. Discard flow-through and collection tub.
7. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl Buffer AE directly onto the Dneasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x g (3000 rpm) to elute.
8. Recommended: For maximum DNA Yield, repeat elution once as described in step

Annex 3. Pictures of sampling area and sample collection

9. Signed Declaration Sheet

I undersigned declared that this thesis is my own and has not been done in other university. All owners of sources used are appropriately acknowledged.

Name
Signature
Date of submission